MUTATION AND LOSS OF HETEROZYGOSITY IN AN INDIVIDUAL
OF THE ROOT-INFECTING FUNGUS ARMILLARIA GALLICA IN A
MIXED HARDWOOD FOREST

by

Stefan Catona

A thesis submitted in conformity with the requirements
for the degree of Master of Science,
Graduate Department of Ecology and Evolutionary Biology
University of Toronto

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MUTATION AND LOSS OF HETEROZYGOSITY IN AN INDIVIDUAL OF THE ROOT-INFECTING FUNGUS *ARMILLARIA GALlica* IN A MIXED HARDWOOD FOREST

Degree of Masters of Science, 2012

by

Stefan Catona, Department of Ecology and Evolutionary Biology, University of Toronto

ABSTRACT

Long-lived individuals of the opportunistic fungal pathogen *Armillaria gallica* arise in single mating events, and then grow vegetatively to occupy large territories including multiple woody substrates. In effect, this leaves a spatial record of mutation, the detection of which would allow new inferences about how fungal individuals grow and infect their hosts. In this thesis, I first identified a large individual of *A. gallica* in eastern Ontario. I then searched for genetic variation within this individual by focusing on the tandemly repeated rRNA gene cluster and four microsatellite markers that are variable in the *A. gallica* population. I discovered a loss of heterozygosity (LOH) in the rRNA gene-cluster region, forming two genotypes that show significant spatial clustering in a Mantel test. My M.Sc. thesis research serves as a baseline for a genome-wide study of the mutational dynamic within the vegetative growth phase of this large and old *Armillaria* individual.
ACKNOWLEDGEMENTS

I would first like to thank my supervisor, Jim Anderson, whose guidance, trust, and “cautious optimism,” have made the past sixteen months a formative experience. I would also like to thank my committee members, Linda Kohn and Sasa Stefanovic, for helping to cast the mould of this project. As well, I am grateful to Cindy Short, Marion Andrew, Lucas Parreiras, Thomas Braukmann, and David Bartfai, for sharing their knowledge with me, and for their friendship. Finally, I extend my heartfelt gratitude to Debbie Yeh, whose intellectual, emotional, and gastronomical support has fueled my every step toward this accomplishment. *Munca e brătară de aur!* 
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INTRODUCTION

In 1992, the discovery of a clonal individual (or genet) of the basidiomycete fungus *A. gallica* of unprecedented size (Smith et al. 1992) garnered the attention of the scientific community and the popular press (Gould 1992). Having arisen from a single mating event, this individual has apparently grown vegetatively for over a millennium, occupying a spatially continuous territory of more than 50 hectares of forest floor, and weighing at least 9,700 kg (Smith et al. 1992). Individuals of this size and age may not be atypical for this genus, as a number of massive individuals of *Armillaria* and other genera have since been documented (Anderson and Kohn 1995; Ferguson et al. 2003). The goal of my M.Sc. thesis project was to investigate the mutational dynamic with large and old individuals of *A. gallica*.

*A. gallica* is a widely distributed opportunistic pathogen, causing root-rot and butt-rot of hardwood trees in temperate forests (Guillaumin et al. 1991). Territories of *Armillaria* genets often include multiple adjacent root systems spread over a wide area (Smith et al. 1992; Rizzo et al. 1995). The hyphae of *A. gallica* have the ability to differentiate into various structures, each of which plays an important role in the life history. These structures are rhizomorphs, pseudosclerotia, and basidiomes (Fig. 1).

The cord-like rhizomorphs unique to *Armillaria* species have a melanized rind and internally differentiated hyphae that act as solute-conducting elements. The interior of the rhizomorph has a lightly packed internal core through which gases can diffuse (Anderson and Ullrich 1982; Cairney et al. 1988; Pareek et al. 2006). Rhizomorphs grow apically at a rate about ten-fold that of unorganized mycelium (Rishbeth 1968) through the forest soil, around roots, and beneath tree bark, persevering in environments inhospitable to
Figure 1: Differentiated structures of *A. gallica*. Hyphae differentiate into rhizomorphs, pseudosclerotia, and basidiomes.
undifferentiated mycelium (Findlay 1951). Their primary role is to explore nearby territory for new sources of nutrition and to promote infection by mechanically and enzymatically penetrating the cortical tissues of potential woody-plant hosts (Garrett 1956).

Interestingly, rhizomorphs are also involved in conducting electrical signals, akin to the membrane depolarizations that propagate along nerve cells, among colonies occupying discrete resource units (Gow et al. 1999). Although the role of electrical signaling in fungi is not known, action-potential-like activity has been elicited in *Armillaria* colonies in vitro as a response to various stimuli, including the introduction of nutrient sources, and mechanical trauma (Olsson and Hanson 1995). A reasonable suggestion is that the size and long persistence time of *Armillaria* necessitates some means of rapid communication over long distances, and that electrical signals fulfill this purpose (Olsson and Hanson 1995).

Pseudosclerotial tissues are characterized by plate-like areas of melanization, and are a common anatomical feature of lignicolous (wood inhabiting) basidiomycetes (Lopez-Real and Swift 1975). The differentiation of these tissues is promoted by stimuli such as oxygen exposure, change in temperature, and mechanical trauma to the hyphae (Lopez-Real and Swift 1977), which leads to the inference that their role is primarily protective (Moore 1998).

The edible basidiomes of *A. gallica* appear abundantly in the fall. Unlike other *Armillaria* species, the basidiomes of *A. gallica* are commonly solitary, arising from woody substrates just under the surface soil. However, they can also be found in clusters on stumps or logs. The pileus is variable in colour, ranging from light brown to pinkish-
brown, and is covered in fine hairs (Berube and Dessureault 1988). The partial veil has a cobweb-like appearance, resembling that of the *Cortinarius* species, and the spore-print is white. The stipe increases in diameter toward the base, creating a bulbous appearance.

At the cellular level, *Armillaria* species possess some peculiar characteristics. The life cycle of *Armillaria*, from karyogamy to sporulation, includes two diploidization, and two haploidization events (Grillo et al. 2000). The first diploidization event occurs during mating of haploids. The first haploidization takes place cryptically in the mycelium immediately preceding the basidiome, or in the basidiome tissues, creating a transient dikaryon in the hymenial layer on which the basidia are formed. The second diploidization, by fusion of two haploid nuclei in the basidium, is followed by the second haploidization, a conventional meiosis, and the formation of four basidiospores (Peabody and Peabody 2000). This means that, for the majority of their life cycle, *Armillaria* species, unlike most other basidiomycete fungi, are diploid rather than dikaryotic (Fig. 2).

**Population structure of *A. gallica***

At the population level, counting unique genets as individuals regardless of their size, it has been demonstrated that allele frequencies in the *A. gallica* population in eastern North America do not deviate significantly from Hardy-Weinberg expectations (Saville et al. 1996). In other words, mating in the *Armillaria* population in eastern North America approximates panmixia. Assuming that *Armillaria* populations have occupied most of the territories available to them, and have resided in these territories for a very long time, panmixia over vast distances suggests either that gene transfer among sub-populations is
Figure 2: The life cycle of *A. gallica*. Haploid nuclei are represented by grey dots; diploid nuclei are represented by black dots. (a) the typical basidiomycete life cycle in which matings of haploids produce persistent dikaryons; (b) the life cycle of a few basidiomycetes, such as *A. mellea* sensu stricto, in which matings form persistent diploids, with no dikaryon in any stage of the life cycle; and (c) the life cycle of most *Armillaria* species with two diploidizations and two haploidizations.
preventing the accumulation of significant differences in allele frequencies, or that current Armillaria populations are descended from a well-mixed ancestral population (Saville et al. 1996).

The size and longevity of Armillaria individuals present unique challenges to the long-term survival of the overall population. For example, asexually reproducing organisms lack a means of purging deleterious mutations from the population. In such cases, there may be selection for a reduced mutation rate, which would be brought about by an increased fidelity of DNA replication (Drake et al. 1998). The reduced mutation rate would, in effect, retard the advance of Muller's Ratchet (Lynch et al. 1993) in small populations and evade the potentially synergistic effect of deleterious mutations in large populations (Kondrashov 1988). For Armillaria, mutation during the asexual, vegetative growth phase is especially relevant at the population level because every vegetative cell in the colony is also a potential germ line during the sexual stage of the life cycle (Buss 1983). Rhizomorphs are formed by hundreds, or thousands, of aggregated hyphae (Motta 1982), but the effective population of hyphae within rhizomorphs may be as few as several dozen cells. The lineage of a rhizomorph is therefore particularly susceptible to Muller's ratchet as it advances over long distances, and to founder effects upon establishment of a new colony. Although mutation rates might be driven downward by selection under these conditions, the associated increase in the fidelity of DNA replication might eventually incur a severe fitness cost (Hodnett and Anderson 2000). Mutation rates in organisms with long asexual phases would therefore be expected to stabilize at some rate higher than zero. Although beyond the scope of my M.Sc. project, these hypotheses about selection and mutation rates could be tested with A. gallica as the experimental system.
**Mutation rate in individuals of *A. gallica***

Hodnett and Anderson (2000) used single-strand conformational polymorphisms (SSCP) to identify alleles at loci among and within *A. gallica* individuals. Their study detected variation in *A. gallica* between, but not within, individuals over a total genomic area of about 10,000 base pairs. Given the large number of mitotic cycles required for *A. gallica* individuals to achieve their large size, up to one km in diameter, the absence of variation in this context was unexpected.

Several explanations have been presented for the surprising genetic homogeneity within asexually reproducing *A. gallica* individuals. The hypotheses that follow are not mutually exclusive, and the possibility that two or more of these mechanisms operate in tandem to reduce the rate of mutation in the vegetative thalli of *A. gallica* individuals deserves consideration.

One plausible explanation for the absence of variation in the study by Hodnett and Anderson (2000) is that diploid *Armillaria* genets do not extend solely by division of the hyphal tip; instead the fragmented hyphae may be carried over short distances with the assistance of microfauna, allowing them to colonize new substrates and expand their territory at a rate higher than mere vegetative growth. This hypothesis may hold if the viability of hyphae dispersed by microfauna is temporally restricted following detachment from a parent colony, resulting in dispersal over short distances of only a few centimeters. Microfaunal dispersal over longer distances would result in multiple, discontinuous patches of territory, a scenario which conflicts with the observation that *Armillaria* individuals each occupy one discrete territory (Hodnett and Anderson 2000).
Another possibility is that *A. gallica* individuals are able to grow to their current dimensions in far fewer cellular generations than was previously estimated, using some means other than the fragmentation and dispersal of hyphae. One such method involves rhizomorphs, which are known to elongate at a rate 5-10 times greater than that of vegetative mycelia (Yafetto et al. 2009; Rishbeth 1968). While vegetative hyphae extend by mitotic cell division only at the hyphal tip, it has been suggested that rhizomorphs may lengthen by the simultaneous division of a number of cells immediately behind the advancing apex, as well as within the apical cell itself (Hodnett and Anderson 2000). This type of protective mechanism is known to operate in the root and shoot apical meristems of plants, and serves to reduce the number of cell generations, and therefore also the number of replication errors, in the apical cell (Grandjean et al. 2004; Reddy et al. 2004). If this is the case for *A. gallica*, calculations equating distance to cellular generations must be adjusted by a factor corresponding to the number of simultaneously dividing cells. This adjustment may be sufficient to explain the absence of SNPs within aged *A. gallica* individuals.

Hodnett and Anderson (2000) sampled multiple points of an *Armillaria* individual, some as far apart as 650 meters, which were estimated to represent at least 6.5X10^7 cell divisions (Saville et al. 1996). At a conservative mutation rate of 10^{-9} to 10^{-10} events per base pair per cell cycle, (Drake 1991), Hodnett and Anderson expected to detect 65 to 650 SNPs in their screening of 10^4 base pairs (Hodnett and Anderson 2000). Instead, they found none. However, if the number of cell divisions is reduced by a factor of ten, that is, if the extension of the rhizomorph tip is accomplished by the simultaneous division of, say, ten cells, only 7 to 65 SNPs are expected from a sample size of 10^4 base pairs. It is
possible that the number of simultaneous cell divisions in the growing rhizomorph is even greater than ten. Under these circumstances, it is conceivable that SNPs may have remained undetected due the insufficient number of base pairs sampled for variation.

**Microsatellite markers and variation within individuals**

Microsatellite markers are tandem repeats of 1 to 6 base pair sequences of DNA present in all known genomes (Schlötterer 2000). Because they are highly polymorphic, microsatellites are extensively utilized in population genetics, disease etiology, and heredity studies (Selkoe and Toonen 2006). My intent in this thesis was to refocus the search for variation within an Armillaria individual on microsatellite regions, which have a much higher rate of fragment-length mutation than ordinary base substitution. Although the mutation rate of microsatellite regions is dependent on factors such as the size of the allele, the repeat unit length, and the zygosity of alleles (Schug et al. 1998, Henke and Henke 1999, Lai and Sun 2003), most microsatellite loci exhibit a mutation rate of $10^{-6}$ to $10^{-3}$ mutations per locus per cell cycle (Schug et al. 1997). Assuming that a meter of rhizomorph elongation constitutes $10^2$ mitoses (a very conservative estimate), and that $10^{-4}$ length mutations occur per mitotic event, it was expected that $10^{-2}$ mutations would occur for every meter of growth. The maximum distance between isolates of the same individual on Exe Island is 190 m, separating Ar9 from Ar20 (Fig. 3). Over this distance, with luck, we might detect one or two mutations. In this search, motif deletions, which involve a reduction in the number of microsatellite repeats, and motif insertions, which increase the number of repeats would both be detectable (Henderson and Petes 1992).
Figure 3: Distribution of Exe Island genotypes in the 2010 and 2011 samples. Isolates belonging to the large individual are numbered in white font. Isolates belonging to the smaller individual are numbered in yellow font. A red dot indicates an isolate exhibiting LOH in the 26S rRNA gene.
In fungi, the occurrence of loss of heterozygosity (LOH) due to gene conversion over short tracts, or mitotic crossing over affecting large tracts of DNA, has been extensively studied (Souza – Junior et al. 2004; Hiraoka et al. 2000; Forche et al. 2009), and linked to physiological and morphological effects. A good example of LOH and spatial sectoring of a diploid mycelium can be seen in Figure 4. The tandemly repeated rRNA gene array is an obvious prospect for finding variation within an *Armillaria* individual because the mitotic instability of this region increases the rate of mutational events leading to LOH (Lindstrom et al. 2011; Andersen et al. 2008). LOH has already been demonstrated in the 26s rRNA gene of the large rRNA subunit of an *A. gallica* individual (Saville et al. 1996). If LOH in the rRNA gene is a common occurrence in the *A. gallica* genome, screening of the Exe Island isolates for LOH using PCR amplification of the 3’ end of the 26 S rRNA gene could reveal variation in genotypes.

**Research objectives**

My goal was to investigate the possibility of genetic variation due to mutation or LOH within an asexually reproducing individual of *A. gallica*. To achieve this, I 1) collected isolates of *A. gallica* from marked locations (Fig. 3), 2) identified individuals by their unique genotypes based on Single Nucleotide Polymorphisms (SNPs), Mating Interactions (MI), Somatic Incompatibility (SI) reactions, and phenotype comparisons, and 3) isolated representatives of an individual from different spatial locations, to sequence their entire genomes, and finally to search the sequence data for SNPs, insertion/deletions, and rearrangements. I prepared DNA from three samples (See Ar7, Ar9, and Ar14 in Figure 3) and the genome sequencing was carried out by the BGI (Formerly Beijing Genomics Institute) in July–December, 2011. Although numerous SNPs were detected among the samples, indicating variation due to mutation, I was not able to pursue this exciting avenue further due to the time constraint of finishing the M.Sc. program.

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1 The ultimate screen for variation would be to isolate representatives of an individual from different spatial locations, to sequence their entire genomes, and finally to search the sequence data for SNPs, insertion/deletions, and rearrangements. I prepared DNA from three samples (See Ar7, Ar9, and Ar14 in Figure 3) and the genome sequencing was carried out by the BGI (Formerly Beijing Genomics Institute) in July–December, 2011. Although numerous SNPs were detected among the samples, indicating variation due to mutation, I was not able to pursue this exciting avenue further due to the time constraint of finishing the M.Sc. program.
Figure 4: *Aspergillus nidulans* colony exhibiting LOH in vitro (source unattributed). LOH has resulted in a mutant sector with different spore colouring. Note: mutant sectors are physically connected to their ancestral cells.
3) assayed a locus containing the tandemly repeated ribosomal rRNA genes, and four microsatellite markers, for variation within the same individual.

*A. gallica* is an ideal candidate for this project because the radial pattern of mycelial extension affords the possibility of tracking the frequency, distribution, and locations of mutant sectors throughout the colony over time. Furthermore, the longevity of *A. gallica* individuals enables the conversion of this study into a multi-year investigation of mycelia turnover within the fungal individual, and this kind of view of growth and mutation is unprecedented in a natural setting.
MATERIALS AND METHODS

Research location

My research was conducted on Exe Island, a 1.2 ha tract of mixed-hardwood forest on Big Rideau Lake, Portland, Ontario. The island suited my purposes for three main reasons. First, the site harbors an abundance of *A. gallica* on woody substrates, mainly dead hardwood trees. Second, several isolates of *A. gallica* were collected from the site in 1992 (Saville et al. 1996) and were genotyped for several polymorphic markers; the multilocus genotypes indicated that a single individual of this fungus occupied most of the area of the island. Third, the forest on the site is undisturbed by human activities such as road building or logging. Exe Island is privately owned and permission to conduct this research was obtained from the owner.

Coarse scale collection of isolates from the study site

In April, 2010, a preliminary sample of rhizomorphs of *A. gallica* was collected from woody substrates, mostly fallen hardwood logs with moisture contact with the underlying soil. Pure cultures were obtained from nine isolates from Exe Island, labeled Ar7, Ar8, Ar9, Ar11, Ar14, Ar16, Ar17, Ar18 and Ar20 (Fig. 3), and two isolates from the mainland nearby, labeled Ar4 and Ar6. Additionally, three fruit bodies were collected from Exe Island in the fall of 2010. From these fruit bodies (Ar21, Ar22 and Ar23), isolates were made of cap or stipe tissues and of single-basidiospores for assay of mating interactions and mating types. Mating-type alleles serve as naturally-occurring, highly polymorphic genetic markers that are diagnostic of individual identity (Guillaumin et al. 1991; Smith et al. 1992). Isolates from fruit body tissues are uniformly diploid and can be treated as the
equivalent of isolates of rhizomorphs; the single basidiospore isolates are all haploid. The coarse-scale collection will from now on be referred to as the “2010 sample.”

**Fine scale collection of isolates from the study site**

In June of 2011, I sampled the large Exe Island individual's territory on a finer scale. Rhizomorphs were collected from hardwood logs around the sites of the 2010 samples on Exe Island. The distances and compass bearings of the fine-scale samples were recorded relative to the positions of the 2010 samples. Geographical coordinates for both the 2010 and the 2011 samples are listed in Table 1. Pure cultures were obtained for 19 fine scale samples retrieved from the study site (Fig. 3). These strains, labeled Ar24, Ar26, Ar29, Ar30, Ar31, Ar35, Ar36, Ar37, Ar38, Ar39, Ar41, Ar42, Ar43, Ar45, Ar46, Ar47, Ar48, Ar49 and Ar50, will from here on be referred to as the “2011 sample.”

**Isolation of axenic cultures**

Segments of rhizomorphs collected from the research site were surface-sterilized in a 2.5% hypochlorite bleach solution for 5 minutes, trimmed to 5 – 10 mm in length, and transferred to water-agar medium. In less than a week, hyphal growth was observed from the trimmed rhizomorph ends. Cubes of mycelium and agar were then transferred to 2% malt-extract agar (MEA) to establish pure cultures.
Table 1: Geographical coordinates of 2010 and 2011 samples from Exe Island, Portland, Ontario.

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Latitude and longitude are displayed in decimal degrees.
**Growth of mycelium for DNA extraction**

The cultures were transferred from MEA to liquid 2% malt extract in petri dishes and incubated in the dark, at room temperature, for a period of three weeks. Five to ten 1 mm cubes of agar and mycelium were floated on the air-liquid interface of the growth medium. The resulting colonies were then dried on filter paper between stacks of paper towels, and collected into 2mL plastic centrifuge tubes. The samples were lyophilized by partial immersion in liquid nitrogen followed by 14 hours of drying in a Speed Vac.

**DNA extraction**

Whole-cell DNA was extracted from the dried mycelia by following the protocol for the Promega DNeasy Plant Mini Kit (50). Purity of the final product was verified using a Thermoscientific Nanodrop 1000 spectrophotometer. Alternatively, extraction of DNA was done with the “miniprep” protocol of (Murray and Thompson 1980). DNA extractions were carried out on the 2010 samples, as well as 8 additional samples of freeze-dried mycelium, which were archived in 1992 (henceforward referred to as the "1992 sample"), but are no longer accompanied by living cultures. No. 629 was from the large individual in northern Michigan and nos. 638, 639, 640, 641, 642, 643 and 644 were all from Exe Island. The expectation was that some of these genotypes should match those of the same resident individual currently sampled (and they did match). The 1992 samples were all included in a previous study (Saville et al. 1996) in which the allelic frequencies of polymorphic nuclear and mtDNA loci were quantified in the eastern North American population.
**PCR and Sanger sequencing**

The following DNA regions were assayed for variation by PCR and Sanger sequencing: C33r, E16f, E75c, I70f, I84c, DP1c, DP4, paMbo38c, J1c, and C15f (Table 2). Regions C33r, E16f, E75c, I70f, I84c are known to encompass variability between individuals (Hodnett and Anderson 2000). DP1 and DP4 were selected because the frequency of their polymorphic regions in the eastern North American *A. gallica* population has previously been characterized (Saville et al. 1996). A limited subset of regions, I70f, E16f, PaMbo38c, and DP1c, was assayed for nucleotide sequence variation in the 2011 sample.

PCR was done in 50 µL reactions; each reaction contained 25 µL of 2 X GoTaq, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 13 µL of distilled, nuclease–free water, and 10 µL of DNA template in a 1: 50, or 1: 100 dilution. The reaction was as follows:

2 minutes at 95 °C

30 seconds st 95 °C

30 seconds at temperature 3 °C below primer – specific Tm

30 seconds at 72 °C

15 minutes at 72 °C

PCR products were cleaned using Wizard® SV Gel & PCR Clean – Up System, and outsourced to The Centre for Applied Genomics for Sanger sequencing, and the resulting data set was trimmed, and analyzed using Sequencher 4.9.
Table 2: Primers used in PCR, Sanger sequencing, rDNA digest genotyping and microsatellite fragment length analysis.

<table>
<thead>
<tr>
<th>DNA region</th>
<th>Primer pair (5'-3')</th>
<th>GenBank</th>
<th>Size (bp)</th>
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<td>paMbo38c</td>
<td>AAGAACATTTTGACTGCCAGG</td>
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<td>272</td>
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<td></td>
<td>ACTGGAACACGATTTGTTC</td>
<td></td>
<td></td>
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<td>C15f</td>
<td>GGTATCGAACTGAGATGGC</td>
<td>AF116296</td>
<td>229</td>
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<tr>
<td></td>
<td>CAGCTACTGACCATTCACC</td>
<td></td>
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<tr>
<td>C33r</td>
<td>GGCTGAACAAACATCTCGAGC</td>
<td>AF116297</td>
<td>291</td>
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<td></td>
<td>AGCAACACGACCAACAGCG</td>
<td></td>
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<tr>
<td>J1c</td>
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<td>CGTAATGACCTCCAAATGGC</td>
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<tr>
<td>DP1c</td>
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<tr>
<td></td>
<td>ATCCACACATTTGATGCACC</td>
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<td>DP4</td>
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<td>I70f</td>
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<td>CCGTTACACCTTCAACCTCG</td>
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<td>I84c</td>
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<td>ACATGCGGTGTGACAGATGC</td>
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<td>LR7RΨ</td>
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<td>GACTTAGAGCGGTTCAG</td>
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<tr>
<td>Arm02</td>
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<td>GQ153965</td>
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<td>GQ153968</td>
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<td>GQ153970</td>
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<td>GQ153971</td>
<td>137-155</td>
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* GenBank accession numbers were not obtainable for asterisked regions.
Ψ Reverse primer was LR12.
Mating type determination

Mating interactions were visualized on MEA using 20 monospore isolates segregated from three basidiocarp isolates fruiting on Exe Island, labeled Ar21, Ar22 and Ar23. The haploid monokaryons were tested in compatibility reactions with one another to determine their mating type. Armillaria species have a “tetrapolar” mating system with two mating-type loci, designated A and B. Individuals must have different alleles at both loci for a successful mating to occur (Korhonen and Hintikka 1974; Casselton and Olesnicky 1998). Multiple alleles exist for both loci in natural populations. Normally, clamp connections and dikaryons are diagnostic of a compatible mating in basidiomycetes. Because the mycelium of Armillaria is diploid and without clamp connections in its fertile, vegetative state, however, the presence of clamp connections cannot be used as a diagnostic indicator. Instead, mating between two Armillaria individuals is confirmed by observing colony morphology. A reduction of fluffy, aerial mycelium in the colony, which takes on a crustose, flat appearance, is indicative of a conversion of the mated haploid mycelia into one uniformly diploid mycelium (Hintikka 1973; Korhonen 1978; Ullrich and Anderson 1978; Anderson and Ullrich 1982).

Somatic compatibility

In Armillaria and most other basidiomycetes (Worrall 1997; Kauserud 2004; Guillaumin et al. 1996), paired colonies fuse when they possess the same alleles at multiple somatic compatibility loci (Fig. 5). All self-self confrontations of diploid mycelia are somatically compatible, without exception. In non-self interactions, allelic difference at the somatic compatibility loci elicits an antagonistic response, resulting in the formation of
Figure 5: Somatic compatibility interactions. In each petri plate, two diploid *A. gallica* colonies are confronted. Compatible colonies fuse (Ar7 X Ar8), while incompatible colonies form a line of demarcation (Ar7 X Ar18).
a line of demarcation at the border between the two plated colonies (Rizzo and Harrington 1993; Worrall 1994). This antagonistic reaction probably involves some form of programmed cell death (Worrall 1997). Even if diploid strains are closely related (e.g., as full or half sibs), they are still unlikely to possess the same vegetative compatibility alleles at each locus underlying this recognition process and be somatically compatible. The pairing of diploid colonies is, therefore, a good indicator of individual identity. Somatic compatibility pairings were arranged on Shaw and Roth's Medium (Harrington et al. 1992) among all strains from the 2010 sample, as well as between all the strains from the 2011 sample and a subset of strains from the 2010 sample (Ar7, Ar9, Ar14 and Ar18). All strains were also self-paired. Following a three week period of incubation at 25°C, the plates were photographed using transmitted light and scored as either compatible or incompatible. The somatic incompatibility tests represented a means independent of the molecular genotyping to ensure that the strains selected for more intensive analysis were indeed representative of the same individual.

Strains from the 2010 sample were tested for somatic compatibility in all pairwise combinations. Somatic compatibility pairings with representatives of the 2010 sample, Ar7, Ar9, Ar14 and Ar18, were arranged to assess the SI grouping of the 2011 sample.

**Morphological analysis of 2010 sample**

If the *A. gallica* collections represent different individuals, then presumably there would be phenotypic differences among them. A further set of experiments was designed to assess morphological variation among the *A. gallica* strains. Thirteen of the genotyped strains were sub-cultured to Weinhold's Medium with 100µL of 30% filter-sterilized EtOH
smeared over each plate to promote rhizomorph formation (Weinhold and Garraway 1966). The addition of alcohol to Weinhold's Medium stimulates rhizomorph growth by preventing the accumulation of growth-inhibiting byproducts formed in a major alternate glycolysis pathway (Carroll and Garraway 1972). The inoculated plates were incubated in the dark at 25°C for a period of three weeks, and were photographed every 24hrs, for a period of 15 days, using both reflected and transmitted light sources. Observations were recorded for the following parameters: 1) total rhizomorph length (Fig. 6), and 2) number of rhizomorph tips. Total rhizomorph length was calculated using image J. Each strain derived from the 2010 samples was represented by three replicates.
Figure 6: Four days of rhizomorph growth. Petri plates containing Weinhold’s Medium with ethanol and a culture of *A. gallica* were photographed using a transmitted light source and the total rhizomorph length was measured each day using Image J.
Probing for variation within the Exe Island individuals

The region of the 26S rRNA gene amplified by the primers LR7R and LR12 (Table 2) is known to exhibit polymorphism for an indel of about 400 bases in the Armillaria population (Saville et al. 1996). Because this region is part of the tandemly repeated rRNA gene cluster, I reasoned that it would have enhanced potential for mitotic crossing over, and array homogenization (Liao 1999; Tsang et al. 1999; Liao 2000) and that this would appear as LOH.

PCR was carried out according to the following specifications, using LR7R and LR12 as forward and reverse primers:

\[
\begin{align*}
2 \text{ minutes at } 95 \degree C \\
30 \text{ seconds at } 95 \degree C \\
30 \text{ seconds at } 50 \degree C \\
2 \text{ minutes at } 72 \degree C \\
15 \text{ minutes at } 72 \degree C
\end{align*}
\]

Amplicons for this reaction were subjected to electrophoresis for 12 hours at 1.1 V/cm on 0.8% agarose gel to resolve allelic size differences in the amplified region. The gel was subsequently bathed for 45 minutes in 500mL of 1X TAE stained with 10 µm EtBr, and photographed under ultraviolet light.

Assaying for length polymorphisms and LOH at four microsatellite regions

The 1992, 2010 and 2011 samples were assayed for length polymorphisms at four microsatellite regions, which are variable in Armillaria populations in Europe (Prospero et al. 2010). PCR using the primers Arm02, Arm11, Arm15, and Arm16 was used to amplify
microsatellite markers (Table 2). The rationale here was that the higher mutation rate and/or potential for LOH would make these regions more likely to reveal variation within individuals than the SNP sites used for the initial genotyping. The forward oligonucleotide primers were labeled with either B-FAM or HEX dye phosphoramidites. PCR was done according to the parameters used in this study to amplify the anonymous regions previously characterized by Saville et al. (1996). The PCR products were outsourced to The Centre for Applied Genomics for fragment length analysis.

**Statistics**

The Kruskal–Wallis, one–way analysis of variance was used for significance testing of differences in continuously varying phenotypic traits across genetic individuals. The distributions for two measurements were tested: 1) total rhizomorph length over a period of 15 days of growth; 2) number of rhizomorph tips generated over a period of 15 days. The Kruskal – Wallis was implemented with JMP 9 statistical software.²

A Mantel test was used to assess the correlation between genetic distance and geographical distance matrices for the 2010 and the 2011 samples. The geographical distance matrix was created using the Geographical Distance Matrix Generator software obtained as shareware from the website of the Center of Biodiversity and Conservation. The Mantel test was performed using Microsoft XLSTAT for Mac 2011 software.

² JMP 9 obtained from www.jmp.com
RESULTS

In this section, I present the evidence from a) multiple SNPs for genotyping, b) mating interactions among single spore isolates (SSIs), c) somatic compatibility reactions, and d) measurement of morphological traits, indicating that there are two genetic individuals of *A. gallica* on Exe Island: a large individual covering most of the island, and a smaller individual in one restricted sector of the island (Fig 3). After identifying the individuals, I show that LOH has occurred in a region of the 26S rRNA gene in the large individual on Exe Island. I then describe a microsatellite marker that is variable among strains unambiguously characterized as belonging to the same *A. gallica* individual.

**Genotyping of the 1992, 2010 and 2011 samples**

Genotyping of the 1992, 2010 and 2011 samples revealed 12 polymorphisms in 5 amplified regions co-segregating 638 and Ar18 from the other Exe Island isolates. Also discovered were 7 polymorphisms in 3 amplified regions co-segregating 638, Ar18, Ar36, Ar37, Ar38, Ar39 and Ar41, from the other Exe Island isolates (Table 3). Our expectation at the outset was that Exe Island was host to one large *A. gallica* individual, whose territory extends over most of the island, and this expectation was confirmed. The majority multilocus genotype from the 1992, 2010, and 2011 samples was an exact match. In addition there is a smaller individual, represented in the 1992 sample by 638, in the 2010 sample by Ar18, and in the 2011 sample by Ar36, Ar37, Ar38, Ar39 and Ar41 (Fig. 3). Additional small individuals may be present, but undetected, on the study site.

The reference strains were isolates Ar4 and Ar6 (from the mainland close to Exe Island), and isolate 629 from Northern Michigan. Ar4 and Ar6 are co-segregated from the
Exe Island isolates, sharing 29 polymorphisms over 8 amplified regions (Table 3). The variation within the 1992 sample presents a situation analogous to that of the 2010 sample, where one isolate, 629, is consistently segregated from the other isolates. Isolate 629 is known to belong to the "Clone One" mentioned earlier (Smith et al. 1992), and is distinct from all other 1992 isolates at 20 sites spanning 7 amplicons (Table 3). The biallelic DP1c amplicon has proven especially valuable for verifying the identity of the Exe Island isolates. This is because only the isolates belonging to the large individual on Exe Island are homozygous for an allele (Table 3) in the DP1c region that has a frequency of only 0.204 in the overall population (Saville et al 1996). The probability of this genotype at the DP1c locus arising independently in the population, therefore, is $0.204^2$, or 0.042.

**Mating type analyses among single-spore isolates from Exe Island**

Interactions among 7 monospore isolates from Ar21 produced some indeterminate reactions, possibly exhibiting only 2 out of 4 mating types (Table A1). Interactions between 6 monospore isolates from Ar22 identified 3 out of 4 possible mating types (Table A1). Isolate Ar23 presented a similar situation, manifesting 3 out of 4 mating types (Table A1). Interactions between monospore isolates from Ar22 and Ar23 demonstrated shared mating types (Table 4). Similarly, the mating reactions among haploid isolates from all the basidiocarps showed incompatibility due to a sharing of mating types (Table 4). These reactions are consistent with Ar21, Ar22 and Ar23 belonging to the same individual and having the same set of mating-type alleles, as would be expected of isolates from the same individual.

<table>
<thead>
<tr>
<th>Amplified regions</th>
<th>E16f</th>
<th>I70f</th>
<th>J1C</th>
<th>DP4</th>
<th>E75f</th>
<th>C15f</th>
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<td>1</td>
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<td>1</td>
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<td>1992 A 629</td>
<td>Y Y Y Y R R A G G T K M R Y</td>
<td>T A C C T T T G T A</td>
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Ar21-2, Ar22-4 and Ar23-2 are haploid monospore isolates.
Empty spaces represent loci that were not assayed.
*Numbers indicate the collection year. Letters A,B,C & D indicate the individual identity of isolate.
Table 3 continued.

Amplified regions

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* *Numbers indicate collection year. Letters A,B,C & D indicate the individual identity of isolate.

Empty spaces represent loci that were not assayed.

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</table>

IUPAC nucleotide code

K = G/T
M = A/C
R = A/G
S = G/C
Y = C/T
W = A/T
Table 4: Mating incompatibility reactions between SSIs isolated from the Ar21, Ar22 and Ar23 basidiocarps.

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+ is indicative of a reduction of fluffy, aerial mycelium to a flat, crustose colony (a transition from haploid to diploid karyotype).
- is indicative of failure to mate due to the presence of similar mating type alleles.
Empty spaces represent crosses that were discarded due to contamination.
Observed Mating types are indicated in brackets. An additional mating type is present, that is neither A1B1 nor A2B2.
Somatic compatibility reactions among the 2010 and 2011 samples

The pairing of diploid strains from the 2010 and 2011 samples is consistent with the presence of two SI groups on Exe Island (Table A2; Table A3). The smaller of the two SI groups includes Ar18, Ar36, Ar37, Ar38, Ar39 and Ar41. The remaining strains from the 2010 and 2011 samples from Exe Island belong to the second SI group. Another SI group was formed by the reference strains Ar4 and Ar6. Self pairings were all compatible.

Morphological analysis of the 2010 sample

A Kruskal – Wallis one – way analysis of variance revealed the presence of more than one distribution in both total rhizomorph length (p < 0.008), and number of rhizomorph tips generated per colony (p < 0.016) after 15 days of growth of Weinhold’s medium + EtOH (Fig. 7). Ar4 and Ar6, isolated from the mainland off of Exe Island, display much higher rhizomorph growth rates (Fig. A1), and rates of rhizomorph tip generation (Fig. A2) than the 11 Exe Island strains assayed in this experiment.

LOH in the 26S rRNA gene

Genotyping of the LR7R / LR12 PCR product revealed a polymorphism attributable to as few as one event of LOH among the individuals on Exe Island (Figure 3). Fourteen of the 26 samples representing the large individual on Exe Island have lost heterozygosity in this segment of DNA (Table 5). How is this variation distributed spatially? A Mantel test excluding data from samples of the smaller individual (Ar18, Ar36, Ar37, Ar38, Ar39 and Ar41) showed that the spatial clustering of the variants is highly significant. The correlation between geographical distance for the two distinct
Figure 7: Phenotypic variation among strains from the 2010 sample. Total rhizomorph length: Mean + standard deviation. 

a- The external reference strains Ar4 and Ar6 generated a greater total rhizomorph length than strains from Exe Island after 15 days of growth. Kruskal – Wallis one-way analysis (p < 0.008).

b- External reference strains Ar4 and Ar6 generated more rhizomorph tips than strains from Exe Island after 15 days of growth. Kruskal – Wallis one-way analysis (p < 0.016).
Table 5: LOH at the 26S rDNA region.

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Allele 1 is distinguished from Allele 2 by the presence of a 400 bp insertion. Ar21-2, Ar22-4 and Ar23-2 are haploid monospore isolates.
+ indicates the presence of an allele.
- indicates the absence of an allele.
Empty spaces represent loci that were not assayed.
genotypes estimated from $10^4$ permutations of the data was strong ($p < 0.0001$). There was no variation in this region of DNA within the smaller Exe Island individual.

**Fragment length analysis of four microsatellite markers**

Amplification and fragment length analysis of 4 *FAM-B / HEX* labeled microsatellite markers disclosed variation between individuals; in one instance, variation was detected within an individual. The Arm02 and Arm15 markers were monomorphic and therefore not informative. The Arm11 marker is nearly monomorphic, but shows a LOH between Ar4 and Ar6, where the 149 bp allele present in Ar4 was lost in Ar6. The Arm16 region is polymorphic and agrees exactly with genotyping by PCR and Sanger sequencing in distinguishing individuals. Ar4 and Ar6 were co-segregated by a 3 bp motif deletion at the Arm16 marker (Table A4). The 1992 Michigan individual representative sample, 629, was segregated from the other 1992 sample, as well as from the 2010 and 2011 Exe Island samples, by a 3 bp deletion at the Arm16 marker, and by heterozygosity at the Arm11 marker.
DISCUSSION

My study system is unique in that ancestral and mutant genotypes are physically connected to one another in their natural settings and are readily analyzed in a spatial context. The radial nature of fungal vegetative growth results in a connected network of somatic cells embedded in their substrate, which is their source of food. In effect, this leaves a spatial record of where the fungus is now, and where it has been in the past. Illustrating this spatial pattern, Figure 4 shows a diploid mycelium of *Aspergillus nidulans*, within which spatial sectors of altered spore colour appear due to LOH and exposure of recessive mutations for spore colour. Could this same pattern persist among longer-lived fungi in nature? If so, then mutant sectors should be physically connected to their immediate non-mutant ancestors and discrete patches representing the mutant and non-mutant genotypes should be visible. This visualization would also allow precise measurements of rates and modes of mutation in a way not possible in populations in which mutants and their immediate predecessors are spatially inter-mixed.

*Armillaria* genets present an especially promising system for revealing the distribution of mutant sectors because a) their size permits sampling over many cell generations, increasing the likelihood of detecting mutations, and b) their longevity makes tracking of mutant sectors over long periods of time a possibility. *Armillaria* is a ubiquitous, opportunistic phytopathogen in temperate forests, and elucidating the dynamic of its vegetative phase may also provide a clearer understanding of how fungal infections spread in space and time. Usually these vital processes are hidden from sight in opaque substrates such as wood or soil, and consequently many fundamental questions of how fungi spread and infect remain unknown.
Unequivocally determining the individual identity of the isolates from Exe Island was a vital, but non-trivial, pre-condition for my investigation of variation within an *A. gallica* genet. An absolute consensus between multiple alternative methods of assessing individual identity, including genotyping by PCR and Sanger sequencing, mating compatibility, somatic compatibility, and morphological assay, resolved the presence of two genotypically and morphologically distinct groups of isolates on Exe Island. This set the stage for subsequent analysis of genetic variation within individuals.

Variation within individuals was detected as a LOH in the rDNA of the larger individual on Exe Island, and a LOH in a microsatellite region of the individual from the mainland. The LOH events must be mitotic because they occur within groups of isolates that share identical genotypes at multiple genomic regions, have a common array of mating specificities, and are somatically compatible with one another. Had the rDNA genotypes originated from independent mating events, other regions known to be polymorphic in the population would also show variation. For example considering the DP1c region alone, the frequency of A genotype is 0.204 in the population (Saville et al. 1996) and so the probability of the large individual of genotype AA for position 34 (Table 3) on Exe Island having originated in two independent matings is $0.204^2 = 0.042$. Using all polymorphic sites for all loci, and estimating their population frequencies from my sample of four unique individuals (total number of alleles counted = eight), this probability becomes much lower (Table A5). Admittedly, four is a small number of individuals from which to estimate allele frequencies in populations, but even with this large uncertainty, each polymorphic locus detected in the small sample of four drives the overall probability of independent origin lower.
Smith et al. (1992) have shown that large, old *Armillaria* individuals grow from a single mating event to cover large territories. During this extended period of asexual growth, mutations presumably occur, and these mutations may leave behind spatially discrete sectors of altered genotype. A comparison of the spatial distribution of the isolates exhibiting LOH at the rRNA locus (Fig. 3) to my predictions about the vegetative spread of mutant sectors (Fig. 8) favours a scenario characterized by the presence of two discrete sectors. One sector, the ancestral genotype before LOH, was heterozygous for the 26S rRNA gene insertion; the other genotype, after LOH, is homozygous for the presence of the insertion. The ancestral sector is in the northern region of Exe Island, and the sectors exhibiting the LOH are clustered to the south of the ancestral heterozygous isolates. Because the individual on Exe Island has likely resided there for hundreds of years, it is impossible to make inferences about the directionality of colonization from the occurrence of LOH. Once the resident individual has established the full extent of its territory, the mycelium should behave as a dissipative structure, with sectors senescing and being replaced on an ongoing basis. The fact that the sectors occur in two obvious clusters may, however, be suggestive of a rapid vegetative expansion, which would preserve the spatial integrity of the two genotypes and the nearly equal fitness of the genotypes before and after LOH within an individual. Alternatively, but less plausibly, Exe Island may be divided by some kind of quasi-allopatric barrier such as frequent disturbance, sub-optimal soil conditions, or the absence of suitable substrates, that keeps the two sectors homogeneous for their genotype with respect to the rRNA insertion.
Figure 8: Potential distributions of mutant sectors revealed by fine scale sampling. The spatial pattern of LOH in the large Exe Island individual closely resembles scenario 2.
CONCLUSIONS AND PERSPECTIVES

Given the distances between isolates in excess of 180m, and the large number of repeats in the microsatellite amplicons, a factor which, in other species, favours deletion over insertion of motifs (Harr and Schlötterer 2000, Lai and Sun 2003), it was somewhat surprising that variation was completely absent from the microsatellite regions of the Exe Island individuals. The absence of SNPs and microsatellite polymorphisms in other large Armillaria individuals has been previously documented (Hodnett and Anderson 2000; Langrell et al. 2001). As with the study of Hodnett and Anderson (2000), the reasons for the paucity of variation in my study could be a much lower than expected number of cell divisions associated with radial growth and with a lower than “normal” mutation rate.

The rate-of-cell-division issue could be resolved with modern techniques of genetic engineering and live imaging. A genetic construct with a fluorescently labeled (e.g., GFP, RFP, or other fluorophore) protein expressed only during mitosis can be visualized over time in a growing rhizomorph. This would make identification of mitotic cells possible without destructive sampling. Also, the availability of serial optical sections of high magnification over time, might make it possible to track individual cell pedigrees within the rhizomorph axis. This experimental scenario may be technically demanding, but it would, if successful, illustrate an important mechanism of protection against mutation – the minimization of apical cell divisions over distance covered – in a new phylum of organisms.

The other critical issue is to investigate is the actual cellular mechanisms that might be responsible for the high fidelity of DNA replication and / or the high efficiency of DNA repair in Armillaria. Much is known about these processes in bacteria and yeast (Kunkel
In addition to considering point mutations, we can also consider changes in microsatellite lengths. In humans, microsatellite instability (MIS) is correlated to the alteration of a number of genes involved in mismatch repair (hMSH2, hMLH1, hPMS1 and HPMS2), and is linked to tumorigenesis (Moslein et al. 1996, Loeb and Loeb 2000, Wu et al. 2011). With an annotated Armillaria genome, it may be possible to locate genes homologous to those involved in mismatch repair in yeast and other organisms. The genes that account for the high fidelity of DNA replication and repair in Armillaria might then be identified in a functional test by deleting them and then measuring the effect on mutation rate in experimental systems. If an unusually high efficiency of DNA repair and/or low rate of mutation were confirmed in the wild type of Armillaria, the ramifications may be broadly significant in biology, including cancer biology.

One limitation of my M.Sc. study is that, since I have identified only one variable marker in the large Exe Island individual, it is impossible to calculate the rate of LOH. Furthermore, the predominance of monomorphic or nearly monomorphic microsatellite markers limits the usefulness of these regions as baseline data points for a long-term study; variable markers would have provided a more interesting foundation. However, part of my contribution to future investigations on variation and mutation rates in A. gallica was to begin the process of obtaining the first fully sequenced genome for the species. To this end, DNA from Ar7, Ar9 and Ar14, was extracted, purified, and sent to the BGI (formerly the Beijing Genomics Institute) for whole-genome sequencing. Tantalizingly, the analysis including a 90 MB genome assembly and SNP identification was finished on Dec 10. The data indicates on the order of a few thousands of possible SNP candidates with the genome
of the larger individual on Exe Island. My thesis work now sets the stage for the exciting work to follow confirming the predicted mutations and analyzing their spatial distributions.

Although mutation rates and numbers of cell division back to most recent common ancestry are confounded with one another, there are two possibilities for moving forward. In one case, the fully annotated genomic data will enable the calculation of the minimum number of cell divisions between isolates. Assuming an average mutation rate of $10^{-9}$ to $10^{-11}$ events per base pair per genome, the number of cellular generations between two points can be obtained by dividing the number of SNPs by the mutation rate and the size of the genome. Knowledge of the number of cellular divisions per unit of space will increase the accuracy of age estimates, and of colonization history models for *Armillaria* individuals. Alternatively, if we knew the number of cell divisions per unit of growth, we could then estimate the true mutation rate of *A. gallica* in nature and assess whether or not it is abnormally low compared to other eukaryotes.

In the context of a fully sequenced genome, it will be possible to design primers specifically targeting known polymorphic sites in order to better resolve the spatial distribution of mutant sectors and, perhaps, to determine the degree of their relatedness to one another. This ongoing phylogeographical investigation promises to provide the first glimpse into the cryptic processes of mycelial turnover and growth in an *A. gallica* individual in its natural habitat. In effect, we will be able to “see” through the opaque substrate of soil and wood.

Fine-scale genotyping of the large Exe Island individual may reveal the following (Fig. 8): 1) That sectors are randomly distributed throughout the territory. In this case, it is difficult to attribute a selective advantage to any of the genotypes, or to determine the
trajectory of their propagation. This data would be used as a baseline for tracking the genotyped sectors over time. 2) That mutant sectors are clustered, with similar genotypes occupying the same region, and becoming increasingly dissimilar with distance. This result would be useful in determining growth dynamics within an individual because sectors with similar genotypes are presumably more closely related. 3) As exhibited by the LOH at the rRNA locus, the territory may be dominated by one or more genotypically distinct sectors. This scenario would imply one or more of the following: a) these dominant genotypes may have undetected, selectively advantageous polymorphisms in important coding or regulatory regions; b) the vegetative mycelium of the dominant sectors might be growing at a much faster rate than anticipated; or c) the vegetative mycelium is currently in the aftermath of a selective sweep.

Hayden et al. (2011) studied the robustness of RNA molecules under stress from mutational load. Their findings suggest that phenotypic plasticity can play a stabilizing role, masking the effects of slightly deleterious mutations, and allowing for the accumulation of variation, which increases the probability of a beneficial mutation (Hayden et al. 2011). When such a mutation does arise, evolution can occur in rapid spurts, driving a selectively advantageous phenotype to fixation in a relatively short period of time, and resulting in a decrease in diversity within a population (Wagner 2008). Rapid evolution is therefore preceded by a neutralist regime characterized by the accumulation of nearly neutral mutations, and an increase in diversity. This biphasic evolutionary mechanism may affect the frequency and distribution of genotypes on Exe Island. Diploidy and LOH may represent, respectively, the buffering and exposure of mutant phenotypes expected under this scenario.
Another consideration with respect to the observed distribution of mutant sectors is that the spatial proximity of isolates may not always be a reliable indicator of their degree of relatedness, especially at the smaller spatial scales. Rhizomorphs forage, encounter uncolonized resource units, and establish new colonies. In turn, these colonies project rhizomorphs radially in search of fresh substrate. Some of these rhizomorphs may grow back in the direction of the progenitor colony, fusing with other rhizomorphs (Lamour et al. 2007), and it is even possible that rhizomorphs from a new colony will anastomose with the progenitor, or another somatically compatible mycelium, strengthening the connectivity between the two colonies, as well as introducing any mutations that have been accumulated along the trajectory of growth. This all hinges on the absence of competition among the mutant sectors of the same individual. Since mutation would only very rarely affect somatic compatibility, this absence of competition is most plausible. If this were the case, then multiple genotypes should be identifiable by fine-scale sampling within connected colonies. The strain-isolation method I have used would fail to detect this type of within-colony variation because a laboratory strain was isolated from a small population of hyphae fanning out from the end of a 0.5 – 1.0 cm section of surface-sterilized rhizomorph. The resulting isolate is therefore not representative of the total genetic composition of the mycelium colonizing the resource unit – furthermore, if variation were present in the laboratory strain in the form of a SNP, there is a possibility that the presence of two homozygous loci would be mistakenly interpreted as one heterozygous locus.

With a sequenced genome, it will be possible to design a large set of primers to test for variation at hundreds or thousands of sites known to be polymorphic within the large Exe Island individual. These primers can be used to assay mycelial colonies occupying
discrete resource units for variation on a very fine scale directly from the environment, sidestepping the stochasticity implicit in the practice of obtaining an axenic strain from rhizomorph isolates. If within-colony variation is a reality, its quantification can be used as a measure of the strength of connectivity between two colonies, because colonies that are weakly joined to the rhizomorph network are expected to exhibit fewer constitutive genotypes than colonies that are strongly connected.

Finally, comparisons between the mutational load of the Exe Island individual with other *A. gallica* individuals under various degrees of stress from environmental pollution is another avenue now open for future researchers. More than three decades ago, Klekowski (1976) considered fern rhizomes in a way analogously to how I am considering fungal rhizomorphs, and discovered that an *Osmunda regalis* individual from a polluted environment carried a higher mutational load than an individual from a comparatively pristine environment. Because *Armillaria* is widely distributed in temperate forests, it would be eminently feasible to reconstitute Klekowski’s experiment with large *Armillaria* individuals instead of ferns. An interesting location for this study would be the temperate forest around the site of the 1986 Chernobyl nuclear accident. As the rate of mutation is known to increase upon exposure to radioactivity (Weinberg et al. 2001; Dubrova et al. 2002; Kovalchuk et al. 2000), the expectation would be that *Armillaria* individuals in the Chernobyl region would carry a higher mutational load than the individual on Exe Island. Not only would it be possible to characterize the kinds of mutations that occurred and their recessivity or dominance, but it would also be possible to estimate when they occurred, i.e., before or after the catastrophic nuclear accident of 1986.
BIBLIOGRAPHY


APPENDIX

Table A1: Mating incompatibility reactions among SSIs isolated from the same basidiocarp.

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+ is indicative of a reduction of fluffy, aerial mycelium to a flat, crustose colony (a transition from haploid to diploid karyotype).
- is indicative of failure to mate due to the presence of similar mating type alleles.
One mating type is present.
Seven SSIs were isolated and mated from basidiocarp Ar21; 6 SSIs were isolated and mated from basidiocarp Ar22, and 7 SSIs were isolated and mated from basidiocarp Ar23. The 21-1 / 21-7 cross was discarded due to contamination.
Table A2: Somatic compatibility reactions among isolates from the 2010 sample.

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+ = compatibility
- = incompatibility
Empty spaces represent missing data points.
Table A3: Somatic compatibility between isolates from the 2011 sample and a subset of isolates from the 2010 sample.

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+ = compatibility  
- = incompatibility  
Empty spaces represent confrontations that were discarded due to contamination.
Table A4: Variable microsatellite markers.

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Note: Ar17 and Ar37 did not amplify
Table A5: Allele frequencies at polymorphic regions. Allele frequencies ($f$) are generated based on a sample size of 4 individuals from this study. Probabilities ($P$) are calculated for the Exe Island “Large individual,” the Exe Island “Small individual,” and the external reference individual “Ar4/Ar6,” genotypes having resulted from 2 mating events.

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<th>Region / Locus</th>
<th>Clone One</th>
<th>Exe Small</th>
<th>Exe Large</th>
<th>Ar4/Ar6</th>
<th>Allele freq.</th>
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(P) two matings: N/A 1.04 x 10^{-4} 1.61 x 10^{-4} 3.75 x 10^{-5}

*The allele frequencies of DP1c are listed as per Saville et al. (1996)
Figure A1: Rate of rhizomorph production. Mean + standard deviation are displayed for each strain in the phenotype assay over 3 replicates (n=3). The total length of rhizomorphs was calculated using Image J.
Figure A2: Rate of rhizomorph tip generation. Mean + standard deviation are displayed for each strain in the phenotype assay over 3 replicates (n=3).
At23

![Graph showing the number of rhizomorph tips over days of growth.](image)

Days of growth

Number of rhizomorph tips