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GENETIC AND GENOMIC INSIGHTS INTO THE SUCCESSIONAL PATTERNS AND REPRODUCTION METHODS OF FIRE-ASSOCIATED *MORCHELLA*

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GENETIC AND GENOMIC INSIGHTS INTO THE SUCCESSIONAL PATTERNS AND REPRODUCTION METHODS OF FIRE-ASSOCIATED *MORCHELLA*

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We recommend acceptance of this thesis paper in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology.

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ABSTRACT

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Burn morels are among the earliest-emerging post-fire organisms in western North American montane coniferous forests, occurring in large numbers the year after a fire. Despite their significant economic and ecological importance, little is known about their duration of reproduction after a fire or the genetic and reproductive implications of mass fruiting events. I addressed these unknowns using post-fire surveys in British Columbia, Canada and Montana, USA in May/June of 2019. To assess fruiting duration, I collected specimens in second-year sites, where burn morels were collected the previous year, and identified them using DNA sequencing. Results demonstrated a predominant shift from burn to non-burn morel species, suggesting rapid changes in soil conditions and/or significant ecological differences between species. To address the implications of mass fruiting, I surveyed first-year post-fire sites, using whole-genome sequences to reveal the spatial extent of individuals and assess population genetic structure within and between sites for two species. Although gene flow appears more inhibited in *Morchella sextelata* than *M. eximia*, both species appear to disperse widely by ascospores and form populations characterized by large numbers of small individuals that persist as dormant structures between fires. My results will lead to a better biological understanding of these commercially and ecologically important mushrooms.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Morel mushrooms (*Morchella* spp.) are among the most renowned mushrooms collected for consumption (McLain et al., 2005; Pilz et al., 2007). In western North America, a multimillion-dollar industry revolves around morel collection in the year immediately following a wildfire, making burn morels one of the most valuable wild mushrooms sold on the market (Pilz et al., 2007). Over 300,000 pounds of dried morels are traded annually across the globe, corresponding to over 3 million pounds of fresh morels and approximately USD $18 million in international commerce; furthermore, morels are an important non-timber forest product contributing to the economy of communities adversely affected by changes in the timber industry (Iqbal, 1993; McLain et al., 2005; Pilz et al., 2004; Pilz et al., 2007; Schlosser & Blatner, 1995). Across North America, every spring people go “mad” for morels with an almost cult-like following, establishing festivals to celebrate their emergence, including competitions to collect the most morels in a short period of time (Weber, 1988). Morels also appear to be ecologically important as early colonizers of disturbed habitats and as mycorrhizal and endophytic symbionts of plants (Baynes et al., 2012; Carpenter et al., 1987; Dahlstrom et al., 2000). Some *Morchella* species are pyrophilous, belonging to a guild of fungi that are among the earliest emerging organisms in the post-fire landscape (Duchesne & Weber, 1993; Larson et al., 2016; Masaphy & Zabari, 2013).

Despite morels’ economic and ecological importance, many basic aspects of

morel biology are not well understood. One of these aspects is the production of large numbers of mushrooms in the year following a wildfire (Larson et al., 2016; Winder & Keefer, 2008). It has been determined that only a limited number of morel species (also known as burn morels, fire-associated morels, or pyrophilous morels) have this biological requirement for fire (O'Donnell et al., 2011). However, the possible ecological interactions between burn morels and non-fire associated *Morchella* species (known as natural morels) and the implications of mass fruiting on the reproduction methods of fire-associated morel species between wildfires have not been extensively researched. This research used field surveys, DNA barcoding, and population genomics to examine these aspects of burn morel biology. Studying pyrophilous fungi can lead to a better understanding of how they interact with their environment and better ways to manage habitats where these beneficial fungi are found (Molina et al., 2001; Pilz et al., 2007).

Morchella (Morchellaceae, Pezizales, Pezizomycetes, Ascomycota) was first described by Christiaan Hendrik Persoon (1794). Taxonomic studies based on macro- and micromorphology resulted in approximately 100 described species (O'Donnell et al., 2011); however, genetic studies subsequently found widespread disagreement between morphological delimitation and genetically defined lineages. The two main causes of this discrepancy are genetically distinct look-alike taxa that do not exhibit significant morphological differences, and single species exhibit that high amounts of variation between individuals (Du et al., 2012; Kuo et al., 2012; O'Donnell et al., 2011; Richard et al., 2014).

O'Donnell et al., (2011) recognized three lineages within *Morchella*: Rufobrunnea, Esculenta, and Elata (FIG. 1.1). These clades are commonly described as

landscape morels, yellow morels, and black morels, respectively. Phylogenetic analyses indicated the Rufobrunnea clade (2 species) as an early diverging basal lineage and the Elata and Esculenta lineages as sister clades (27 and 36 species, respectively). The majority of morels exhibit high continental endemism in the northern hemisphere (O'Donnell et al., 2011; Richard et al., 2014). Fire association is found only within the Elata clade, in only four species globally – *Morchella exuberans*, *M. eximia*, *M. tomentosa*, and *M. sextelata* (FIG 1.2; Kuo, 2008; Richard et al., 2014). All four species are found in the Northern Hemisphere, and *Morchella tomentosa* and *M. sextelata* are endemic to western North America (Masaphy & Zabari, 2013; O'Donnell et al., 2011; Richard et al., 2014). In the United States, pyrophilous morels most typically occur in mountainous forests in the western states; however, *Morchella exuberans* was identified after a wildfire in the Great Smoky Mountains National Park in Tennessee, demonstrating a wider distribution than previously known (Miller et al., 2017). According to phylogenetic analyses, fire association has evolved convergently, i.e., burn morels do not form a monophyletic group exclusive of non-fire-associated species (FIG. 1.2; O'Donnell et al., 2011; Richard et al., 2014).

Fire-associated morels are likely to play an important role in post-fire regeneration. Observational research in post-fire habitats has shown that some fire-associated species of morels do exhibit mycorrhizal characteristics with various species of trees, such as true fir and spruce, at varying levels of elevation (Keefer, 2005; McFarlane et al., 2005; Pilz et al., 2007). A mycorrhizal habitat would be consistent with many *Morchella* species (the Rufobrunnea clade notwithstanding) appear to form facultatively mycorrhizal symbioses with different species of trees ranging from

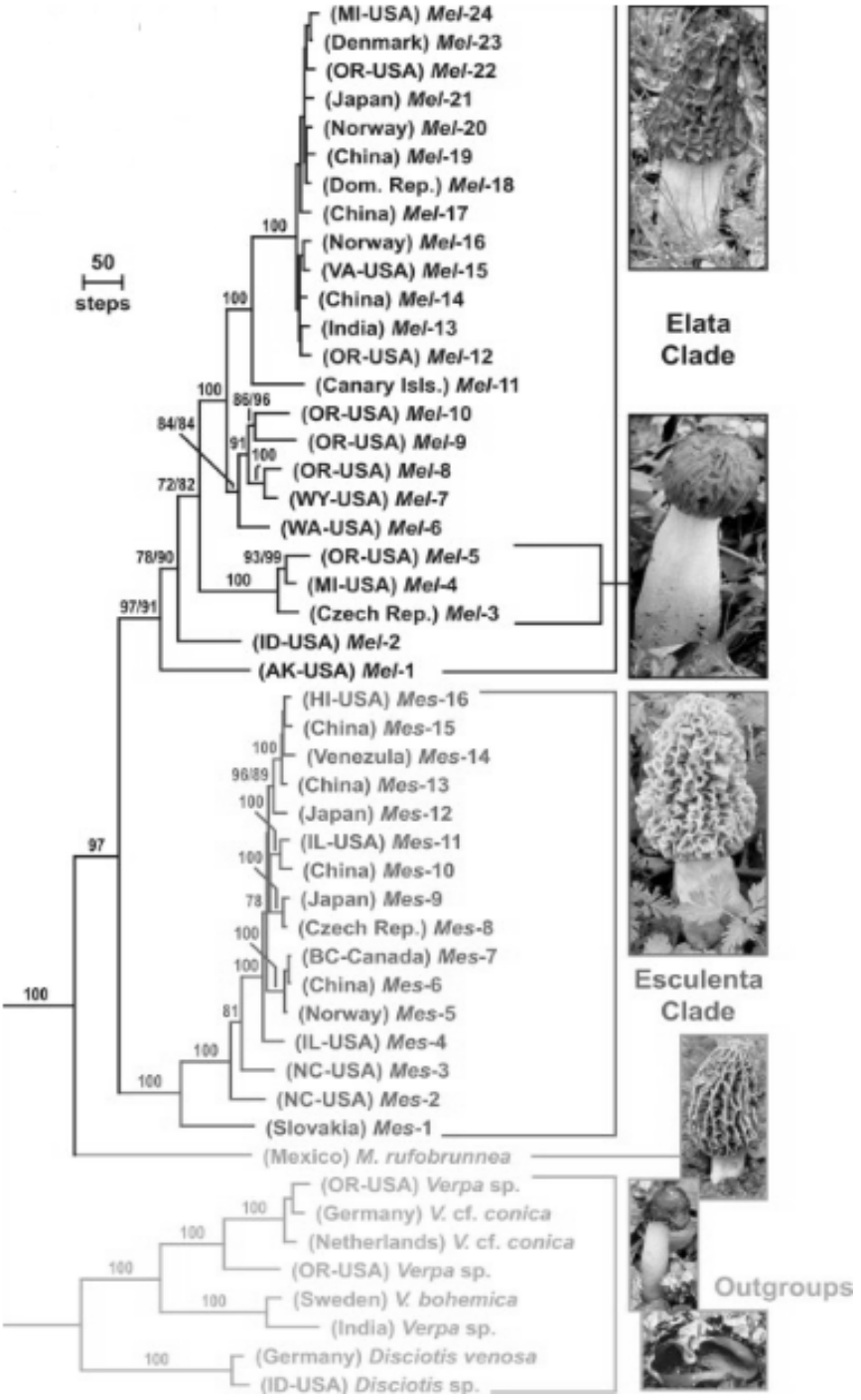

Figure 1.1. Phylogeny demonstrating the 3 recognized clades within the genus *Morchella*. The Rufobrunnea clade is represented in this tree by a single species, *M. rufobrunnea*. Maximum parsimony bootstrap values based on 1000 pseudoreplicates of the data are indicated above internal branches (*Mes*- designates species in the Esculenta clade; *Mel*- designates species in the Elata clade; Modified from O'Donnell et al., 2011).

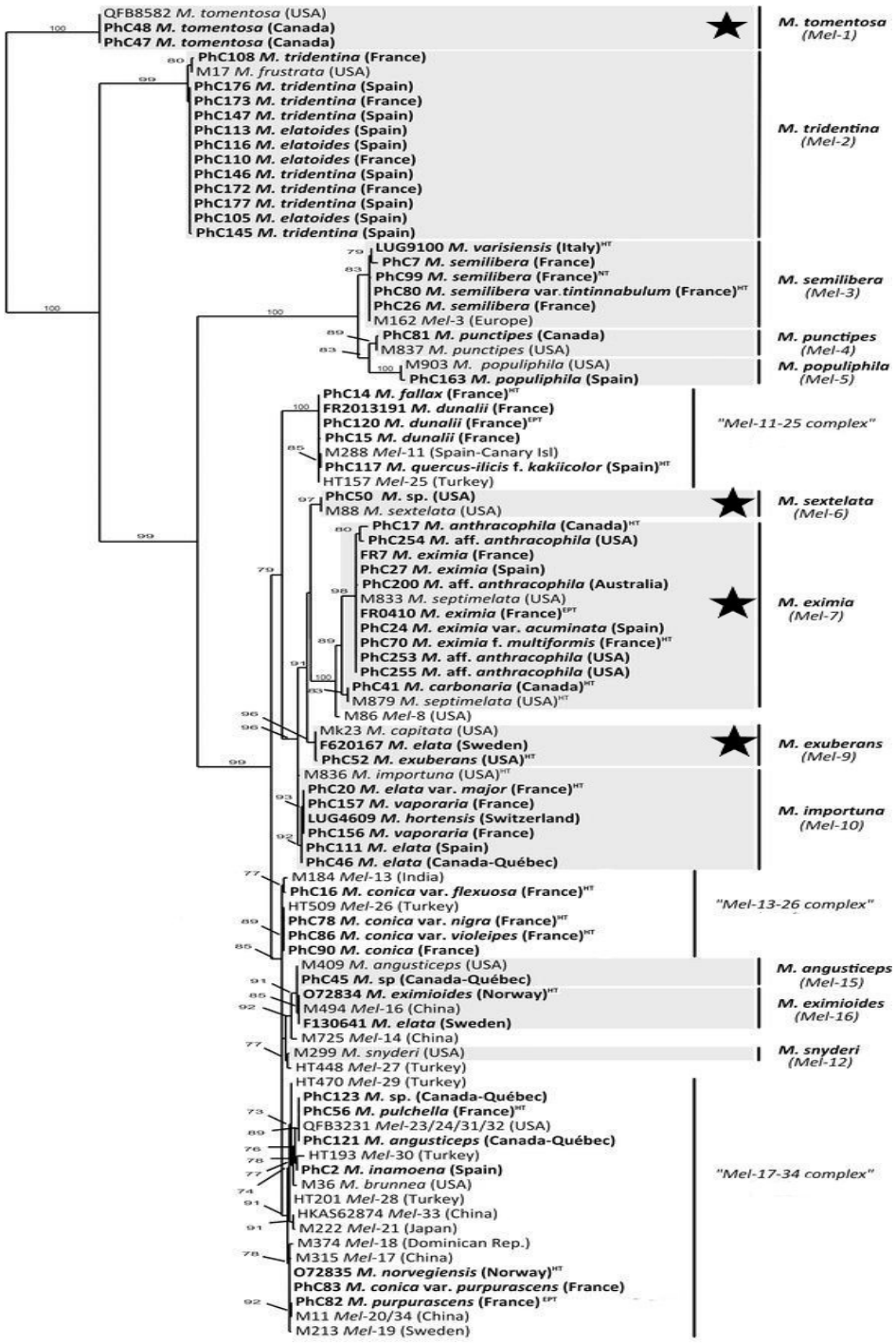

Figure 1.2. Phylogeny of Elata clade (black morel) species of *Mel*-1 – *Mel*-34, demonstrating convergent evolution of fire adaption in *Morchella* spp. designated with stars. *M. tomentosa* (*Mel*-1) diverged prior to the other known fire-associated species (*Morchella sextelata* (*Mel*-6), *M. eximia* (*Mel*-7), *M. exuberans* (*Mel*-9)). Numbers by nodes represent branch support above 70%, as assessed by the SH-aLRT statistical test (*Mel*- designates *Morchella* Elata clade; Modified from Richard et al., 2014).

hardwoods in the Midwest to conifers in the west (Harbin & Volk, 1999; W. Liu et al., 2019; Miller et al., 1994; Pagliaccia et al., 2011; Schmidt, 1983). However, fire-associated morels also appear to be partially saprotrophic based on carbon isotopic profiles (Hobbie et al., 2016). It is therefore possible that they may not simply be mycorrhizal or saprotrophic, but may be mycorrhizal mutualists that respond to the death of their hosts by adopting a saprotrophic habit. This possibility raises questions about how burn morel mycorrhizal networks establish, and whether the mycelium is primarily active soon after a fire or during the interval between fires.

Beyond mycorrhizal symbioses, burn morels also appear to play other important roles in plant establishment and maintenance. It is common for burn morel ascocarps (fruiting bodies) to harbor newly sprouted seedlings, potentially functioning as a "nurse" substrate for plant regeneration (T. Osmundson & L. Evans, personal communication). One study detected *Morchella sextelata* (a fire-associated species) and *M. snyderi* as endophytes in a fire-resistant invasive prairie grass, suggesting a mutualistic association that may facilitate fire survival by both species (Baynes et al., 2012).

Raudabaugh et al. (2020) did not observe *Morchella* in endophytic (in moss) or endolichenic associations in pre- or post-fire habitats in a survey in the Great Smokey Mountains National Park. They found the fire-associated species *Morchella exuberans* as a soil saprobe in February 2017 (the year following a 2016 wildfire), but did not find it present in their March 2018 samples, despite the prolific fruiting that occurred in April of 2017 (Miller et al., 2017). Though this evidence comes from a region where fire-associated fungi, and more specifically morels, are not common, it is interesting to note the lack of burn morel mycelia present in as little as two years post-fire. Though absence

of evidence is not evidence of absence, this finding leads to more questions about the persistence of burn morels in the soil prior to, during, and after wildfire disturbances.

Morchella spp. are not the only genus within the order Pezizales that produce ascocarps after a fire disturbance; therefore, comparison with these other species may provide clues to the biology of pyrophilous morels, functional traits associated with fire adaption, and the roles these organisms play in pre- and post-fire habitats, as well as highlight important differences across fire-associated species. Fujimura et al. (2005) observed ascocarps of five genera of post-fire Pezizales, but research found no root-tip colonization at the site surveyed, implying that fire-associated morels are mostly saprotrophic in post-fire habitats. According to Motiejūnaitė et al. (2014), many species of fungi emerge in wildfire disturbed habitats, filling different ecological roles, such as decomposing woody debris and enhancing nutrient availability for newly sprouted seedlings.

While the previous study concentrated on ecological niches, Carlsson et al. (2012) focused on the ability of some wood fungi mycelia to withstand the varying heat levels of wildfires by possibly accumulating heat shock proteins (HSPs). These HSPs are found in a wide range of organisms, but the role of HSPs in fungal heat tolerance is not known (Gupta, 1995). They are believed to play a significant role in fungi ability to withstand gradually increasing heat in fire-disturbed habitats (Carlsson et al., 2012). While the exact role of HSPs in heat resistant fungi is unknown, some species of fungi rely on heat for spore germination (Glassman et al., 2016; Peay et al., 2009; Plesofsky-Vig & Brambl, 1985). Glassman et al. (2016) looked at ectomycorrhizal fungal spore bank recovery after high intensity forest fires. Certain species, like *Rhizopogon olivaceotinctus*, a fire-adapted

hypogeous fungus found in the order Pezizales, increased in abundance after severe wildfires. *Neurospora crassa,* an ascomycete used as a model organism for genetic analyses, demonstrated a shift to HSP production when exposed to elevated temperatures (Plesofsky-Vig & Brambl, 1985). Though the exact functions of HSPs in fire-associated fungi has yet to be discovered, it can be concluded that fire adaptations associated with mycelial persistence and spore germination are found across many taxa. Fire-associated morels have not been tested for the presence of HSPs. Based on their massive fruitings, and subsequent massive ascospore dispersal events post-fire, it would be interesting to investigate if this fire-adaptation is present in either their mycelia or ascospores, and how it relates to their post-fire fruiting events.

One of the most intriguing aspects of fire-associated morel biology is the mass production of ascocarps in the year following a forest fire. To examine the genetic implications of these mass fruiting events, it is important to understand aspects of the life cycle of these species (FIG 1.3; Volk & Leonard, 1990). *Morchella* species, like other sexually reproducing ascomycetes, produce haploid ascospores that are products of karyogamy – the fusion of two haploid nuclei – followed by meiosis. The haploid ascospores germinate and form haploid primary mycelia. Primary mycelia have demonstrated the ability to produce asexual conidiospores in artificial cultivation; however, conidial formation in both the laboratory and natural habitats are not widely reported in most species, and its importance in natural regeneration and dispersal is unknown (W. Liu et al., 2018). Two sexually compatible primary mycelia fuse to form a heterokaryotic secondary mycelium that ultimately produces ascocarps. Rather than forming heterokaryotic ascocarps, the secondary mycelium may form a resting structure

called a pseudosclerotium (differing from true sclerotia by lacking the differentiated complex tissues such as a rind and medulla found in the latter), that may increase survival in harsh environmental conditions (FIG. 1.3; Volk & Leonard, 1990). It has been proposed that the primary mycelium may also form pseudosclerotia and fruiting bodies, but neither form has been observed in laboratory experiments (Ower, 1982; Ower et al., 1985; Volk & Leonard, 1990). The occurrence of some *Morchella* species and other fire-associated fungi raises the possibility of survival within plant tissues, but the overall effect of endophytism in *Morchella* living as endophytes is not known (Baynes et al., 2012; Raudabaugh et al., 2020).

Research on life stage development and nutritional requirements has led to recent advancements in the morel cultivation industry and may shed light on how morel mycelia function in nature (Q. Liu et al., 2018; W. Liu et al., 2019). Although many efforts at morel cultivation have failed to produce ascocarps of significant size, some species of *Morchella* have successfully been cultivated: one using a method that provides or restricts available nutrients at different points in the life cycle, and another using a method of creating a mycorrhizal association with tree seedlings (W. Liu et al., 2019; Ower et al., 1986; Miller, 2005).

This variety of cultivation methods reflects the different hypothesized ecological roles of *Morchella* species in nature, from ruderal disturbance-associated saprotrophs to mycorrhizal mutualists. However, one aspect that the different cultivation methods have in common is that pseudosclerotia have been observed, although their role in the ascocarp production is still unknown. Pseudosclerotia play a role in nutrient storage for survival in harsh conditions or over wintering, with high lipid and carbohydrate concentrations (He

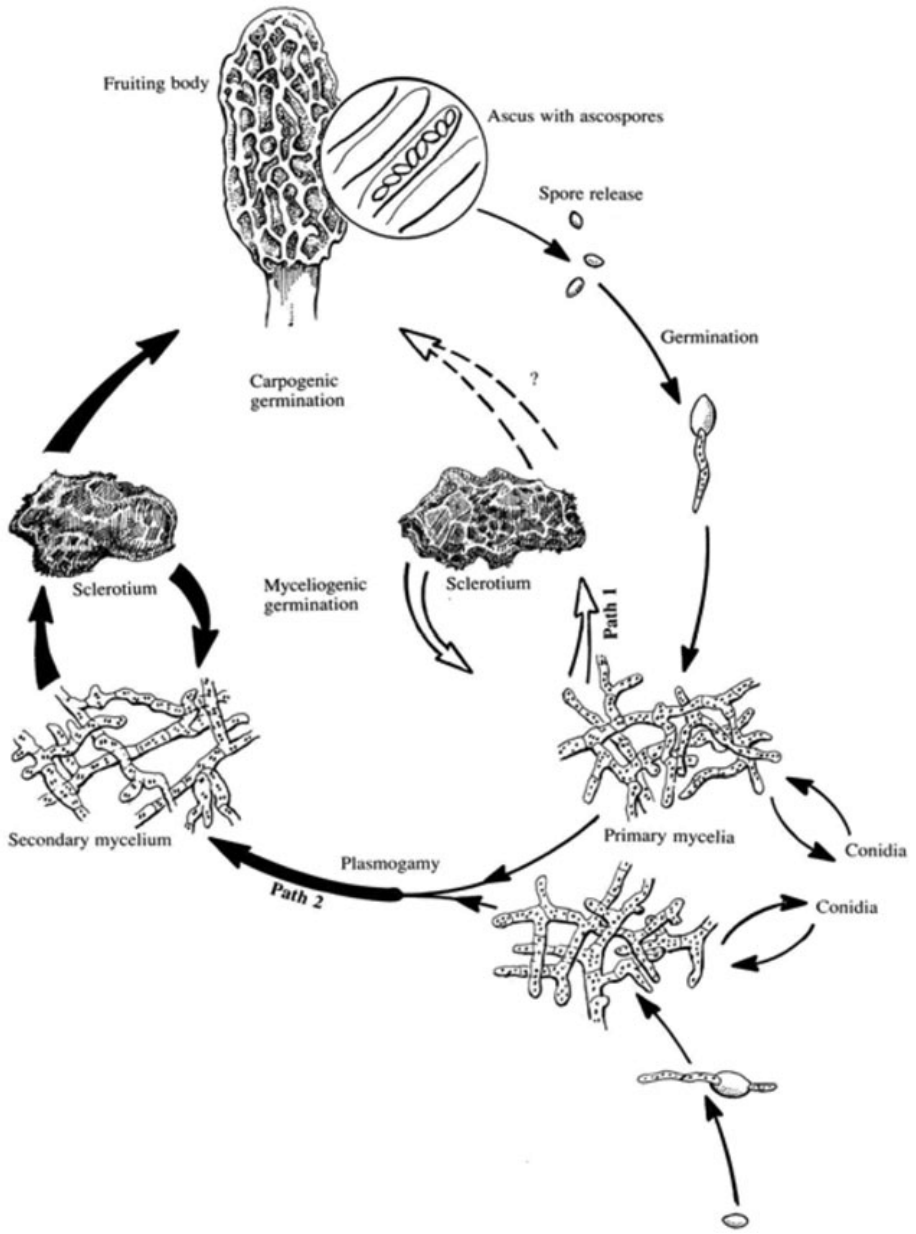

Figure 1.3. Life cycle of *Morchella* spp. (Volk & Leonard, 1990; used with permission of Dr. Thomas Volk).

et al., 2018). Therefore, it is possible that pseudosclerotial formation provides essential signals of surviving unfavorable environmental conditions, similar to the requirement for overwintering prior to seed germination in many plants (or cold stratification of seeds for artificial cultivation). The ability of pseudosclerotia to sense environmental cues is

suggested by the observation that nitrogen (urea) addition stimulates the growth of pseudosclerotia, and subsequent removal of the nitrogen source is necessary to stimulate fruiting body formation. Interestingly, nitrogen levels found in wildfire disturbed habitats also show variations, with much higher levels recorded in the first year after fire and dropping off in subsequent years (DeLuca & Zouhar, 2000). *Morchella tomentosa*, one of the four fire-associated morel species, has had documented pseudosclerotia formation associated with fruiting, coined radisciscl erotia (FIG. 1.4; Stefani et al., 2010). Though evidence for pseudosclerotia formation is undocumented for the three other fire-associated morel species in natural habitats, their presence has been shown for both *Morchella eximia* and *M. sextelata* in artificial cultivation settings (Q. Liu et al., 2018).

How does the life cycle of the fire-associated morel species occur under field conditions? Which aspects are most closely associated with mass fruiting? What effects do these associations have on population genetic diversity? Although laboratory studies have provided important information about the life cycle of *Morchella* spp., understanding the applicability of this knowledge to wild populations is difficult due to the cryptic nature of fungal mycelia in soil. However, population genomics offers powerful tools to answer questions about the ecology and evolution of these organisms (Douhan et al., 2011; Grünwald et al., 2016; McDonald, 1997; Xu, 2006).

Genetic studies have been conducted on species in both the Esculenta and Elata clades. These studies suggest a wide variety of reproductive strategies within the genus, including heterothallic mating (Chai et al., 2017; W. Liu et al., 2018; W. Liu et al., 2019; Pagliaccia et al., 2011; Volk & Leonard, 1989), with cases of inbreeding (Du et al., 2016; Volk & Leonard, 1989) and outcrossing (Dalgleish & Jacobson, 2005). This variation

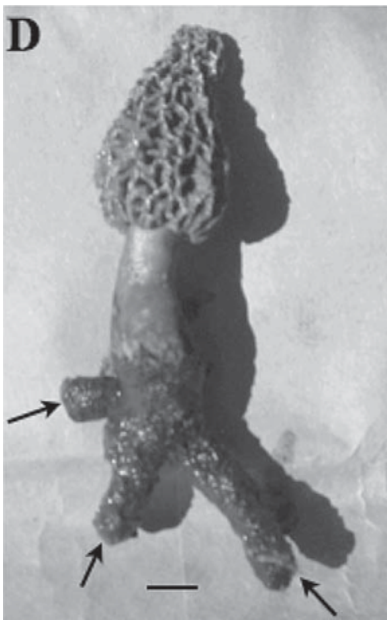

Figure 1.4. *Morchella tomentosa* radisciscleroti um at the base of the fruiting body. Scale bar equals 1 cm (Modified from Stefani et al., 2010).

leads us to wonder how fire-associated morels reproduce and demonstrate the need for more population level studies using genomic analyses. Although several studies have used whole genome analyses to examine the population structure of morel mushrooms, only one study has involved a fire-associated species (*M. sextelata*), and under cultivated conditions; studies of natural populations of fire-associated morels have not previously been conducted.

The fruitings of burn morel species in enormous numbers in the year after a wildfire is not only the key to their economic appeal, but also presents some interesting ecological questions about the role of ascospores in the persistence and evolution of these species. Key to such questions is the role of the secondary mycelium during the interval between fires. Following ascospore germination, it is possible that mycelial expansion occurs in either the primary mycelium or in the secondary mycelium following mating between two primary mycelia. If expansion occurs over a long time period (with or

without subsequent pseudosclerotial formation), then it is likely that competition between adjacent mycelia occurs and that populations are composed of a limited number of large genetic individuals, or genets (Dahlberg & Stenlid, 1995). However, it is also possible that ascospores either survive as a dormant spore bank or form primary or secondary mycelia that undergo growth to only a limited extent before forming pseudosclerotia that survive in a dormant state until the next fire event. These mycelial networks would therefore be much smaller and show higher genetic variation between individual specimens.

The size, distribution, and overlap of individual genets, within or across species, has been documented in fungal population studies based on sampling fruiting bodies and/or mycelial networks (Anderson & Kohn, 1998; Branco et al., 2013; Branco et al., 2015; Dahlberg & Stenlid 1995; Dunham et al., 2006; Hortal et al., 2012; Yoon et al., 1990). With recent advances in molecular methods for the investigation of fungal population structure and ecology, it is possible to not only distinguish unique individuals within a population, but also to uncover clues about their modes of reproduction and spatial distribution patterns (Branco et al., 2013; Dahlberg & Stenlid, 1995; Douhan et al., 2011; Dunham et al., 2006; Hortal et al., 2012; McDonald, 1997; Xu, 2006).

Objectives

This research draws upon the collection and analysis of genetic data to identify species (DNA barcoding) and examine population genetic diversity (population genomics). The data collected from this research will shed new light on a very popular mushroom for which many aspects of its basic biology are poorly known. Pioneering information on reproduction methods and emergence patterns for fire-associated morels

will be of interest to the mycological community and the commercial industry and could aid in further research on burn morels and other fire-associated fungal species.

Objective 1: How long do burn morels continue to produce mushrooms after a wildfire disturbance, and what is the timing of succession (species shift) to non-fire associated species? One aspect of morel ascocarp production often noted by collectors is that production levels drop precipitously on burn sites after the first year. It is often assumed that these smaller subsequent fruitings represent additional reproduction of fire-associated species. However, preliminary data suggest that second year post-fire ascocarps belong to "natural" or non-fire associated *Morchella* species based on phylogenetic evidence (Osmundson, Burchard, Evans, & Garbelotto, unpublished; Pilz et al., 2004). In other words, ecological succession appears to occur on a short time scale.

Successional studies have been conducted on species that emerge after a wildfire for some plants and for some species of fungi (Barker et al., 2013; Hewitt et al., 2017; Mediavilla et al., 2014; Salo et al., 2019; Segarra-Moragues & Ojeda, 2010), but not for morels. Multiple studies have demonstrated the prolific fruiting events of fire-associated *Morchella* spp. in the first year after a wildfire, but our knowledge of fire-associated morel fruitings is based solely on anecdotal evidence (Duchesne & Weber, 1993; Greene et al., 2010; Larson et al., 2016; Masaphy & Zabari, 2013; Pilz et al., 2004). With the accumulation of evidence presented by Richard et al. (2014) denoting the cryptic nature of some *Morchella* species, it is not surprising that current literature is lacking any documented succession of genetically identified morel species in the years following a wildfire.

Although unpublished data (Osmundson, Burchard, Evans, Garbelotto,

unpublished) and the study by Pilz et al. (2004) suggest that non-fire-associated morel species can be found as early as the second-year post fire, the extent and generality of this second-year shift have not been established due to the previously limited amount of data available. To increase the validity of these inferences by increasing their geographical scope and sample size, as well as more rigorously documenting whether succession has occurred, the same sites used by T. Osmundson, L. Evans, and colleagues to collect first-year specimens in 2018 were resampled for second-year post-fire specimens in 2019.

In Chapter 2, I describe the use of DNA barcoding and phylogenetic analyses of four molecular loci (ITS/LSU, TEF1, RPB1, RPB2) to identify the species found in 2019 and determine whether they represent known fire-associated or non-fire-associated morel species. Based on the prior information described above, I hypothesized that morels found on these sites will predominantly be non-fire-associated ("natural") species. This understanding of successional shifts, combined with on-going genomic studies could provide greater insight into how different morel species function in, and/or respond to, changes in the post-fire environment over a relatively short period of time.

Fourteen specimens were identified to species. Results indicated that there is a shift from fire-associated to non-fire associated morel species in as early as the second-year post-fire. Specimens analyzed were predominantly *Morchella snyderi* and *M. brunnea*, both found within the Elata clade but not believed to be obligately fire-associated species. One specimen was found to be a fire-associated species, *M. tomentosa*, and was the only specimen found at that site. It is important to note that this was the only site where salvage logging took place prior to the second spring after the wildfire, suggesting a role for soil disturbance in promoting ascocarp production.

Objective 2: Based on the results from Objective 1, fire-associated morel fruiting numbers drop precipitously in the second spring and later after a wildfire disturbance. How does this significant shift in fruiting habits relate to their life cycle in nature? What is the most likely reproduction method for post-fire fruiting events? Sexual reproduction in fungi has several potential advantages, including increasing genetic diversity within the population, allowing the production of resting structures, and promoting dispersal. The mass production of ascocarps in fire-associated morels would appear to be consistent with a high level of population genetic diversity. However, if all ascocarps in a site derive from a single secondary mycelium, population genetic diversity may instead be quite limited (i.e., all specimens collected would be part of a single genet, and all of their resulting offspring would therefore be siblings from the same parent). In this case, the energy expenditure of producing thousands of ascocarps functions more in increasing the chance of repopulating the habitat and/or promoting spatial dispersal rather than promoting genetic diversity. Levels of diversity and dispersal can be readily inferred using population genetics approaches. In the apparent absence of genetic diversity or gene flow, it may be reasonable to assume that the primary function of mass fruiting is to regenerate the mycelium from older resting structures (pseudosclerotia) and produce new ones, thereby serving as a means of "resetting" one or several dominant mycelial clones.

Population studies focusing on genet size and population structure have been conducted on numerous species, but not for morels. The morel life cycle provides details on which structures are most important for regeneration and about the relative importance of clonal vs. sexual propagation in the life cycle of these organisms. When a new genetic individual establishes, it will propagate as a monokaryotic mycelium, eventually making

asexual structures, such as conidia or dormant pseudosclerotia, and/or sexual reproductive structures (ascocarps all part of one genet). If clonal reproduction is the predominant mode of reproduction, a site should be characterized by having a smaller number of larger clones; if sexual production of new individuals is important, a larger number of small clones should be evident (Dahlberg & Stenlid, 1995).

It is currently unknown which of these models best explains the regeneration of fire-associated morels, or what role pseudosclerotia play in the development of ascocarps. If ascocarps collected in a certain site show genetic identity (clones of one individual) among contiguous samples, we can assume they are all derived from the same individual mycelium (or from resting structures thereof). If the ascocarps collected show higher levels of genetic diversity (in the most extreme case, each ascocarp belonging to a genetically distinct individual mycelium), we can assume that sexual reproduction and ascospore dispersal play a major role in post-fire regeneration, perhaps mediated by survival in a spore bank between fire disturbances or by formation of dormant pseudosclerotia (Glassman et al., 2016). This aspect of the research addresses an intriguing question: fire-associated morel species appear to expend significant resources on mass production of sexual reproductive structures after a fire disturbance (some fire intervals lasting over 100 years), but how does this expenditure translate to ascospore dispersal and the level of genetic diversity (and, by extension, the capacity for evolution and speciation) in these fire-associated species?

In Chapter 3, I describe the use of population genetic analyses of field-collected ascocarps to determine the size of genetic individuals and examine the level of genetic variation within the population to infer which reproductive mode is most likely. Because

of the potential for distinct individuals to be closely related, (e.g., siblings produced from ascospores by the same parent), I sequenced the full genomes of *Morchella sextelata* and *M. eximia* specimens used for this portion of the study. Despite the proposed importance of pseudosclerotia in the lifecycle and reproduction of *Morchella* spp., the primary focus of this objective is to collect and analyze fruiting bodies and their genomic variation, and therefore collection of pseudosclerotia was outside the scope of this project. Because I am interested in the spatial distribution of their underground mycelial networks, I used GPS data to locate exactly where ascocarps were collected.

Comparisons of genetic similarity both within and between collection sites were analyzed by using principal component analysis, and the examination of migration potential was analyzed via admixture plots. Due to previous studies indicating limited population level diversity within sites, I hypothesized that I would not observe genetic differences between spatially proximate mushrooms, indicating that reproduction occurs from existing mycelial networks that either persist between fire disturbances or produce resting structures such as pseudosclerotia. If samples within sites are genetically distinct, further examination of individual genotypes could distinguish inbreeding (siblings from same parent mycelium) vs. outcrossing to determine the extent to which sexual reproduction promotes genetic diversity in these populations.

Species distribution patterns in first-year wildfire sites indicate that multiple individuals of each species were present, providing evidence against the hypothesis of substantial spread of vegetative mycelia leading to low numbers of genetically distinct individuals. Population structure in *M. eximia* was not observed in the population genomic analyses, indicating widespread migration of ascospores and a lack of

geographic barriers to reproduction; results showed a high degree of genetic variation within sites and little to no differentiation between the sites surveyed. *M. sextelata* showed two genetically-defined populations across the four sites surveyed, with one population corresponding to the most geographically separated site and the other consisting of individuals from fires separated by over 300 kilometers. These results lead to the idea that plasmogamy is occurring frequently in nature, and that ascospore dispersal is not limited to geographic regions in close proximity to one another.

With climate change predicted to result in an increase of wildfires in many parts of the western U.S. and globally, the study of fire-associated species can contribute to an increased overall understanding of how ecosystems recover from fire and how they could benefit from changes in management of wildfire-prone lands (Parks et al., 2016; Pausas & Keeley, 2009; Riley & Loehman, 2016). Parks et al. (2016) looked at how fire severity has changed over the last few decades, and predicted benefits and failures of our current land management practices on the mitigation of wildfires. Pausas & Keeley (2009) state that the shift from high frequency, low intensity fires to low frequency, high intensity fires are outside the historical variability of these fire-dependent habitats, demonstrating our need for a better understanding of how fire-disturbed ecosystems are influenced by biotic and abiotic factors.

Increasing our understanding of post-fire morel biology will inform collection practices and tools to aid in predicting their emergence (McLain et al., 2005; Pilz et al., 2007). By genetically testing mushrooms that emerge after a fire, accompanied by plotting the exact location of samples, it is possible to determine the spatial extent of individual mycelia and therefore shed light on the role of ascospores in their reproduction

efforts. Using these data along with other genetic analyses, I will provide evidence on how fire-associated morels continually emerge in the year following a wildfire and the potential succession of morel species in fire-disturbed habitats.

CHAPTER II

SUCCESSIONAL PATTERNS OF *MORCHELLA* IN POST-WILDFIRE HABITATS

Introduction

Morel mushrooms (genus *Morchella*) are widespread across the Northern Hemisphere, producing ascocarps in a variety of habitats ranging from riparian hardwood forests to montane coniferous forests and human influenced habitats such as landscaping. They frequently fruit after disturbances such as logging, insect infestation of trees, and wildfire (Kuo et al., 2012; O'Donnell et al., 2011; Pilz et al., 2004; Pilz et al., 2007; Richard et al., 2014; Weber, 1988). There are approximately 65 accepted species of *Morchella*, 22 of which occur in North America (TABLE 2.1; Richard et al., 2014). They are an economically important group of mushrooms, supporting a multimillion-dollar commercial industry with over 300,000 pounds of dried mushrooms traded annually, totaling approximately USD $18 million in international commerce (Iqbal, 1993; Pilz et al., 2007). Much of this industry relies upon the collection and sale of fire-associated morels that fruit in large numbers after a wildfire has moved through a mountain forest. Four species are believed to be obligately fire-associated: *Morchella tomentosa*, *M. exuberans*, *M. sextelata*, and *M. eximia* (TABLE 2.1; Richard et al., 2014).

According to O'Donnell et al. (2011), fire-associated morels (commonly known as burn morels) belong to the Elata clade and, according to phylogenetic analyses, fire association has evolved convergently (i.e., burn morels do not form a monophyletic

Table 2.1. *Morchella* species found in North America. *Mel*- designates elata clade species (Richard et al., 2014).

Species	Clade	Occurrence
M. rufobrunnea	Rufobrunnea	Transcontinental
M. americana	Esculenta	Transcontinental
M. diminutiva	Esculenta	Eastern NA
M. sceptriformis	Esculenta	Eastern NA
M. ulmaria	Esculenta	Eastern NA
M. prava	Esculenta	Continental NA
M. angusticeps	Elata	Eastern NA
M. punctipes	Elata	Eastern NA
M. septentrionalis	Elata	Eastern NA
Mel-36	Elata	Eastern NA
M. brunnea	Elata	Western NA
M. populiphila	Elata	Western NA
*M. sextelata**	Elata	Western NA
M. snyderi	Elata	Western NA
*M. tomentosa**	Elata	Western NA
Mel-8	Elata	Western NA
Mel-18	Elata	Western NA
*M. eximia**	Elata	Transcontinental
*M. exuberans**	Elata	Transcontinental
M. importuna	Elata	Transcontinental
M. tridentina	Elata	Transcontinental
Mel-19	Elata	Transcontinental

* designates fire-associated species

group exclusive of non-fire-associated species). These post-fire fruiting events are massive, capable of producing an average of 2600 ascocarps per hectare in the first year after a wildfire (Winder & Keefer, 2008). Fire-associated morels are some of the first organisms to emerge after a wildfire, promoting soil stabilization, nutrient cycling, and possibly aiding in the establishment of new vegetation and tree seedlings (Greene et al., 2010; Hobbie et al., 2016; Larson et al., 2016; Salo et al., 2019; Winder & Keefer, 2008).

A conspicuous aspect of *Morchella* ascocarp production, often noted by collectors, is that production levels drop precipitously on burn sites after the first year. It would thus appear that burn morels are classic r-selected pioneer species that reproduce in large numbers after a disturbance and are then succeeded by other species. The rate at

which this successional transition occurs can tell us not only about the species involved but also about changes in the habitat itself. It is often assumed that the smaller fruitings of morels that occasionally appear in subsequent years after the first post-fire year represent additional reproduction of fire-associated species (Pilz et al., 2007). However, it is also possible that these subsequent fruitings belong to so-called "natural," or non-fire-associated, species. These two possibilities have different implications for understanding the pace of fungal secondary succession after wildfire.

Previous work by Pilz and colleagues (2004) characterized five putative species (PS A-E) in healthy, burned, and insect-damaged forests of northeastern Oregon, USA, three of which only fruited in the first spring following a wildfire (PS B, PS C, and PS D), and two of which fruited in non-burned forests (PS E) or in the second year following a wildfire (PS A, and PS E). Identification used a two-step molecular genetic approach: samples were first analyzed via RAPD-PCR, and representatives from each of the of the putative species groups based on RAPD profiling were further assessed via RFLP-ITS using four restriction enzymes. In a later study investigating the utility of ITS rDNA locus in identifying morels to species, 22.6% of the *Morchella* specimens needed additional loci for more accurate identification of species (Du et al., 2012).

Using a four-locus molecular phylogenetic analyses for species descriptions from Kuo et al. (2012) and Richard et al. (2014), we concluded that Pilz and colleagues (2004) putative species A corresponds to *M. brunnea*, *M. snyderi*, *M. angusticeps*, and/or *M. septrentrionalis* (natural black morels; this taxon fruited prolifically in non-burned, insect-killed forests and was the most abundant morel in their second-year wildfire sites. Putative species B and C correspond to *M. sextelata* and/or *M. eximia*, (fire-associated

morels) and were found in early season first-year wildfire sites. Putative species D corresponds to *M. tomentosa* (late fire-associated morel) and were found late in the fruiting season in the first year after a wildfire. Putative species E corresponded to *M. tridentina* (mountain blond morel) which were found in unburned forests or unburned stands within the perimeter of their fire sites.

Pilz and colleagues (2004) also reported that burn and nonburn morel species did not seem to fruit at the same time and suggested further research be conducted to determine if two crops of morels could be produced by staggering thinning and burning activities at least a year apart. Our own preliminary data suggest that non-fire-associated morel species are found as early as the second year post-fire (Osmundson, Burchard, Evans, & Garbelotto, unpublished), but the extent and generality of this second-year shift have not been established due to the previously limited amount of data available and limited collections from the unpublished data. In other words, ecological succession appears to occur on a short time scale.

Due to cryptic speciation as well as intraspecific morphological variability within the Elata clade, the identity of both first year and subsequent ascocarps has been uncertain. However, recent molecular phylogenetic studies have significantly advanced our understanding of *Morchella* taxonomy and diversity; these works provide the foundation necessary to address the question of successional patterns more rigorously than has previously been possible (Du et al., 2012; Kuo et al., 2012; O'Donnell et al., 2011; Richard et al., 2014).

This study is focused on the second year and later post-fire fruitings of *Morchella* species. We performed second year resampling of field sites (TABLE 2.2) that were

Table 2.2. *Morchella* specimens used in the analyses with loci successfully sequenced and included in the phylogenetic analysis. Nine specimens were collected in second-year post-wildfire sites in Montana, US in 2019 where fire-associated morels were found in the spring of 2018. Five additional specimens were obtained from second-year, fourth-year, and fifth-year post-wildfire sites in Montana, California, and Washington states (Osmundson et al., unpublished).

Specimen	Wildfire	Year of Fire	Year Collected	Included Loci
CC1	Cub Creek Fire, MT	2017	2019	ITS/LSU, TEF1, RPB1, RPB2
DC1	Deep Creek Fire, MT	2017	2019	RPB1, RPB2
DC2	Deep Creek Fire, MT	2017	2019	ITS/LSU, TEF1, RPB1, RPB2
DC3	Deep Creek Fire, MT	2017	2019	ITS/LSU, TEF1, RPB2
DC4	Deep Creek Fire, MT	2017	2019	TEF1, RPB1, RPB2
DC5	Deep Creek Fire, MT	2017	2019	TEF1, RPB1, RPB2
DC6	Deep Creek Fire, MT	2017	2019	TEF1, RPB1, RPB2
DC7	Deep Creek Fire, MT	2017	2019	RPB2
DC8	Deep Creek Fire, MT	2017	2019	RPB1, RPB2
Morel14a	Chippy Creek Fire, MT	2007	2009	TEF1, RPB1, RPB2
Morel14b	Chippy Creek Fire, MT	2007	2009	TEF1, RPB1, RPB2
Morel64	(unknown), CA	unknown	4th year	ITS/LSU, TEF1, RPB1, RPB2
Morel96	Rim Fire, CA	2017	2019	ITS/LSU, TEF1, RPB1, RPB2
Morel97	Table Mountain Fire, WA	2014	2019	ITS/LSU, TEF1, RPB1, RPB2

sampled in 2018 (for a study of first-year post-fire *Morchella*) to more rigorously establish whether a shift in species composition has occurred between the first and second year. Our analysis also included collections of second-year, fourth-year, and fifth-year post-fire *Morchella* specimens from our previous research and from other collectors (Osmundson et al., unpublished). We conducted a phylogenetic analysis using sequences of four loci previously used in studying *Morchella* systematics (O'Donnell et al., 2011) to identify the second-year and later collections and test the hypothesis that morels found on these sites will predominantly be non-fire-associated species. Our results suggest a rapid pace of succession and could provide greater insight into how different morel species function in, and/or respond to, changes in the post-fire environment over a relatively short period of time.

Methods

Site Selection and Specimen Collection

Emergence of second year ascocarps was assessed in June 2019 by resampling Montana field sites over a one-week period. Collections of the fire-associated species (presumed based on their occurrence in the first year in large numbers; Osmundson et al., unpublished) were made in May 2018 following wildfires that occurred in 2017. Criteria for original site selection included locations for establishing a gradient of geographical separation (>80 kilometers), size of the fire (>2000 hectares), and site accessibility. Resampling was conducted at the Cub Creek Fire and Deep Creek Fire near Thompson Falls, MT and the West Fork fire near Libby, MT.

Due to the severe decrease in *Morchella* occurrence after the first post-fire year, sampling was conducted with teams of 3-4 individuals using general (non-plot-based) collecting to cover as much ground area as possible, and all specimens identified as *Morchella* spp. using basic macromorphological characteristics were collected. Specimens were successfully acquired from two of the second-year fires: Cub Creek (n = 1) and Deep Creek (n = 8). We acquired additional second-year post-fire (n = 3), fourth-year post-fire (n = 1), and fifth-year post-fire (n = 1) specimens from our own collections and donations from other collectors (TABLE 2.2).

Sample Preparation

Fresh specimens were stored in silica desiccant to preserve DNA quality in addition to retaining dehydrated fungarium voucher specimens. Vouchers were deposited in the UWL fungarium collection; voucher collection information will be made publicly available through the Mycological Collections Portal (mycoportal.org). In addition, we

attempted to obtain ascospore collections (spore prints) to facilitate genetic analyses since ascocarp tissues can contain bacteria and other species that can complicate (though not prevent) genetic analyses. Immediately after returning from field sites, specimens were prepared for spore printing and dehydration. Individual ascocarps were cut in half, one half was placed within a wax paper pouch and dehydrated at approximately 45 °C for approximately 2 hours to facilitate release of ascospores onto the wax paper, and one half was placed on the dehydrator for 12 hours at 45 °C. Dried samples and spore prints were placed in individual plastic bags and labelled with date and collection site.

DNA Extraction and Sequencing

DNA was extracted from 14 dried collections using a rapid NaOH extraction based on several published sources (Osmundson et al., 2013). Using sterile technique, a 3 mm^3 piece of dried ascocarp tissue was removed from the hymenium layer, placed in a 2 ml reinforced screw cap tube with 4-6 2.7 mm glass beads (Bio-Spec Products, Bartlesville, OK, USA), and homogenized for 30 seconds in a BioSpec Mini Beadbeater homogenizer. A 200 µl volume of 0.5 M NaOH was added to the tube and vortexed until a slurry was formed. The sample was heated in a 95 °C heating block for 6 min, then mixed by vortexing. A 5 µl volume of slurry was added to 495 µl of 100 mM pH 8.0 Tris HCl and vortexed for 10 seconds. Final extracts were stored at -20 °C.

Morchella specimens were identified to species level using a phylogenetic analysis of the four loci previously established as useful for distinguishing species within the genus: ITS rDNA (internal transcribed spacer), and partial sequences of LSU rDNA (large ribosomal subunit), RPB1, RPB2 (RNA polymerase II, subunits 1 and 2), and TEF1 (translation elongation factor 1-α) (Du et al., 2012; O'Donnell et al., 2011).

Primers (TABLE 2.3) and PCR conditions followed O'Donnell et al. (2011). The PCR products were electrophoresed on a 1.2% low melting agarose gel. To remove primer dimers and any non-specific amplicons, the target band was excised from the gel and purified using the Axygen Biosciences Gel Extraction Kit (Axygen Inc., Union City, CA, USA) following the low melting point extraction protocol. The resulting products were quantified using the Qubit dsDNA HS (high sensitivity) Assay Kit (Invitrogen, Waltham, MA, USA), and any samples that failed to produce over 20 ng/µl of DNA were reamplified using the same PCR parameters. The PCR product cleanup reagent ExoSAP-IT (Applied Biosystems, US) was used to remove primers and unincorporated nucleotides using 0.5 µl reagent to 10 µl of sample. Samples were Sanger sequenced by Eurofins Genomics (Louisville, KY, USA) using sequencing primers from O'Donnell et al. (2011) (TABLE 2.3).

Table 2.3. Primers used for PCR amplification and sequencing (O'Donnell et al., 2011).

Loci	Primer	Sequence (5'-3') [a]	PCR	Sequencing
ITS/LSU				
	ITS5	GGAAGTAAAAGTCGTAACAAGG	X	X
	NL4	GGTCCGTGTTTCAAGACGG	X	X
	NL1	GCATATCAATAAGCGGAGG		X
RPB1				
	RPB-1A	GARTGYCCDGGDCAYTTGG	X	
	RPB-1C	CCNGCDATNTCRTTRTCCATRTA	X	
	RPB-A2	GTTAGATGAAGTGAGACACAC		X
	RPB-C2	GMAGAACMGTAATCACCATCC		X
RPB2				
	fRPB2-7cF	ATGGGYAARCAAGCYATGGG	X	
	RPB2-3053R	TGRATYTTRTCRTCSACCATRTG	X	
	RPB2-9F	CAAATGGGCRATTGTCATACG		X
	RPB2-3R	GCATYGGTATGCAGGTTGTGG		X
TEF1				
	EF-2F	AACATGATSACTGGTACYTCC	X	X
	EF-2218R	ATGACACCRACRGCRACRGTYTG	X	X

[a] IUPAC degenerate nucleotides: D: AGT; H: ACT; M: AC; N: ACGT; R: AG; S: CG; Y: CT.

Phylogenetic Analysis

Fourteen specimens were included in phylogenetic analyses (TABLE 2.2). Editing and alignment of sequences was performed with Geneious Prime 2019 v2019.2.1. The BLAST searches confirmed that all sequences aligned with existing *Morchella* sequences in GenBank. The combined data matrix included partitions for the loci TEF1 (1043 bp), ITS/LSU (597 bp), RPB1 (738 bp), and RPB2 (874 bp). Positions excluded from the analysis due to alignment ambiguity were 54-96, 358-407, and 1009-1060 within the TEF1 locus. Sequences from O'Donnell et al. (2011) were included as references for identification, and *Morchella rufobrunnea* (Rufobrunnea clade) was used as an outgroup to root the phylogeny.

A maximum likelihood phylogenetic analysis was performed with RAxML (Randomized Accelerated Maximum Likelihood) version 8 (Stamatakis, 2014) via the CIPRES Science Gateway (Miller et al., 2010). The maximum likelihood tree was determined using 20 distinct starting trees and a GTRCAT substitution model partitioned by locus. Branch support was determined using 1000 multi-parametric bootstrap replicates. A random seed value of 12345 was used for both beginning tree searches and bootstrapping. The final tree figure was prepared using FigTree v1.4.4 and Inkscape 0.92.4. All sequences obtained will be deposited in GenBank, all sequence alignments in TreeBASE, and accession numbers will be cited in any subsequent publications.

Results

In sharp contrast to the extensive *Morchella* fruitings observed in 2018, fruiting was extremely sparse in 2019 with a total of nine ascocarps observed from only two of the sites in the second year after a wildfire (TABLE 2.2). Although one collection

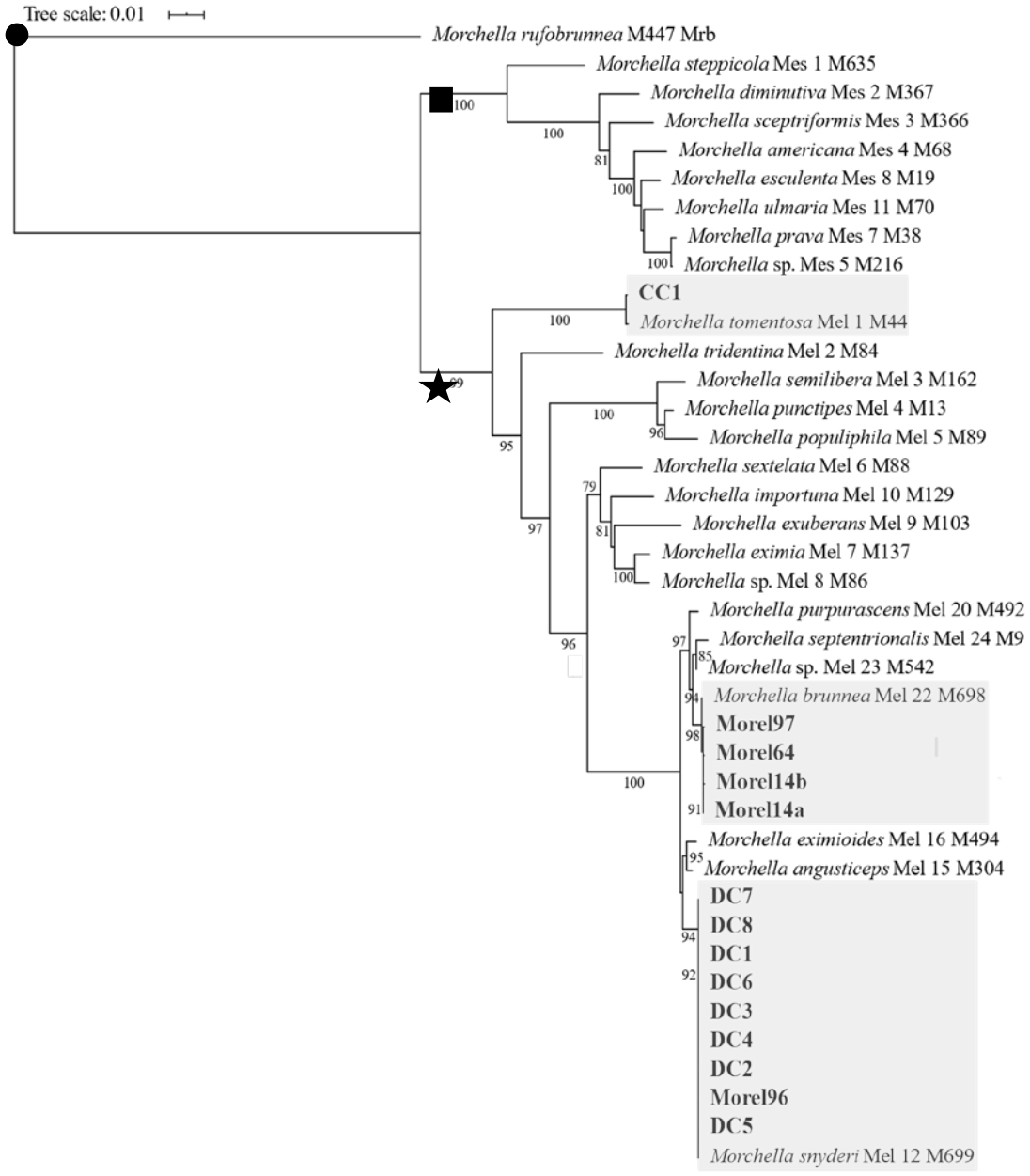

Figure 2.1. Maximum likelihood phylogeny of second- through fifth-year post-fire morels and reference taxa using ITS/LSU, TEF1, RPB1, and/or RPB2 sequence data (see TABLE 2). Numbers adjacent to branches indicate bootstrap branch support (values above 70% shown). Branch lengths represent the mean number of nucleotide substitutions per site on that branch. The circle indicates the Rufobrunnea clade, the square indicates the Esculenta clade, and the star indicates the Elata clade. Shaded clades represent the species identified in the phylogenetic analysis with the specimens analyzed in bold text.

belongs to the fire-associated species *M. tomentosa*, the rest of the second-year collections belong to the non-fire-associated species *M. brunnea* and *M. snyderi* (TABLE 2.2, FIG. 2.1). All of these placements are strongly supported, with bootstrap support values ranging from 94-100%. Although both of the older collections (4th-year and 5th-year post-fire) belong to *M. brunnea* and all of the 2019 collected 2nd-year specimens belong to *M. snyderi*, there is not a clear distinction between the species in terms of occurrence, as two 2nd-year specimens collected in 2009 also belong to *M. brunnea*. There is also no clear difference in geographical distribution, as both species include specimens from Montana and California.

Discussion

Wildfires have increased in frequency and intensity in the past century, primarily in western North America and are predicted to increase in the coming years (Parks et al., 2016; Pausas & Keeley, 2009; Riley & Loehman, 2016). Understanding how these habitats respond to and recover from fire is increasingly important, and studies of secondary succession play an important role in gaining that understanding (Barker et al., 2013; Hewitt et al., 2017; Mediavilla et al., 2014; Segarra-Moragues & Ojeda, 2010).

Fire-associated morels, as well as a number of other apothecium-producing ascomycetes and agarics (gilled fungi), undergo rapid colonization and mass fruiting on burned soils in the year after a fire (Duchesne & Weber, 1993; Fujimura et al., 2005; Greene et al., 2010; Larson et al., 2016; Pilz et al., 2004; Salo et al., 2019). Whereas sporocarps of some species, such as the Discomycete *Geopyxis carbonaria*, can continue to occur at high levels in at least 1-2 subsequent years (personal observation), fruiting of *Morchella* drops to very low levels in the second-year post-fire. When *Morchella* do fruit

on these sites, their occurrence may represent either a “last ditch” effort at reproduction by fire-associated species or may signal the presence of non-fire-associated species; these two possibilities suggest different scenarios regarding the pace of changes in the soil.

Based on sampling in three Oregon forests over two field seasons and identification using less precise molecular methods (n = 200; RAPD, PCR-RFLP), Pilz et al. (2004) demonstrated that morels emerging in second-year and later post-wildfire sites were predominantly non-fire associated species and that fire and non-fire associated species do not seem to fruit in the same areas at the same time. Osmundson et al. (unpublished) found similar results, though these observations were based on a limited sample size (n = 5). The purposes of our study were to further examine the generality of these finding by expanding the geographic scope of sampling, and to place specimens from second-year and later burned habitats into the current phylogenetic framework for the genus based on a 4-locus sequence dataset.

The evidence from this study demonstrated that succession of morels after wildfires can occur on a short timeframe, with a species shift from fire-associated to predominantly non-fire-associated morel species in the second year after a wildfire (FIG 2.1). Our results suggest that two taxa predominate, *Morchella snyderi* and *M. brunnea*; based on our inferences, these are the same taxa (PS A, morels fruiting on all soil types the second year following a fire) identified by Pilz and colleagues (2004). We did not observe a temporal or geographical distinction in the occurrence of the two species. However, we also did not in any cases observe both species at the same site at the same time. Similarly, in the one case where we observed documented fire-associated species (*M. tomentosa*) in a second-year post-wildfire site, none of the other *Morchella* species

were observed. These data suggest that the two species may respond differently to other fire-associated fungi, to one another, or differences in soil chemistry or other aspects of habitat.

Based on our results and those of Pilz et al. (2004), it might be assumed that drastic changes in soil and flora present in the first year after a wildfire stimulate the fruiting of burn morels, but not naturals. Research has not thus far documented natural morels fruiting in the first year after a wildfire. According to Kuo et al. (2012), *Morchella snyderi* fruits under non-burned, montane conifers from April-June, while *M. brunnea* appears under hardwoods and is believed to fruit in non-burned conifer forests in the early spring. Pilz et al. (2004; 2007) described "natural black morels" (PS A) as common on non-burned soils, or when found on burned soils, fruiting no sooner than the second spring after an intense wildfire; this was the most abundant putative species on their non-burned and second-year post-fire plots.

Both Kuo et al. (2012) and Pilz et al. (2004;2007) note that second-year and later post-fire fruitings are drastically decreased compared to first year surveys, despite the dominance of "natural blacks" within these habitats. Carris et al. (2015) concluded that both mitosporic (anamorphic) and ascocarp stages of *M. snyderi* were present in habitats that were selectively logged in the year prior and that this was the most common black morel found in their study sites. Larson et al. (2016) concluded that both *M. snyderi* and *M. brunnea* are common in the forests of western North America, but their relationship to fire, if any, is not clear. Identifying *M. brunnea* in second-, fourth-, and fifth-year post-fire habitats supports previous assumptions that this species, along with *M. snyderi*, both fruit in second-year and later wildfire disturbed habitats.

Based on limited data on the nutritional requirements of these "natural black" morel species, it might be assumed that the drastic changes in post-wildfire habitats (i.e. soil chemistry, flora, etc.) promotes the fruiting of these species. It is interesting to note that the sampling location of the *M. tomentosa* collection was the only one of our sites where salvage logging had been conducted in the year following the fire (TABLE 2.2); this finding could suggest that prolonged disturbance may prolong the fruiting activity of fire-associated species, though it is also important to note that only a single collection of *M. tomentosa* was made on the site despite extensive logging activity there.

Both previous data and those from the present study are limited in sample size and geographic and temporal scope, and therefore establishing conclusions on the occurrence or ecology of these species with high confidence is difficult. However, the results suggest a hypothesis that can be tested further using occurrence data and more detailed ecological data: that significant changes in the post-fire soil environment (e.g., pH, nitrogen, carbon) occur on short time scales and that different *Morchella* species respond differently to these changes. In order to create a more complete study of morel succession after wildfires, increasing the number of second year and later wildfire sites across western North America, as well as European and Asian wildfire sites, would provide more species and habitat types for comparison. Resurveying post-wildfire sites from the present study could provide further evidence that natural morels continue to fruit in the years after a wildfire, as well as provide evidence of temporal fruiting habits for *M. snyderi* and *M. brunnea* and other species that might fruit later in the season.

A second – and more difficult to answer – set of questions arises from these results: *how* do the different *Morchella* species respond to the post-fire environment?

Fungi are notoriously difficult to study in their natural settings due to their unpredictable fruiting behaviors and predominantly vegetative mycelial form, spore bank, and/or production of dormant structures (i.e. pseudosclerotia found in *Morchella* spp.). Modifying how and when field sites are surveyed by implementing soil and root tip sampling would aid in answering questions surrounding morel mycelial interactions (presence of both burn and natural morel mycelia), a morel spore bank, presence and persistence of sclerotia, and their nutritional modes both prior to and after a fire disturbance.

Because the mycelium is likely to be active prior to fruiting, and because sporocarp production may be triggered to occur near the end of the individual's life cycle, it is difficult to infer from sporocarp occurrence whether fire-associated and non-fire associated taxa coexist in the soil either prior to or after fruiting (i.e. their mycelium may start growing at the same time but fruit at different times, or they may start growing at different times relying on their spore bank, fruit at different times, but remain active together in the soil). Understanding the nutritional requirements and environmental responses of *Morchella* species has implications not only for better understanding fire ecology but also for developing improved strategies for cultivating these highly sought-after edible fungi.

CHAPTER III

POPULATION LEVEL DIVERSITY AND REPRODUCTION OF FIRE-ASSOCIATED *MORCHELLA*

Introduction

In northwestern North America, morel mushrooms (genus *Morchella*) fruit prolifically in the first year after a wildfire in montane coniferous forests, with studies reporting over 8000 fruiting bodies emerging in a single hectare (Winder & Keefer, 2008). These massive fruitings promote a multimillion-dollar industry surrounding their collection and sale, with over 300,000 pounds of dried morels (3 million pounds fresh) traded annually across the globe, generating over $18 million USD in international commerce (Iqbal, 1993; Pilz et al., 2007). Though all *Morchella* species are considered edible, the commercial industry largely depends upon fruitings of fire-associated morels, consisting of four species of black morels (Elata clade) that form ascomata only in the year after a forest fire: *Morchella tomentosa*, *M. sextelata*, *M. eximia*, and *M. exuberans* (FIG. 3.1; Kuo et al., 2012; O'Donnell et al., 2011; Richard et al., 2014).

Despite the economic importance of these mass fruiting events, many questions still remain about the extent to which mass ascospore production promotes population level genetic diversity, facilitates dispersal, and contributes to gene flow (Chai et al., 2017; Du et al., 2016; W. Liu et al, 2018; Pilz et al., 2004; Winder & Keefer, 2008). Billions of ascospores released during the massive fruiting events provide multiple opportunities for sexual reproduction to occur, increasing genetic diversity. Ascospores

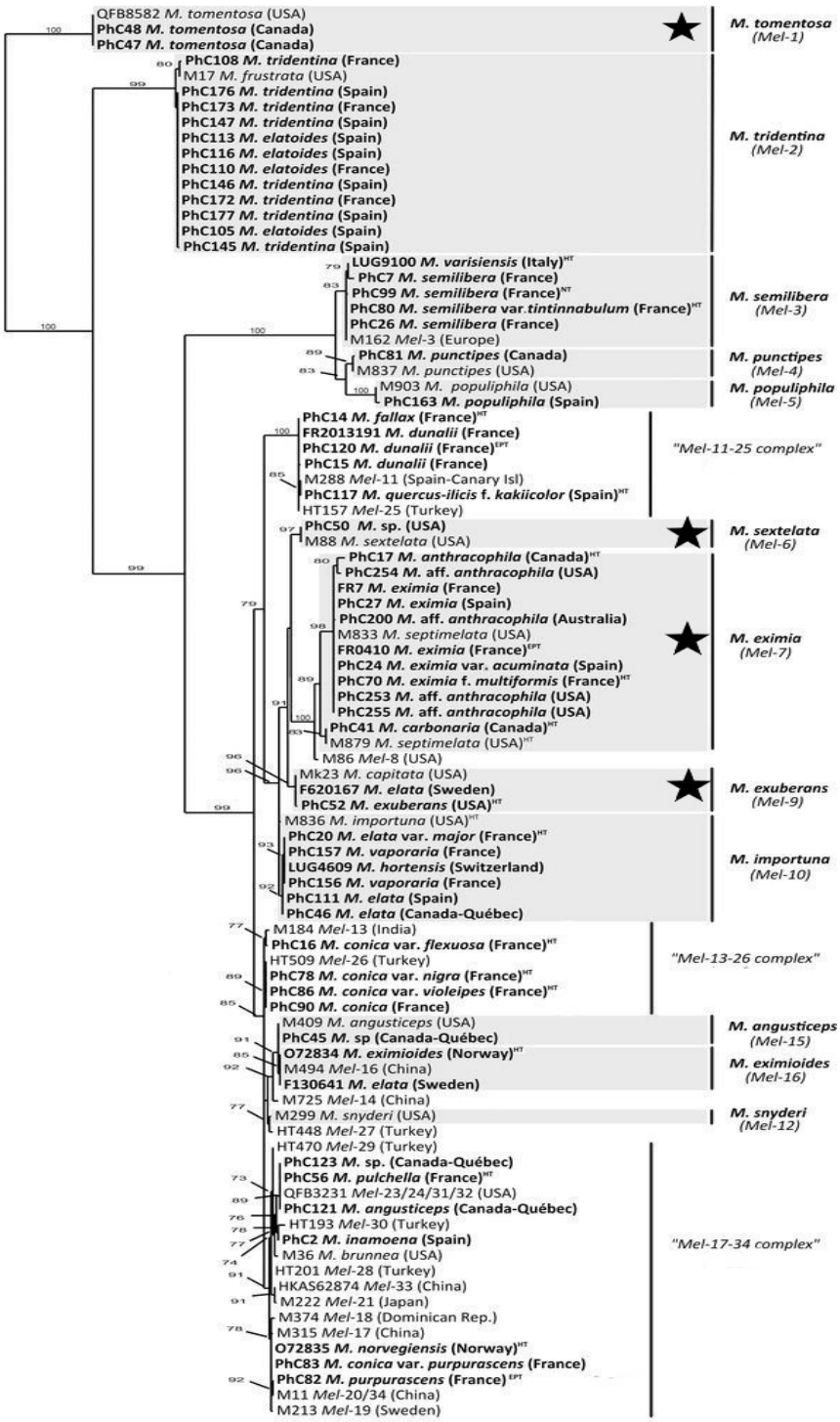

Figure 3.1. Phylogeny of *Mel*-1 – *Mel*-34 (*Mel*- designates Elata clade species) demonstrating convergent evolution of fire adaption in *Morchella* spp. (fire-associated species designated with stars). *M. tomentosa* (*Mel*-1) diverged prior to the other known fire-associated *Morchella* spp. (*Morchella sextelata* (*Mel*-6), *M. eximia* (*Mel*-7), and *M. exuberans* (*Mel*-9)). Numbers by nodes represent branch support above 70%, as assessed by the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) (Modified from Richard et al., 2014).

may facilitate dispersal, migration, and gene flow over significant distances, or may drop mostly locally, limiting these effects. However, vegetative spread of mycelia between fire events would likely limit diversity through resource competition and exclusion of some individuals.

Key to these questions is the behavior of the secondary mycelium in the life cycle of these species between fire events (FIG. 3.2). Due to the cryptic nature of the vegetative mycelium, ephemeral fruiting habits, and tendency to fruit in remote fire sites that can be extremely difficult to access, studying the reproduction methods of these fungi in nature has proven to be difficult. However, mating and culture studies have revealed many aspects of the life cycles in *Morchella* species that can guide the understanding of these species in natural habitats. The morel life cycle has been studied across taxa with varying conclusions on pseudosclerotium form and function (Stefani et al., 2010), conidia production (Q. Liu et al., 2018), and whether fruiting bodies are produced from secondary and/or primary mycelia (Volk & Leonard, 1990).

The fruiting bodies produce sexual ascospores, derived from the meiosis of diploid nuclei and a subsequent mitotic division to produce eight ascospores per ascus, with sister spores forming a tetrad (Volk & Leonard, 1989). Ascospores germinate to form a primary mycelium, and the plasmogamy of two primary mycelia form a heterokaryon, or secondary mycelium, that can undergo indeterminate growth. Under laboratory conditions, the secondary mycelium forms pseudosclerotia, tightly woven, underground hyphal masses believed to be dormant structures to survive overwintering and harsh conditions. Ower (1982) proposed that the primary mycelia formed from the germination of the haploid ascospore can also produce pseudosclerotia and possibly

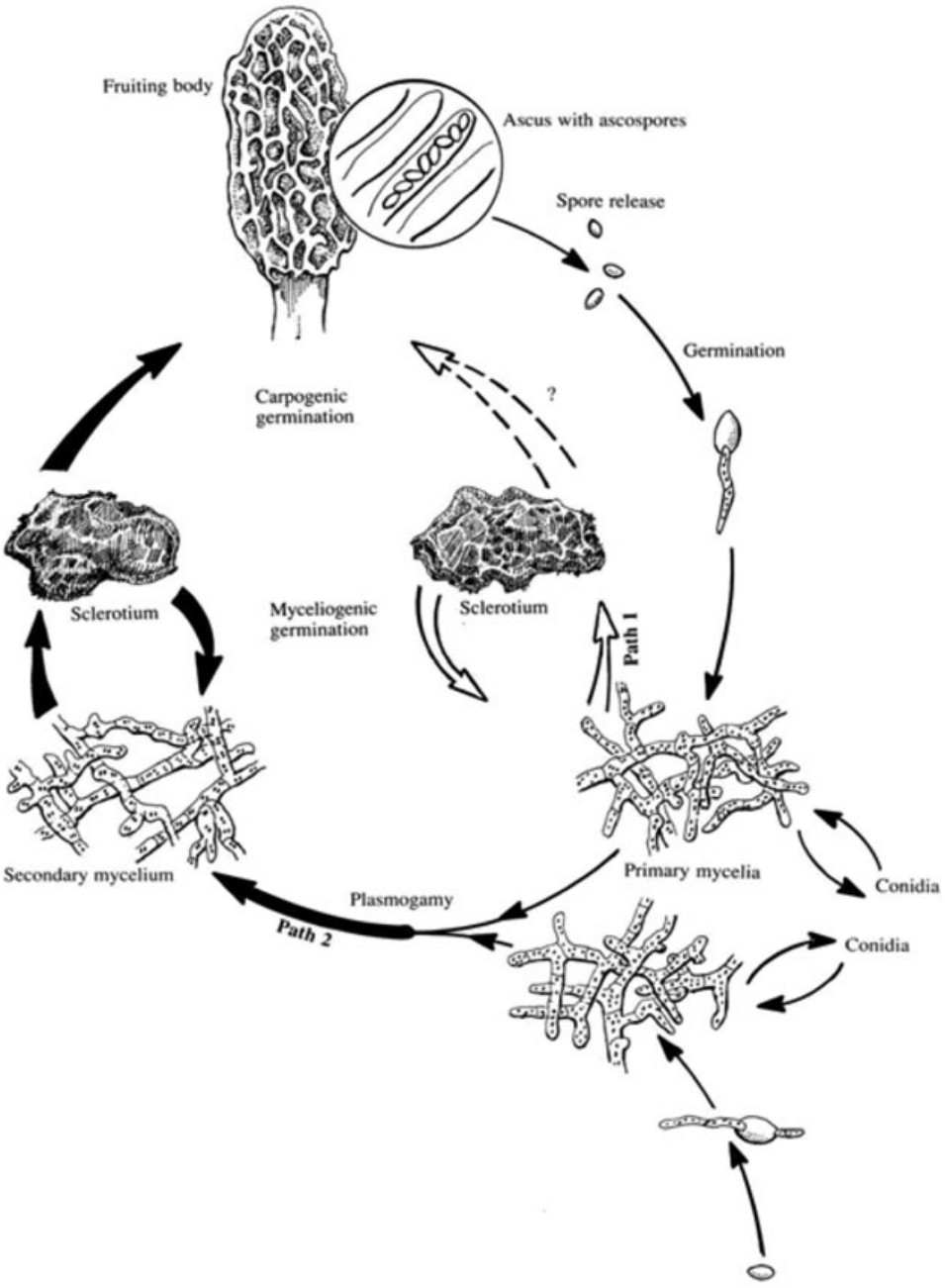

Figure 3.2. Life cycle of *Morchella* spp. (Volk & Leonard, 1990; used with permission of Dr. Thomas Volk).

fruiting bodies. Although it has been proposed that pseudosclerotia serve as necessary precursors to ascocarp formation and/or storage structures through carpogenic germination (Volk & Leonard, 1990), their production and importance in nature is not well understood. Conidial formation from primary mycelia has also been observed in

cultivation studies, but the conidia do not germinate (Q. Liu et al., 2018; Ower, 1982). The function of conidia in the natural life cycle of *Morchella* is currently unknown.

The duration of the spread of the vegetative mycelia (whether primary or secondary) in post-fire morels is also poorly understood. Long periods of spread may allow competition and exclusion of some individuals, leaving fewer, larger individuals. For example, some species in the basidiomycete genus *Armillaria* exhibit the ability to extend a single mycelium across very large areas, covering over 15 hectares (Smith et al., 1992). If burn morel ascomata in a site follow vegetative growth patterns seen in fungi such as *Armillaria*, population genetic diversity may be quite limited (e.g., multiple fruiting bodies from the same parental mycelium). In this case, the energy expenditure of producing thousands of ascomata functions more in increasing the chance of repopulating the habitat and/or promoting spatial dispersal rather than promoting genetic diversity. Alternatively, shorter periods of spread, whether intrinsic to these species or caused by temporal physical changes to the post-wildfire environment, could result in populations composed of many diverse individuals. In short, the vegetative spread of mycelia could impact population level diversity as much as, or even more than, ascospore production.

Population genetics approaches can be used to examine the life cycle of fire-associated *Morchella* by examining the genetic structure of populations from the individual to the landscape scale. Population genetics studies have investigated genetic variation, spatial extent of mycelia, and the role of sexual and asexual reproduction in several macrofungi (Bergemann & Miller, 2002; Branco et al., 2015; Dahlberg & Stenlid, 1995). Dahlberg and Stenlid (1995) suggest that sexual reproduction by ascospores produces many small heterokaryotic individuals that undergo vegetative expansion, and

competition between individuals results in a smaller number of larger individuals over time; however, the size of the individual mycelia can vary based on species, resource availability, and importance of frequent ascospore establishment vs. vegetative spread.

Using whole genome analyses, Branco et al. (2015) detected population structure and low levels of migration in the ectomycorrhizal basidiomycete, *Suillus brevipes*. They concluded that despite the prolific production of basidiospores (on the order of trillions of basidiospores per km^2), population structure shows evidence of limitations in basidiospore dispersal and/or colonization. These limitations could be due to physical barriers to the movement of basidiospores or inhibition of basidiospore germination and mycelium persistence in new habitats. A study of *Russula brevipes*, another ectomycorrhizal basidiomycete, found evidence for temporal and spatial persistence of genets over time, with variations in genet size from 3-18 m (Bergemann & Miller, 2002). These results supported the idea proposed by Dahlberg and Stenlid (1995) that the presence of small genets aligns with recent colonization by basidiospores, that local populations were reproducing by sexual production by basidiospores, and larger genets could colonize by mycelial expansion. Both studies demonstrate how geographic and temporal barriers to reproduction and the dynamics of vegetative mycelial spread and spore dispersal shape population structure in ectomycorrhizal basidiomycetes.

Morchella species vary significantly in their ecology (including both mycorrhizal and saprotrophic species as well as possible shifts between modes), life cycles, and fruiting behavior (Chai et al., 2017; Dahlstrom et al., 2000; Du et al., 2016; Hobbie et al., 2016; W. Liu et al., 2018). Multiple species within the Elata clade are heterothallic (i.e. require different mating types for plasmogamy to occur; Chai et al., 2017; W. Liu et al.,

2018), though high rates of inbreeding and evidence of clonality have been observed in some wild morel populations (Du et al., 2016). In contrast, studies of "natural" (non-fire-associated) species in the Elata clades found no heterozygosity in *M. eohespera* fruiting bodies, suggesting that the fruitings were derived from haploid primary mycelia, showing a pattern of variability consistent with clonal propagation (Du et al., 2016). The study of Elata clade species *Mel*-13 and *M. eohespera* by Du and colleagues revealed significant genetic differentiation between sites but no clear relationship between genetic and geographical distance, suggesting that intrinsic factors (e.g. inbreeding and clonality) play a more significant role than extrinsic factors (e.g. geographic barriers to reproduction) in shaping genetic structure of populations. Because of such variation within the genus, it is difficult to predict from these previous results how, and how often, plasmogamy is occurring in burn morels and how massive ascospore production in the first-year post-fire fruitings affects diversity of populations and their life cycles in nature.

How do the mass fruitings in the first year after a fire and the drastic decrease in fruiting numbers in the subsequent years relate to burn morel reproduction in nature? Are the massive amounts of ascospores contributing to increased sexual reproduction, and therefore increased genetic variability in the vegetative mycelium and fruiting bodies? Or are ascospores primarily repopulating the same habitats from a limited number of individuals, starting a new cycle of vegetative growth until the next fire moves through the habitat? Are ascospores contributing to gene flow, creating massive populations of fire-associated morels across the western mountain ranges and/or recolonizing on a more local or regional scale?

These interesting questions are what spurred this research, which uses analyses of

genome-wide SNP (single nucleotide polymorphism) data to determine the spatial extent of vegetative mycelia, distinguish genetic individuals, assess relatedness between individuals, and characterize population genetic structure in order to draw inferences about the role of mass fruitings, vegetative expansion, and dormancy (e.g. as pseudosclerotia or spore banks) in the biology of fire-associated morels.

Surveys of two fire-associated morel species (*M. eximia* and *M. sextelata*) in four sites burned by wildfires in the previous year (three in southern British Columbia, Canada and one in northwestern Montana, USA) were used to assess two alternative hypotheses. The first is that low genetic variation would be observed within sampling sites, with high variation between sites. These observations would be consistent with the occurrence of long periods of mycelial survival and vegetative spread between fire events, producing few, large individuals. The second hypothesis is that high variation would be observed within sites and low variation between sites. These observations would be consistent with limited mycelial spread, producing large numbers of individuals that delay fruiting for long periods of time between fire events. In both cases, whether mycelial spread involves the primary or secondary mycelia could be assessed by measuring individual heterozygosity. Our results indicate that ascospore production plays an important role in the biology of these species from local to landscape scales and that burn morel species may differ in terms of their level of gene flow between distant sites.

Methods

Site Selection and Specimen Collection

Montana, USA and southern British Columbia, Canada were selected as the best locations for sampling due to an existing collaborator network to facilitate access to field

sites. Criteria for site selection included establishing a gradient of geographical separation (>15 km), size of the fire (>600 hectares), and site accessibility. Sampling of the fire-associated morel species *M. eximia* and *M. sextelata* was conducted on the Gottfriedsen Mountain Creek Fire (642 hectares, 1 plot, n = 6), Blazed Creek Fire (6798 hectares, 4 plots, n = 14), and Randal Creek Fire (1181 hectares, 2 plots, n = 11) in British Columbia, Canada, and the Gold Hill Fire in Montana, USA (2711 hectares, 1 plot, n = 6) in late May/early June of 2019 (TABLE 3.1, FIG. 3.3, FIG. 3.4). Characteristics of the fires varied in terms of trees present prior to or after the fire (tree species could not be distinguished in the high intensity Gottfriedsen Mountain Creek fire site), aspect, fire severity, and evidence of prior collecting activity (TABLE 3.2).

Sampling was conducted with teams of 3-5 individuals and all specimens were identified as *Morchella* using basic macromorphological characteristics (*M. eximia* and *M. sextelata* cannot be reliably distinguished from one another using these characteristics). Transects were implemented in order to determine the spatial extent of individual mycelia and to ensure future collections could be made in the exact locations to aid in continued research (FIG. 3.5). Transects were 50 meters in length, with the starting point set at a fruiting body. Samples were collected along the transect within 2 meters perpendicular to the transect line on either side. Distances of each sample along the transect line and perpendicular to it were noted. Global Positioning System (GPS) points were recorded for the beginning and end of each transect and for each collection made outside of a transect.

Table 3.1. *Morchella sextelata* (n = 24) and *M. eximia* (n = 13) specimens collected in the spring of 2019 at four first-year post-wildfire sites in southern British Columbia, Canada, and northwestern Montana, USA.

Wildfire	Plot	Specimen	Species
Blazed Creek Fire, BC, CAN	1	BC2^	*M. eximia*
	1	BC3*	*M. sextelata*
	1	BC4^	*M. sextelata*
	1	BC7	*M. sextelata*
	2	BC8	*M. sextelata*
	2	BC9^	*M. sextelata*
	2	BC10	*M. eximia*
	2	BC11	*M. sextelata*
	3	BC12	*M. sextelata*
	3	BC13^	*M. sextelata*
	3	BC14^	*M. eximia*
	4	BC15^	*M. eximia*
	4	BC16*	*M. sextelata*
	4	BC17*	*M. sextelata*
	4	BC18*	*M. sextelata*
	4	BC19*^	*M. sextelata*
	4	BC22^	*M. sextelata*
	4	BC26^	*M. sextelata*
	4	BC29^	*M. sextelata*
Gottfriedsen Mountain Creek Fire, BC, CAN	1	BL1^	*M. sextelata*
	1	BL6^	*M. eximia*
	1	BL9^	*M. sextelata*
	1	BL15	*M. eximia*
	1	BL25^	*M. sextelata*
	1	BL34^	*M. eximia*
Gold Hill Fire, MT, USA	1	GH1^	*M. sextelata*
	1	GH3^	*M. eximia*
	1	GH6	*M. sextelata*
	1	GH9	*M. sextelata*
	1	GH12^	*M. eximia*
	1	GH14^	*M. eximia*
Randal Creek Fire, BC, CAN	1	Y1	*M. sextelata*
	1	Y3*^	*M. sextelata*
	1	Y6^	*M. sextelata*
	1	Y8*	*M. eximia*
	2	Y10	*M. sextelata*
	2	Y11	*M. sextelata*
	2	Y12	*M. eximia*
	2	Y13	*M. eximia*
	2	Y14^	*M. sextelata*
	2	Y15^	*M. eximia*
	2	Y16	*M. sextelata*
	2	Y17^	*M. sextelata*
	2	Y18	*M. sextelata*

*specimens not included in final analyses, ^TEF1 sequence confirmed

Table 3.2. Wildfire sites surveyed in late May/early June 2019 in southern British Columbia, Canada and northwestern Montana, USA. Site details include trees present, aspect, fire severity (L = low, M = moderate, H = high), and whether the site showed signs of previous morel collecting activity (e.g., cut mushroom "stumps") prior to our surveys.

Fire	Trees	Aspect	Severity	Prior collection
Blazed Creek	Spruce, Fir, Hemlock, Cedar	South	L/M	Yes
Gottfriedsen Mountain Creek	unknown	South	H	No
Gold Hill	Lodgepole	West	M	Yes
Randal Creek	Lodgepole, Larch	North	M	Yes

Figure 3.3. Map of four 2018 wildfire sites in southern British Columbia, Canada and northwestern Montana, USA that were sampled in 2019: Gottfriedsen Mountain Creek Fire, Blazed Creek Fire, Randal Creek Fire, and Gold Hill Fire. The two most distant fires were separated by approximately 360 kilometers (Google Earth, v. 9.3.110.3; 460 km x 220 km).

Sample Preparation

Tissue samples from fresh specimens were stored in silica desiccant to preserve SDNA quality, and the remainders were dehydrated to serve as fungarium voucher specimens. Vouchers were deposited in the UWL fungarium collection; voucher collection information will be made publicly available through the Mycological Collections Portal (mycoportal.org). In addition, ascospore collections (spore prints) were

obtained to facilitate genetic analyses since ascocarp tissues can contain bacteria and other species that can complicate (though not prevent) genomic analyses.

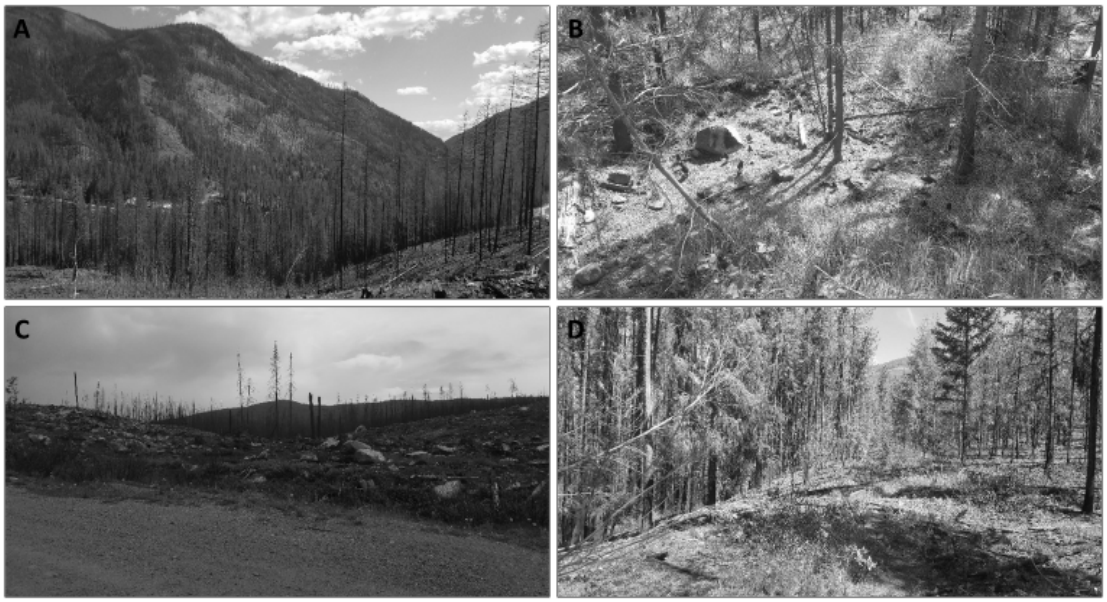

Figure 3.4. Pictures of the four wildfire sites surveyed: A) Blazed Creek Fire, British Columbia, Canada, B) Randal Creek Fire, British Columbia, Canada, C) Gottfriedsen Mountain Creek Fire, British Columbia, Canada, and D) Gold Hill Fire, Montana, USA.

Figure 3.5. Gold Hill Fire transect. *M. sextelata* samples include GH1, GH6, and GH9. *M. eximia* samples include GH3, CH12, and GH14. Distance from GH1 to GH14 is 50 m.

Specimens were prepared for spore printing and dehydration immediately after returning from the field sites. Individual ascocarps were cut in half; one half was placed within a wax paper pouch and dehydrated at 45 °C for approximately 2 hours to facilitate release of ascospores onto the wax paper, and one half was placed on the dehydrator for approximately 12 hours at 45 °C to dehydrate it completely. Dried samples and spore prints were placed in individual plastic bags and labelled with date and collection site.

DNA Extraction, Sample Screening and Selection for Whole-Genome Sequencing

Final species-level identifications were based on molecular evidence. DNA was extracted from 44 dried collections using a cetyl trimethyl ammonium bromide (CTAB)/phenol chloroform extraction followed by column purification using the GENECLEAN purification kit (MP Biomedicals, Irvine, CA, USA) following Ivors et al. (2004). Using sterile technique, a 3 mm^3 piece of ascocarp tissue was removed from the hymenium layer, placed in a 2 ml reinforced screw cap tube with 4-6 2.7 mm glass beads (Bio-Spec Products, Bartlesville, OK, USA), and homogenized for 30 seconds at 5000 RPM in a BioSpec Mini Beadbeater homogenizer. Pulverized tissue was incubated in 300 µl CTAB buffer at -80 °C for 8 minutes, then moved to a 75 °C heating block for 2 minutes. This freeze-thaw cycle was repeated two more times with a final incubation at 75 °C for 20 minutes. A 350 µl volume of 25:24:1 phenol:chloroform:isoamyl alcohol was added and samples were vortexed for 1 minute to create an emulsion. Samples were centrifuged at 13,300 g for 15 minutes, and 200 µl of the aqueous phase was recovered. DNA was purified from the aqueous phase using the GENECLEAN kit following the manufacturer's instructions. Samples were eluted with 40 µl of elution buffer and extracts were stored at -20 °C.

Morchella specimens were identified to species level using a phylogenetic analysis of TEF1 (translation elongation factor 1-α) gene sequences. Primers and PCR conditions followed O'Donnell et al. (2011). The PCR products were electrophoresed on a 1.2% low melting agarose gel. To remove primer dimers and any non-specific amplicons, the TEF1 target band was excised from the gel and purified using the Axygen Biosciences Gel Extraction Kit (Axygen Inc., Union City, CA, USA) following the low melting point extraction protocol. The resulting products were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA), and any samples yielding less than 20 ng/μl of DNA were reamplified using the same PCR parameters. For samples not requiring gel purification, ExoSAP-IT (Applied Biosystems, US) was used to remove primers and unincorporated nucleotides using 0.5 μl reagent to 10 μl of sample. Samples were Sanger sequenced by Eurofins Genomics (Louisville, KY, USA) in a single direction using the sequencing primer EF-2F (AACATGATSACTGGTACY TCC; O'Donnell et al., 2011).

Geneious Prime 2019 v2019.2.1 was used to confirm species identification of 24 successfully sequenced specimens via maximum likelihood phylogenetic analysis using RAxML v8.2.11 with sequences from O'Donnell et al. (2011) as reference sequences (TABLE 3.1). The multiple sequence alignment from this analysis was also examined in Geneious Prime to design a PCR-RFLP assay for additional screening of the 20 specimens that originally failed to sequence. An in silico digest with the MseI enzyme was determined to distinguish the two species based on the sequenced specimens. Reactions were prepared consisting of 0.4 μl *MseI* restriction enzyme (New England Biolabs Inc., Ipswich, MA, USA), 2 μl 10x buffer, 7 μl DNA (40 ng) and 10.6 μl sterile

water to reach a total volume of 20 µl per sample. The samples were incubated at 37 °C for 1 hour, then the temperature was increased to 65 °C for 20 minutes. The RFLP products were electrophoresed on 1.2% low melting agarose gel at 70 V for 75 minutes using 4 TEF1 sequence confirmed specimens as controls.

Dual indexed sequencing libraries for the 44 samples were prepared using the NEBNext Ultra II Library preparation kit (New England Biolabs Inc., Ipswich, MA, USA). The resulting products were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA), pooled with equimolar concentration per sample, and size selected to a 400 bp (base pair) mean insert size using AMPure Beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Paired-end 150 bp sequences were generated on one lane of an Illumina NovaSeq 6000 SP flow cell. Size selection and sequencing were performed by the University of California, Berkeley Vincent J. Coates Genomic Sequencing Laboratory.

De Novo Assembly of Reference Genomes

Reference genome assembly and read mapping used a multi-step pipeline (FIG. 3.6). Illumina sequencing produced over 400 million raw reads across 43 of the 44 samples. FastQC v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used for an initial assessment of the quality of the raw data. Fqtrim v0.9.7 (https://ccb.jhu.edu/software/fqtrim/) was used for filtering and 3’ quality trimming of reads, with a Phred quality score cut-off value of 15, minimum post-trimming length of 60 bp, and maximum of 50% low complexity sequence content. Adapter trimming was performed using Trimmomatic v.1.2.14 (Bolger et al., 2014). A final quality check of the filtered and trimmed reads was performed using FastQC v0.11.5. A total of 37 specimens

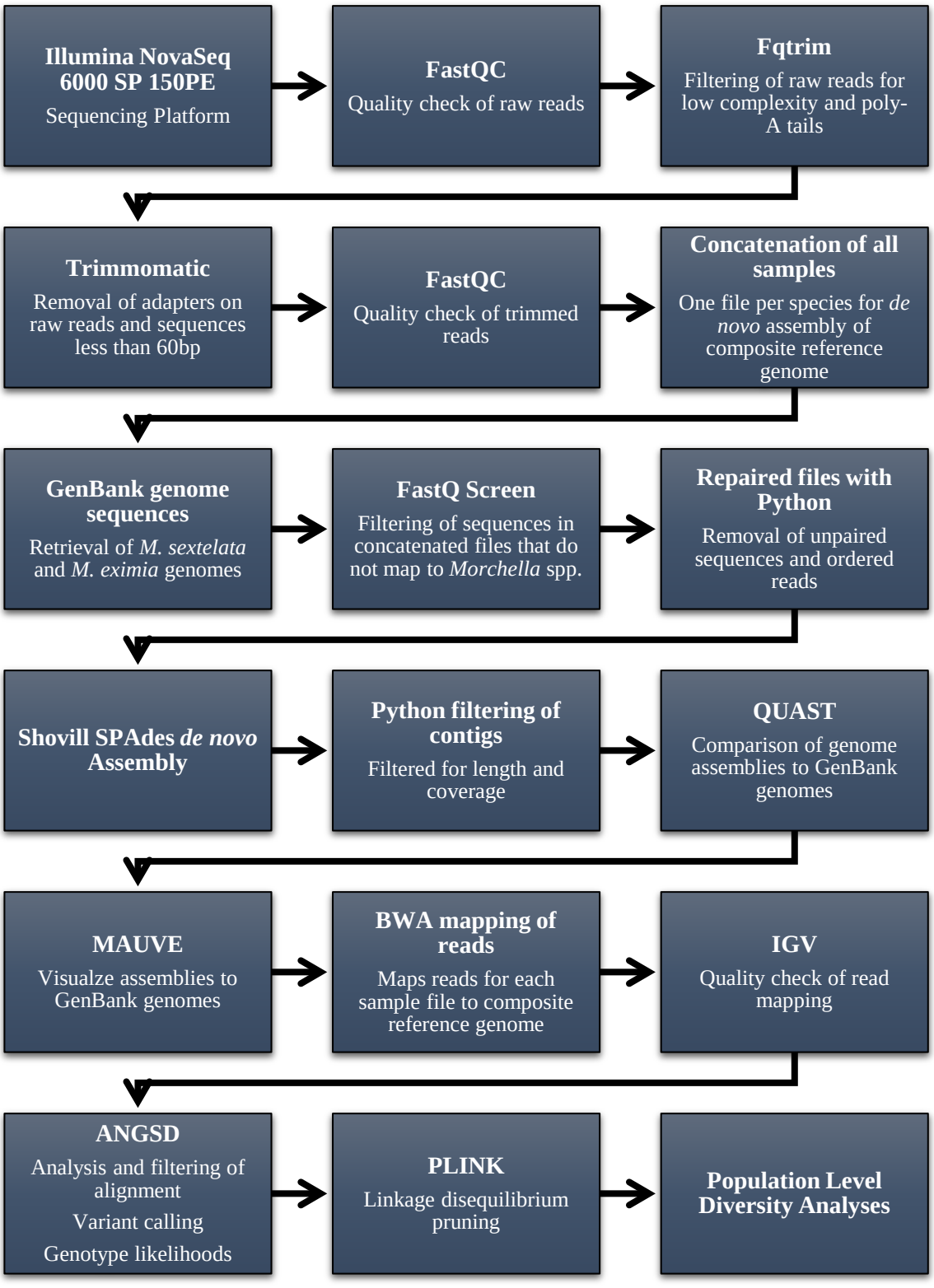

Figure 3.6. Pipeline used for quality control of the raw and assembled data, filtering, de novo assembly of reference genomes, and mapping of sample reads to the reference genomes.

were retained for further analysis (TABLE 3.1).

A composite reference genome most representative of western North American specimens was constructed using forward and reverse read files concatenated from the individual sample read files for each species. Potential contaminant sequences were filtered from the concatenated read files using FastQ Screen (Wingett & Andrews, 2018) by eliminating any sequence that did not map (uniquely or multiple times) to the NCBI reference genomes QMFK01.1 (*M. eximia* strain MG90) or SDUUO1.1 (*M. sextelata* strain NZTD180501373). The excluded read files were examined using a local BLASTN search (Altschul et al., 1990) against the NCBI nuccore database; spot checking of the results confirmed the excluded reads to be from bacteria or other contaminating organisms. The filtered concatenated read files were repaired using the python script interleave_pairs.py (http://seqanswers.com/forums/showthread.php?t=6140; https:// github.com/lexnederbragt/denovo-assembly- tutorial/blob/master/scripts/interleave _pairs.py) by removing unpaired sequences and matching the order of reads in the forward and reverse paired read files.

Shovill (https://github.com/tseemann/shovill) was used to perform a SPAdes (Bankevich et al., 2012) de novo assembly using a 150x upper depth threshold for each species. Python scripts were used to filter and discard contigs under 1000 base pairs in length and under 3x coverage (https://github.com/tinybio/filter_contigs). Quality checks and visualization of the de novo assemblies were conducted using QUAST (Gurevich et al., 2013) and by aligning to the corresponding NCBI reference genomes using MAUVE v2.4.0 (Darling et al., 2004).

Read Mapping and Variant Calling

The composite reference genome assemblies were indexed, and forward and reverse reads for each individual sample were mapped to the corresponding composite reference using the bwa-mem algorithm in BWA v0.7.15-r1140 (Burrows-Wheeler Aligner; Li & Durbin, 2009). BAM output files were sorted and indexed using SAM-tools v1.8 (Li et al., 2009), deduplicated using Picard v2.22.4(http://broadinstitute.github.io/picard/), then reindexed using SAMtools. Visual quality checks of the mapped reads for each species were performed using the Integrative Genomics Viewer (IGV; Robinson et al., 2011).

Single nucleotide polymorphism (SNP) calling (Kim et al., 2011) and calculation of genotype likelihoods (Li, 2011) were performed using ANGSD (Analysis of Next Generation Sequencing Data; Korneliussen et al., 2014) with the following filters: retain reads with a minimum mapping quality of 60; retain SNPs found in a minimum of approximately 70% of the samples (9 for *M. eximia* and 18 for *M. sextelata*); retain SNP positions with a SNP p-value of 0.01 or less and a minor allele frequency of 0.05 or greater; score SNPs for individual samples when that sample has a read depth of 10 or more sequences covering that SNP. Linkage disequilibrium pruning of the SNP data was performed using PLINK v1.9 (Chang et al., 2015) using independent pairwise correlation with a sliding window of 50 base pairs and an r^2 threshold of 0.64.

Analyses of Population Genetic Structure

Population structure was analyzed for each species via principal component analysis (PCA) in PCAngsd v0.95 (Meisner & Albrechtsen, 2018). Final proportions for PC1 and PC2 were calculated from the resulting covariance matrix, and final graphs were

produced using R v3.6.1 (R Core Team, 2019). ADMIXTURE v1.3.0 (Alexander & Lange, 2011) was used to calculate population admixture proportions of each sample for each species. Simulations were run at levels of 1-10 for K, the number of inferred ancestral populations. Cross validation error values were plotted against K values in R, with the lowest cross validation error value considered in selecting the best-fit K value. An admixture graph for *M. sextelata* was generated using R software.

Results

Over 400 million raw Illumina reads were obtained from the pooled library from 44 morel samples. After filtering the raw sequence data, 7 samples were eliminated due to low read counts, resulting in a final dataset representing 24 *M. sextelata* and *13 M. eximia* samples (TABLE 3.1). The composite reference genomes consisted of 51Mbp for *M. eximia* and 45Mbp for *M. sextelata* (TABLE 3.3). Filtering and linkage disequilibrium pruning resulted in final datasets of 221,446 SNPs for *M. eximia* and 125,679 SNPs for *M. sextelata* (TABLE 3.4).

Table 3.3. Metrics for QUAST analyses, including total genome size, total number of contigs, N50, and L50 values.

Species	Total genome size (bp)	Total contigs	N50	L50
M. eximia	51,687,173	5976	17,871	808
M. sextelata	45,459,805	1802	52,323	263

Table 3.4. Metrics for SNP analyses, including total base pairs analyzed, number of SNP positions remaining after ANGSD quality checks and filtering, and number of SNP positions remaining after linkage disequilibrium (LD) pruning that were included in the population genomic analyses.

Species	Total base pairs	After first round of filtering	After LD pruning
M. eximia	51,553,291	603,180	221,446
M. sextelata	45,442,408	302,900	125,679

The PCA plot for *M. eximia* showed variants distributed across PC1, indicating a

lack of population structure (FIG. 3.7). The results suggest that *M. eximia* has high levels of within site diversity and that samples from within the same transect correspond to distinct individuals. ADMIXTURE analysis of ancestral populations plotted against the cross validation (CV) errors for *M. eximia* supported the observations in the PCA. Minimum CV errors were obtained at K = 1 and K = 10. CV errors steadily declined with K values larger than 5, suggesting that CV errors would have continued to decline if K values larger than 10 had been tested. These results are consistent with a lack of population structure among the sites (FIG. 3.8).

The PCA plot of *M. sextelata* showed evidence of two genetically distinct clusters (FIG. 3.9). The Gottfriedsen Mountain Creek Fire (GMCF) samples formed a distinct genetic cluster with two samples from Randal Creek Fire (RF). However, other RF samples clustered with all specimens from the Blazed Creek and Gold Hill fires in a second cluster. Many of the second clustered samples are similar but genetically distinct from one another, suggesting that clonality does not occur widely in this species, though inbreeding may be present. The K vs. CV error plot for *M. sextelata* supported these results, with a minimum CV error at K =2 (FIG. 3.10). A bar-plot of the admixture proportions from the K = 2 ADMIXTURE analysis indicates two distinct populations corresponding to the clusters in the PCA analysis, with the same GMCF samples and 2 RCF samples falling into one population, and the Blazed Creek Fire, Gold Hill Fire, and 5 of the 8 samples from RCF falling into a second population (FIG. 3.11). Two of the RCF samples are fully assigned to the first population and may represent migrants; one RCF sample (Y10) shows an 80/20 admixture proportion of population 2 and population 1, respectively, indicating potential admixture between populations.

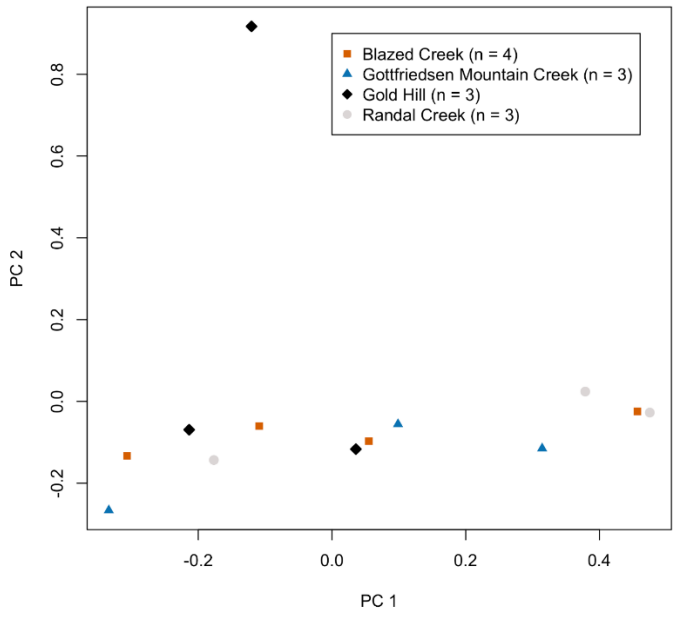

Figure 3.7. Principal component analysis (PCA) plot of *M. eximia* samples based on analysis of 221,446 single nucleotide polymorphisms. Fire sites are shown in different colors and symbols. Sample sizes for each fire site are indicated in the figure key, with a total sample size of n = 13. PC1 explains 18.1% of the variation and PC2 explains 9.7%.

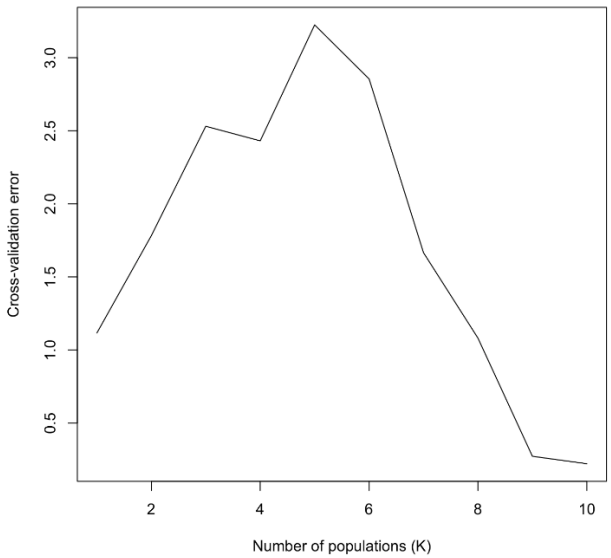

Figure 3.8. *Morchella eximia* ADMIXTURE-generated cross validation (CV) errors for each value of K, the number of inferred ancestral populations for all samples. As the value of K increases, the CV error value approaches zero, and therefore no optimal K value was selected.

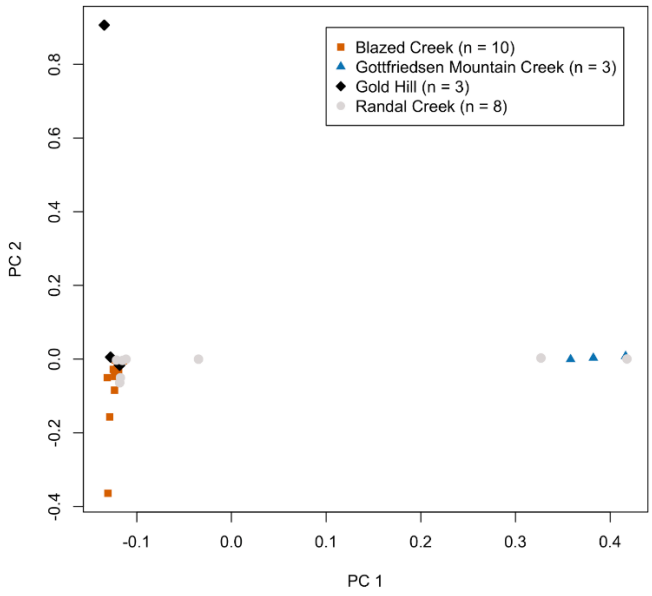

Figure 3.9. Principal component analysis (PCA) plot of *M. sextelata* samples based on analysis of 125,679 single nucleotide polymorphisms. Fire sites are shown in different colors and symbols. Sample sizes for each fire site are indicated in the figure key, with a total sample size of n = 24. PC1 explains 15.2% of the variation and PC2 explains 5.7%.

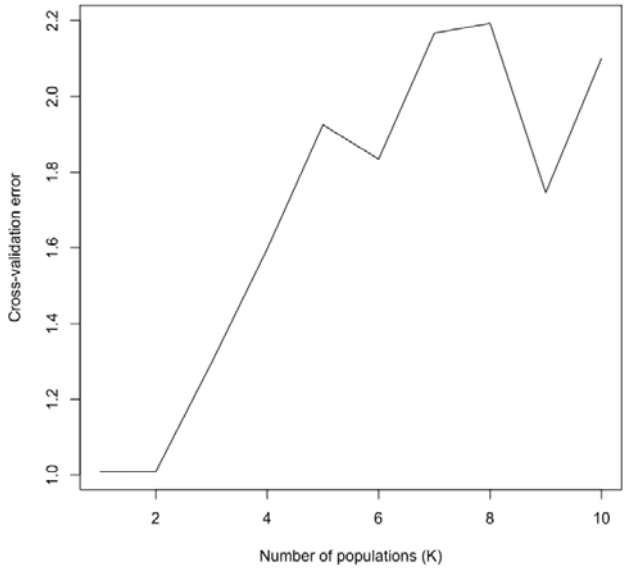

Figure 3.10. *Morchella sextelata* ADMIXTURE-generated cross validation (CV) errors for each value of K, the number of inferred ancestral populations for all samples, suggesting an optimal value of K = 2. A plot of admixture proportions is shown in FIG. 10.

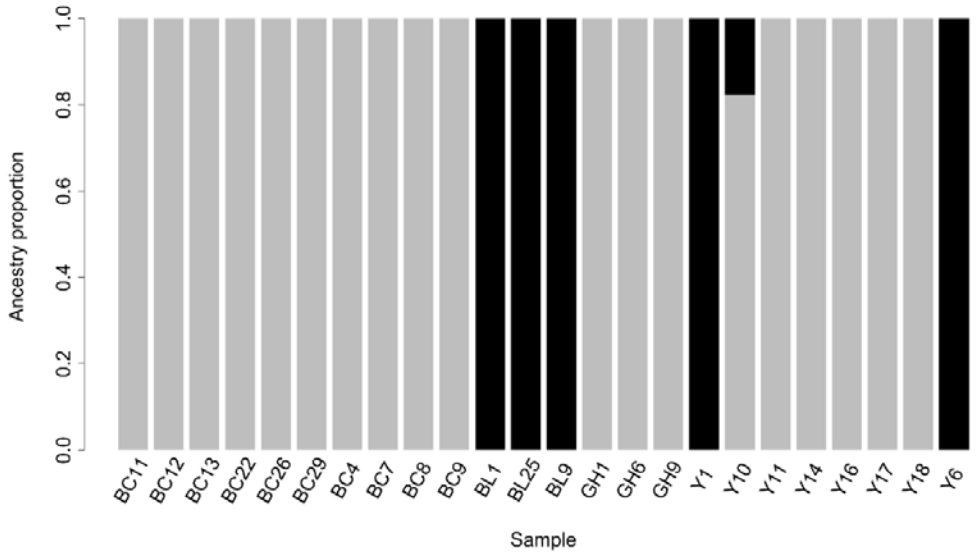

Figure 3.11. Admixture proportions for *M. sextelata* samples as inferred by ADMIXTURE with K = 2. Sample numbers and sites correspond to Table 2, with each sample represented by a vertical bar. Ancestry proportions are shown in black for population 1 and grey for population 2.

Discussion

Based on laboratory studies by Volk & Leonard (1989; 1990), meiotic ascospores produced by *Morchella* ascomata germinate to form homozygous primary mycelia. These mycelia could form ascomata or fuse with primary mycelia from sexually compatible ascospores to form secondary mycelia; the latter method is considered more likely. These secondary mycelia can then form ascomata. Previous research suggests that the formation of ascomata from primary or secondary mycelia is interrupted by a dormant stage in the form of pseudosclerotia, and are derived from the carpogenic germination of pseudosclerotia (FIG. 3.2). Which of these variations occur in nature, and for how long, has major implications for population genetic structure. While mass fruiting is the most obvious feature of the life cycle of fire-associated morels, this is just a small part of the cycle. Other parts are less easily observed, but perhaps no less important. In this study, we used genome-wide SNP data to make inferences about the behavior of these

organisms between fire events, and the effects of these behaviors on population genetic structure.

Several different scenarios could describe the biology of fire-associated morels between fire events. First, ascospores form primary mycelia; these mycelia then form ascomata, either with or without a dormant (pseudosclerotial or conidial) stage. In this scenario, we would expect to observe no heterozygosity within individuals since the primary mycelia are derived from a single haploid ascospore and are therefore homozygous. Second, ascospores may germinate and undergo plasmogamy, forming secondary mycelia, but the secondary mycelium only spreads for a short time period before entering a dormant (pseudosclerotial or mycorrhizal) stage. The pseudosclerotia are then stimulated by changes in conditions caused by the next fire event, possibly undergoing another short period of myceliogenic spread before forming ascomata in the spring/summer of the year after the fire. This scenario would lead to high within-site variation. Third, the secondary mycelia may spread over longer periods of time, perhaps even as long as the entire between-fire interval, or spread through successive episodes of dormancy and clonal repopulation. The spread could create massive mycelial networks leading to low within-site diversity with ascomata located in the same site more likely to be produced by the same mycelial individual. Finally, rather than immediately germinating, ascospores may form a dormant spore bank, only germinating after a fire event, forming fruiting bodies from the myceliogenic metabolism of nutrients released from the fire disturbance. This scenario would lead to high within-site variation and low variation between sites. For all scenarios, the degree of between-site variation is determined by the level of ascospore dispersal and gene flow between sites.

The results of this study provide evidence for a critical role of ascospores in establishing new individuals, dispersal, and gene flow between geographically separated populations (FIG. 3.7, FIG. 3.9, FIG. 3.11). The data showed that each sample analyzed was from a distinct individual mycelium, providing evidence that repeated establishment of many new individuals, possibly in combination with long-term survival of a few old and persistent ones, is the adaptive strategy employed by these fungi in response to fire (FIG. 3.7, FIG. 3.9). We observed heterozygosity within individuals (through quality checking read mapping and variant calling in the Integrative Genomics Viewer), indicating that fire-associated morel ascocarps are produced from secondary rather than primary mycelia. Evidence of high genetic diversity within sites and the lack of evidence of clonality between samples, even within the same transect (FIG. 3.5), indicates that these secondary mycelia undergo limited vegetative spread between fire events (FIG. 3.7, FIG. 3.9). It is likely that these species survive in a dormant stage between fires as either resting structures such as pseudosclerotia (with or without mycorrhizal formation) or as ascospores within a soil spore bank, only producing fruiting bodies after abiotic and/or biotic changes stimulated by wildfires.

Though the genetic analyses cannot distinguish between the scenarios that involve limited mycelial expansion, results from previous studies suggest that initial mycelial expansion followed by pseudosclerotial dormancy is more likely than spore bank survival. Laboratory studies on the life cycle (Volk & Leonard, 1990) and cultivation (Ower, 1982; Ower et al., 1985; 1986) of morels provide evidence demonstrating the importance of pseudosclerotial formation from secondary mycelia. The presence of pseudosclerotia was noted in the outdoor cultivation of morels, but did not establish

whether they were necessary for fruiting body development (Q. Liu et al., 2018).

Pseudosclerotia have been observed in two field studies of fire-associated *Morchella*. Stefani and colleagues (2010) observed a unique underground structure (coined “radiscisclerotium” due to their solid, compact hypogeous structure) connected to the stipes in the fire-associated morel *Morchella tomentosa*, and suggested that in comparing these structures to other *Morchella* pseudosclerotia observations, both form and function imply that *M. tomentosa* structures are a time resistant resource reservoir allowing fruiting over multiple years. However, they argued that these structures are unlikely to persist throughout the entire fire rotation period (FIG. 3.12).

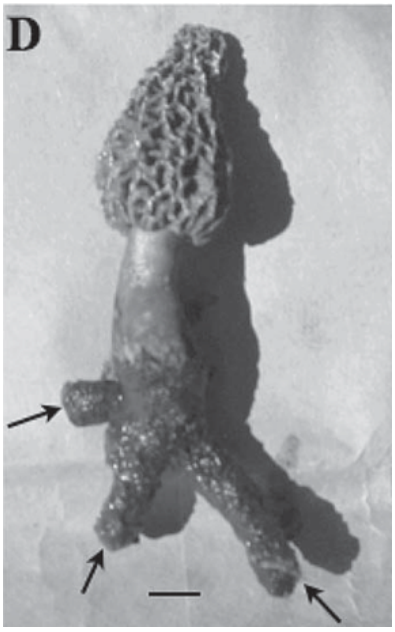

Figure 3.12. *Morchella tomentosa* radiscisclerotium at the base of the fruiting body. Scale bar equals 1 cm (Modified from Stefani et al., 2010).

Miller et al. (1994) found evidence of *Morchella* pseudosclerotia present in significantly higher numbers in the first year after a burn when compared to same site in the second year. However, due to the difficulties in observing pseudosclerotia in a natural setting, it was not determined whether these were newly produced structures. The timing

of the production of pseudosclerotia and their length of dormancy cannot be inferred with confidence from these data. The same study observed sclerotia of the ascomycete *Cenococcum geophilum*, noting that the condition of these sclerotia suggested that they were produced before rather than after the fire, which suggests that at least some fungi use sclerotia to survive between fires.

Greene and colleagues (2010) looked at morel sporocarp emergence after wildfires and suggested that two scenarios could lead to massive post-fire fruitings, based on observations from Pilz et al. (2007) as well as length of time between the fire event and fruiting body formation. The first is that dormant pseudosclerotia undergo rapid metabolism and myceliogenic growth, which then supports the formation of fruiting bodies. The second is that pseudosclerotia retain enough nutrition and immediately undergo carpogenic growth in the formation of fruiting bodies. Though they believed vegetative mycelia could undergo metabolism during the winter months (first scenario), they assumed the simpler second scenario (fruiting directly from pseudosclerotial nutritional reserves) was more likely. Though many studies have suggested the presence of pseudosclerotia is needed for fruiting body formation, some research has looked at the possibility of a morel ascospore bank. In a study of post-fire ectomycorrhizal spore banks using tree seedling bioassays, Glassman et al. (2016) did not observe *Morchella*, suggesting that post-fire morels are not ectomycorrhizal and/or do not leave resistant spores in the spore bank.

Our results suggest that ascospore production promotes local reestablishment and genetic diversity, while also contributing to long-distance dispersal and gene flow between sites separated by 250 kilometers or more (FIG. 3.3, FIG. 3.11). *Morchella*

eximia showed high genetic diversity within sites, but often low diversity between sites, consistent with a lack of barriers to gene flow (FIG. 3.7, FIG. 3.8). *Morchella sextelata* showed a similar pattern of high genetic diversity within and low diversity between sites (FIG. 3.9, FIG. 3.10). However, evidence of isolation by distance was observed in *M. sextelata* between the most geographically separated population at Gottfriedsen Mountain Creek Fire (GMCF) and the rest of the sites (FIG. 3.9, FIG. 3.11). This isolation is not complete, as both migration (occurrence in the GMCF population of individuals assigned genetically to the population that encompasses the non-GMCF sites) and admixture between populations (sample Y10 in the Randal Creek Fire site) was detected (FIG. 3.11).

Chai and colleagues (2017) used genomic sequencing and identified mating types in *M. importuna, M. purpurascens*, and *M. sextelata*, supporting previous claims on the heterothallic mating system of morels. Other studies have indicated that some species of morels might be more prone to inbreeding, as well as the possibility that fruiting from primary mycelia might be occurring due to observations of high homozygosity found in wild populations (Dalgleish & Jacobson, 2005; Du et al., 2016). The population structure of both *M. sextelata* and *M. eximia* observed in our study imply that both species exhibit heterothallism, and that the formation of heterokaryotic mycelia is occurring frequently between fire events (FIG. 3.7, FIG. 3.9).

Our results raise a number of interesting questions for future research. How are ascospores dispersing across the western mountain ranges of North America (i.e. wind, animals, commercial harvesters)? Does *M. eximia* form a single panmictic population throughout the western US, or do isolation by distance and/or barriers to gene flow exist?

If so, at what scale, and what represents an effective barrier? At what scale, if any, does *M. sextelata* exhibit isolation by distance? Can it be confirmed whether these fungi survive as pseudosclerotia or as ascospores within the spore bank? Are closely located but genetically distinct individuals produced by outcrossing or inbreeding?

Further analysis of the data collected, as well as increasing the sample size and fire sites surveyed could help answer the questions generated from this research. Analyses planned for this dataset include calculation of individual heterozygosity metrics to supplement our qualitative observations of heterozygosity, calculation of kinship coefficients to quantify the extent of inbreeding within sites, and calculation of F_{ST} statistics to quantify the level of genetic differentiation between sites. Closer examination of haplotypes and application of migration models (Beerli, 2008) may allow further insight into the processes of migration and gene flow in these species. Additional types of sampling could also provide further evidence to examine burn morel life cycles in nature.

Collecting soil samples and surveying for underground growth structures before, during, and after fruiting events could provide evidence of persistence of resting pseudosclerotia as well as whether morel ascospores are present in the spore bank and, if so, for how long. Generating a more complete reference genome by combining the short reads from Illumina sequencing and long reads from MinION sequencing (in progress) could provide thousands more variants across the sample set, which would allow for higher resolution in our analyses as well as provide a basis for further research into functional traits and genes involved in mating in fire-associated morels. Our work is far from finished, but our results thus far provide a new window into the ecology and reproduction of morel mushrooms in wildfire disturbed habitats.

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