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I am submitting herewith a thesis written by Shannon M. Tieken entitled "*Artomyces pyxidatus* (Auriscalpiaceae, Fungi) in the Great Smoky Mountains National Park: Individuals and Populations." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

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ARTOMYCES PYXIDATUS (AURISCALPIACEAE, FUNGI) JÜLICH IN THE GREAT SMOKY MOUNTAINS NATIONAL PARK: INDIVIDUALS AND POPULATIONS

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ABSTRACT

Artomyces pyxidatus is a North-temperate saprophytic coral fungus found in Europe, Asia, and North America. Collections from the Great Smoky Mountains National Park were used in this study to define the size of an individual and elucidate gene flow between three locations within the park (Greenbrier, Sugarlands, Cataloochee). Molecular techniques were used to determine the relationships between populations and among individuals. Two inter-simple sequence repeat (ISSR) primers amplified segments of DNA scattered throughout the genome and together produced 26 variable bands. ISSR analysis appeared to be a reliable technique for defining individuals, but should be used in conjunction with other techniques that are able to eliminate ambiguity and ensure accuracy. The nuclear ribosomal ITS 1-5.8S-ITS 2 (ITS) repeat provided a more defined genetic region and was studied using restriction fragment length polymorphisms (RFLP). An insertional element located within a Group I intron contained by the ribosomal 18S subunit was found in most eastern North American *A. pyxidatus* collections and was amplified and used to supplement the other molecular analyses by verifying the identity of individuals (Lickey 2002).

Analyses based on genetic characterization of multiple fruitbodies from single logs indicated that several *A. pyxidatus* individuals could occupy a single substrate and that the mycelia could reach a measured size of 1.9 meters. Data also showed that the three GSMNP populations were not in Hardy-Weinberg equilibrium and were, therefore, not undergoing random gene exchange. A deficiency of heterozygotes suggested that the cause was inbreeding. The Greenbrier population appears to be the most genetically diverse based on Neighbor Joining and Principle Coordinates analyses. Both techniques

indicated that the three populations were interrelated and had genetic characters that produced little phenetic clustering based on collection location. Unique ISSR alleles, however, were found in all three populations, suggesting some level of isolation.

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CHAPTER ONE

INTRODUCTION

The fungus *Artomyces pyxidatus* is usually found in cove hardwood forests of the United States. It is particularly common in the Great Smoky Mountains National Park (GSMNP). Little is known about fungal spore dispersal methods, individual size, or population structure. *Artomyces pyxidatus* was selected for the purpose of defining the size of a fungal individual. Although this species could not be considered a representative of all fungi, it did establish a standard of comparison for saprobic fungi. This study was also intended to evaluate the genetic interaction of separated populations of *A. pyxidatus* through the use of molecular techniques. The presence of high and low altitude ridges between the populations provided a means to evaluate limitations to gene flow due to geographic barriers.

Species characteristics - *Artomyces pyxidatus* Jülich is characterized by fruitbodies with branches in ranks of two to five, occasionally dichotomous, with tips that are pointed or cup-shaped. Basidiomata are generally yellow in color and acrid in taste. The species is separated from the other members of the genus based on narrow spores (< 3.0 μm wide) and variably shaped gloeocystidia with rounded or acute apices (Dodd 1972). *Artomyces pyxidatus* is a lignicolous species found primarily on deciduous wood such as *Liriodendron tulipifera*, *Quercus*, and *Acer saccharum*. It is distributed throughout the temperate forests of Asia, Fennoscandia, northern Europe, and eastern North America (Wu 1995). Collections from Costa Rica and Mexico have also been verified as being *A.*

pyxidatus (Lickey 2002). *Artomyces pyxidatus* recently replaced the former taxonomic name of *Clavicorona pyxidata* (Lickey 2002).

What constitutes an individual? – Little is known about what constitutes an individual in Basidiomycete fungi. Anderson and Kohn (1995) concluded that fungi with dikaryotic or diploid mycelia should be defined as individuals (genets) using the same system as that for plants. This system describes a genet as all vegetative structures derived from a parent mycelium whether connected or detached from the parental structure. Since fungal mycelia are underground or obscured by substrate, estimates of individual size must be made by examining fruitbodies and determining if two or more fruitbodies represent a single genetic mycelium (i.e. they have the same nuclear and mitochondrial genes). A few studies (Anderson *et al* 1998, Dahlberg and Stenlid 1994, Gherbi *et al* 1999, Paavolainen *et al* 2001, Redecker *et al* 2001, Smith *et al* 1992) have used molecular techniques to circumscribe an individual. In *Armillaria gallica*, Smith *et al* (1992) used restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNAs (RAPD), and mitochondrial DNA to show that a single individual invaded more than 30 acres of soil in northern Michigan and, by statistically analyzing the yearly growth rate, concluded that the fungus took approximately 1500 years to grow to the observed size. In contrast, studies by Kay and Vilgalys (1992) with *Pleurotus ostreatus* using mating antagonism reactions, suggested that individuals were much smaller and that, for this fungus, at least 15 individuals could occupy the same log. This latter example resembles the behavior of *A. pyxidatus* because both of these fungi are

saprobes and may be what Schmitt (2001) refers to as unit-restricted fungi. These fungi appear to be restricted to ephemeral patches of substrate and do not propagate through vegetative growth to new substrates. Sexual reproduction and sore dispersal can increase gene flow between populations and may produce small isolated genets (Anderson *et al* 1998, Zhou *et al* 1999). A population with a large clonal mycelial distribution suggests a mature asexual colonization such as that of *A. gallica* (Smith *et al* 1992). A comparison of various fungi and their putative sizes is presented below.

Laccaria amethystina	$\leq$ 1.5 m	Gherbi et al 1999
Russula cremoricolor	1.27 m	Redecker et al 2001
Amanita francheti	4.7 m	Redecker et al 2001
Lactarius xanthogalactus	7.3 m	Redecker et al 2001
Cortinarius rotundisporus	$\leq$ 30 m	Sawyer et al 1999
Suillus bovinus	20-40 m	Dahlberg and Stenlid 1994
Armillaria bulbosa	30+ acres	Smith et al 1992

Preliminary studies with *A. pyxidatus* collections from a 10 m^2 plot in Wisconsin suggests that a single *A. pyxidatus* individual can occupy several adjacent logs (K. Hughes pers. comm.).

Population studies – A number of molecular approaches have been used to examine population variability in fungi including sequence analysis of the ribosomal intergenic spacer regions (James *et al* 2001) and internal transcribed spacer (ITS) regions (Hughes *et al* 1999), RAPDs (Cole and Kuchenreuther 2001), RFLPs (Kerrigan *et al* 1993, Methven *et al* 2000, Kretzer *et al* 2000), inter-simple sequence repeats (ISSR) (Zhou et al 1999, Longato and Bonfante 1997) and combinations of two or more of these techniques (Anderson *et al* 1998). In each of these studies, molecular techniques were utilized to

better understand the genetic interaction and relationships within and between populations. These interactions are different depending on the fungus.

There must be a transfer medium (air, water, animal or insect transport) in which fungal spores can travel for gene flow to occur between populations. Gene flow may also occur through mycelial contact between two individuals of separate populations. Rishbeth (1959) found that air currents dispersed the spores of *Fomes annosus* and *Peniophora gigantea* approximately 200 miles from their source. Oceanic currents and human activities were suggested as the medium for distributing genetic material between *Pleurotopsis longinqua* populations located in Chile and New Zealand (Hughes *et al* 1998). Once the spores have been distributed and a population has been established, it is free to exchange spores with another population if the dispersals intersect (parapatric populations). Low level gene flow between two areas permits the divergence of populations, whereas high gene flow favors a single cohesive population (Petersen and Hughes 1999).

Effective barriers to gene flow are not well identified for fungi. Sometimes allopatric populations separated by continental and oceanic obstructions exhibit common genetic characters so that distant populations appear genetically similar (Methven *et al* 2000, Kerrigan *et al* 1993, Kerrigan *et al* 1995). A preponderance of evidence, however, suggests that this is unusual and that large environmental barriers like mountain ranges and oceans are effective deterrents to gene flow (Methven et al 2000, Hughes *et al* 1999, Vilgalys and Sun 1994, Kay and Vilgalys 1992, E. Grand pers. comm.). In contrast,

populations with little geographic obstruction are most often homogenous (Kerrigan *et al* 1999, Smith *et al* 1992).

Molecular techniques -

ISSR analysis – Within the genome of organisms including fungi are segments of DNA called microsatellites that vary in the number and length of repeated sequences. The ISSR technique involves the use of complementary primers that amplify the intervening DNA sequence. This process theoretically samples from the entire genome of an organism [microsatellites tend to be concentrated near telomeres and centromeres which means most of the amplified DNA fragments are also from these regions (A. Wolfe pers. comm.)]. After the DNA from every collection is amplified and electrophoresed on an agarose gel, the bands are correlated and individually scored based on presence or absence of a band. From the data generated by this process, a Neighbor Joining (NJ) tree and Principle Coordinates Analysis (PCA) (Rohlf 2002) can be constructed to analyze relationships based on character similarity.

Analysis of ISSR bands has been deemed a reliable method for determining genetic variability within and between species of ectomycorrhizal fungi (Longato and Bonfante 1997). Zhou *et al* (1999) used ISSR analysis to determine genets of *Suillus grevillei* and predict their method of proliferation. Smith *et al* (1992) used random amplified polymorphic DNA (RAPD) analysis to determine the size of *A. gallica* and the reproductive mechanism utilized by the fungus, but results from this method are difficult to duplicate (Burt *et al* 1994, Longato and Bonfante 1997). Analysis of ISSRs, therefore,

is a more suitable method due to its reliability, efficiency, minimal requirement for prior knowledge of the specific genetics of the organism, sensitivity, and established ability to determine relationships between and among species (Longato and Bonfante 1997, Zhou *et al* 1999).

RFLP analysis – Restriction fragment length polymorphisms identify individuals based on differences in size and number of DNA fragments generated when digested with specific restriction endonucleases. One of several advantages to using RFLP analysis is the diversity of applications. Previous studies using *Hae*III and *Bst*F51 in *Flammulina,* distinguished major species within the Northern Hemisphere while further digestion of *F. velutipes* with *Bgl*I and *Bst*UI distinguished specific biogeographical patterns (Methven *et al* 2000). Bidartondo *et al* (2000) identified ectomycorrhizal groups using RFLP analysis of the (ITS) region with *Alu*I, *Hinf*I, and *Cfo*I restriction enzymes to determine that the relationship between *Sarcodes sanguinea* and *Rhizopogon ellenae* is mutualistic rather than parasitic. The restriction enzyme *Taq*I was used to digest ITS in *Pleurotopsis longinqua* so that estimates regarding genetic divergence between allopatric populations could be made and sequence differences could be compared (Hughes *et al* 1998).

In this study, RFLP analysis was used to detect polymorphisms within the ribosomal ITS region. Characterization of this region was intended to identify enough individual differences in sequence and fragment size that conclusions could be drawn about the size of the individuals and gene flow between populations.

18S ribosomal subunit analysis – A character unique to *A. pyxidatus* that was useful in determining individuals was the presence of a Group I intron in the 18S ribosomal repeat (Hibbett 1996). Complicating this insertion further was the presence of an insertion within the intron of eastern U.S. *A. pyxidatus* collections (Lickey 2002). Although the ribosomal repeat is generally regarded as a conserved segment of DNA, heterogeneity may not be uncommon. Gowan and Vilgalys (1991) found variability in the length of repeated copies of ribosomal DNA within *Xylaria magnoliae* individuals, so this finding could be a reasonable explanation for the broad DNA banding patterns. The data from this technique was therefore used only as a supplement to ISSR data to identify individual clones.

CHAPTER TWO

MATERIALS AND METHODS

Collections and Sampling - Collections of *A. pyxidatus* were made from May 17, 2001 to July 8, 2001 at three locations in the Great Smoky Mountains National Park (Fig. 4.1). The sites chosen were Greenbrier, Tennessee (to provide data for determination of individual size), Sugarlands, Tennessee (west of Greenbrier and separated by low ridges) and Cataloochee, North Carolina (separated from the other two areas by the crest of the Smoky Mountains). Each site was surveyed for fruitbodies by searching for fallen logs in groves of hardwood trees. When a fruitbody was located, it was collected in its entirety, described in a field book, and transported back to the lab where it was cultured, dried, and stored. Collections with proliferative and uncontaminated cultures were recorded in a database (CULTENN). For the Greenbrier site only, logs with several distinctly separated fruitbodies were located and the position of each fruitbody on the log was measured and recorded. Several individual basidiomes were collected from the same log. On two logs measuring 9.5 meters and 7 meters, 16 and six individual basidiomes formed, respectively. The position of collections were measured and mapped on the log (Fig. 3.7). Several fruitbodies were collected from single substrates to assess the clonal expansion of individuals. These substrates were visited and sampled again whenever new fruitbodies developed. The majority of the collections from the Cataloochee site were made by previous fieldworkers. Since a sample size of approximately 25 collections was desired, only 6 fresh collections were made to supplement the 19

Table 2.1 List of Collections

Field Book No.	Location	Collection Date
1616	Cataloochee	30.VI.89
1617	Cataloochee	30.VI.89
6189	Greenbrier	17.V.01
6190	Greenbrier	17.V.01
6191	Greenbrier	17.V.01
6192	Greenbrier	23.V.01
6193	Greenbrier	23.V.01
6194	Greenbrier	23.V.01
6195	Greenbrier	23.V.01
6196	Greenbrier	23.V.01
6197	Greenbrier	23.V.01
6198	Greenbrier	23.V.01
6199	Sugarlands	23.V.01
6200	Sugarlands	23.V.01
6609	Cataloochee	29.V.93
6611	Cataloochee	29.V.93
6612	Cataloochee	29.V.93
6614	Cataloochee	29.V.93
6615	Cataloochee	29.V.93
6616	Cataloochee	29.V.93
6617	Cataloochee	29.V.93
9239	Cataloochee	14.VI.97
9240	Cataloochee	14.VI.97
9243	Cataloochee	14.VI.97
9244	Cataloochee	14.VI.97
9245	Cataloochee	14.VI.97
9246	Cataloochee	14.VI.97
9277	Cataloochee	29.V.98
9278	Cataloochee	29.V.98
9279	Cataloochee	29.V.98
9281	Cataloochee	29.V.98
9350	Sugarlands	23.V.01
9351	Sugarlands	23.V.01
9352	Sugarlands	23.V.01
9354	Greenbrier	4.VI.01
9355	Greenbrier	4.VI.01
9356	Greenbrier	4.VI.01
9357	Greenbrier	4.VI.01
9358	Greenbrier	4.VI.01
9359	Greenbrier	4.VI.01
9360	Greenbrier	4.VI.01
9361	Greenbrier	4.VI.01
9362	Greenbrier	4.VI.01
9363	Greenbrier	4.VI.01
9364	Greenbrier	4.VI.01
9365	Greenbrier	4.VI.01
9366	Greenbrier	4.VI.01

Table 2.1 continued

9367	Greenbrier	4.VI.01
9368	Greenbrier	4.VI.01
9369	Greenbrier	4.VI.01
9370	Greenbrier	4.VI.01
9371	Greenbrier	4.VI.01
9372	Greenbrier	4.VI.01
9373	Greenbrier	4.VI.01
9374	Sugarlands	4.VI.01
9375	Sugarlands	4.VI.01
9376	Sugarlands	4.VI.01
9377	Sugarlands	4.VI.01
9379	Sugarlands	4.VI.01
9380	Greenbrier	14.VI.01
9381	Greenbrier	14.VI.01
9382	Greenbrier	14.VI.01
9383	Greenbrier	14.VI.01
9384	Greenbrier	14.VI.01
9385	Greenbrier	14.VI.01
9386	Sugarlands	19.VI.01
9387	Sugarlands	19.VI.01
9388	Sugarlands	19.VI.01
9389	Sugarlands	19.VI.01
9390	Sugarlands	19.VI.01
9391	Sugarlands	19.VI.01
9392	Sugarlands	25.VI.01
9393	Sugarlands	25.VI.01
9394	Sugarlands	25.VI.01
9395	Cataloochee	29.VI.01
9396	Cataloochee	29.VI.01
9397	Cataloochee	29.VI.01
9398	Cataloochee	29.VI.01
9399	Cataloochee	29.VI.01
9400	Cataloochee	29.VI.01
9863	Sugarlands	8.VII.01
9864	Sugarlands	8.VII.01
9865	Sugarlands	8.VII.01

previously collected. Collections were made every 5-10 days according to rainfall patterns. Fruitbodies generally formed 2-3 days after significant amounts of rain, so collecting was scheduled within that time frame. A list of collections is given in Table 2.1.

Culture techniques and storage - Collections were wrapped in wax paper to prevent desiccation and transported to the lab. In a sterile Laminar flow hood, an inner branch of the fruiting structure was excised and affixed with Vaseline to the upper surface of a Petri lid. This procedure minimized contamination that could be present on the tissue surface and allowed spores to fall on the nutrient agar. Each collection was cultured on malt extract agar (MEA; see Appendix) in 50 x 16 mm Petri plates. Plates were placed nearly vertical against a support to allow a diffuse spore drop over the surface of the agar. The tissue was removed from the lid after 24 hours. Spores on the agar surface germinated, resulting mycelium proliferated and anastomosed to create dikaryotic mycelia. To further minimize contamination, a portion of the agar containing growth was removed after 2-3 days and positioned on a fresh MEA plate to continue growing. All plates were sealed with two layers of Parafilm to prevent outside contamination by mites or airborne spores. For collections retrieved from cold room storage, a piece of the agar slant was excised and deposited directly in a jar containing potato dextrose broth (PD; Appendix). For collections cultured on MEA through spore prints, a portion of the agar containing mycelium was cut out and deposited in the jar of PD broth. Dikaryotic isolates were stored in two ways: 1) approximately six squares of agar were cut from the plate and

transferred to a 2 ml microvial filled with 1.8 ml of sterile distilled water and stored at room temperature (Burdsall and Dorworth 1994); and 2) two plugs of mycelia were removed and transferred to two MEA agar slants. Slants were maintained at room temperature until mycelia colonized the agar surface and then one was stored at 11 C while the other remained at room temperature as a backup.

DNA extraction - Samples of *A. pyxidatus* growing in liquid media were grown until a thick mycelial mat covered the entire upper surface of the liquid. The contents were then poured through a thin mesh material that retained sample tissue and allowed the PD broth to drain into an Erlenmeyer flask. The initial agar inoculum plug was removed and the tissue was blotted with paper towel to absorb excess liquid and weighed on a balance. Approximately 300 mg of tissue was used for DNA extractions. The remainder of each sample was frozen and stored at –80 C. Tissue was ground with a mortar and pestle in Carlson Lysis Buffer (1991), transferred to a 1.5 ml microfuge tube, and heated for 30-60 minutes at 74 C with inversion every 10 minutes. Samples were centrifuged for 10 minutes at 10,000 rpm in a microcentrifuge and the supernatant decanted into a clean microfuge tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the solution, the tubes were vortexed, and the samples were again centrifuged for 10 minutes at 10,000 rpm. The supernatant containing the DNA was removed and measured and an equal volume of isopropanol was added to precipitate nucleic acids. The samples were incubated for approximately 15 minutes at room temperature, centrifuged at 10,000 rpm for 10 minutes, and the nucleic acid pellet washed with 200 µl of ice cold absolute

ethanol. The sample was centrifuged and the pellet was dried until all traces of alcohol were evaporated and the pellet was resuspended in 50 μl Tris EDTA buffer. Two microliters of RNAse diluted 1:25 were added and the DNA was examined by gel electrophoresis. Extracted DNA was stored at –20 C.

Gel electrophoresis - Each sample of extracted DNA was quantified by agarose gel electrophoresis. A 1.5% agarose gel was prepared (Appendix). The mixture was heated in a microwave until it began to boil. Boiling was allowed to continue for 30 seconds at which time the flask was removed from the microwave and the mixture was examined for complete melting of agarose. After a slight cooling, the gel was poured and allowed to solidify around a plastic comb. After solidification, the comb was removed and 5 μl of the DNA extract combined with 2 μl of 10X loading dye (Appendix) was loaded into a well. A ladder standard using λ *Hind* III digestion (Promega) was loaded in a separate well as a molecular size standard. Electrophoresis was performed at 100 volts until the bromphenol dye marker had migrated approximately ¾ the length of the gel. The gel was then viewed under UV illumination and photographed with a Kodak DC120 camera using Kodak 1-D gel documentation system.

ISSR - ISSR analysis was performed on each of the 84 collections. Thirty-one ISSR primers were initially examined to determine if they gave sufficient resolution. Selection was based on gel resolution of primed regions, amplification success and complexity, and intracontinental variation between samples. A list of tested primers is given in Table 2.2.

Table 2.2 ISSR primers examined. ISSR primers listed with sequence, intracontinental and intercontinental variation, and amount of amplified background noise. Highlighted primers were selected for use in the ISSR analysis.

Primer	Sequence	Intracontinental	Intercontinental	Noise
805	$(TA)_8$-C	no amplification	no amplification	-
807	$(AG)_8$-T	no amplification	no amplification	-
810	$(GA)_8$-T	yes	yes	yes
811	$(GA)_8$-C	no	yes	no
817	$(CA)_8$-C	yes	no	no
818	$(CA)_8$-G	no amplification	no amplification	-
823	$(TC)_8$-C	yes	no	-
825	$(AC)_8$-T	yes	yes	no
834	$(AG)_8$-YT	yes	yes	yes
835	$(AG)_8$-YC	yes	yes	no
836	$(AG)_8$-YA	no amplification	no amplification	-
839	$(TA)_8$-RG	no amplification	no amplification	-
855	$(AC)_8$-YT	no amplification	no amplification	-
858	$(TG)_8$-RT	no amplification	no amplification	-
861	$(ACC)_6$	yes	no	no
862	$(AGC)_6$	yes	yes	yes
864	$(ATG)_6$	yes	no	no
867	$(GGC)_6$	smeared	smeared	-
871	$(TAT)_6$	no amplification	no amplification	-
872	$(GATA)_4$	no amplification	no amplification	-
873	$(GACA)_4$	yes	yes	no
875	$(CTAG)_4$	large fragment	large fragment	-
876	$(GATA)_2(GACA)_2$	no amplification	no amplification	-
880	$(GGAGA)_3$	no	yes	no
881	$(GGGTG)_3$	no amplification	no amplification	-
890	VHV-$(GT)_7$	yes	yes	no
$(GTG)_5$	$(GTG)_5$	no	no	yes
MANNY	$(CAC)_4$-RC	yes	yes	no
MAO	$(CTC)_4$-RC	no	yes	no
OMAR	$(GAG)_4$-RC	yes	yes	no
TERRY	$(GTG)_4$-RC	yes	yes	yes

Five *A. pyxidatus* controls were used to test each of the primers. The field numbers and locations for each were 4289-Switzerland; 4091-Highlands, NC; 9235- Metcalf Bottoms, TN; 6601-Greenbrier, TN; 6602-Greenbrier, TN. The selected primers were MAO and MANNY (Wolfe 2001). The PCR protocols were: initial melting cycle of 94 C for 3 minutes, 35 repetitions of a three step amplification cycle of 94 C for 1 minute, 52 C for 1 minute, and 72 C for 1 minute, ending with an extension cycle of 72 C for 1 minute. The components of each PCR sample were: 32.5 μL ddH_2O, 5 μL 10X PCR buffer without MgCl (Promega), 6 μL MgCl (25 mM; Promega), 4 μL dNTPs (10 mM), 2 μL primer (10 μM), 1 μL bovine serum albumin (400 ng/μL), and 0.5 μL *Taq* polymerase (2 units; Promega).

Each sample was electrophoresed on a 1.5% agarose gel at 100 volts. All gels included a control and a standard (ϕX *Hae*III digestion; Promega) for comparison when scoring. Composite gels consisting of 2-3 samples from each gel were run to further correlate band identity between gels. To score data, the following steps were performed: 1) each sample was individually scored for presence or absence of bands; 2) bands identified from each gel were correlated with all of the other gels; 3) one gel that correlated well with many of the other gels and thus required the least amount of extrapolation was selected as the standard; and 4) a final table of band numbers was produced and each sample was recorded as 0 (absence) or 1 (presence) based on the correlations.*ITS 1-5.8S-ITS 2* - The nuclear ribosomal ITS region was amplified using forward primer ITS 5 and reverse primer ITS 4 (White *et al* 1990). PCR amplification was performed according to the same parameters as listed for ISSR analysis. The ITS

region was quantified in a 1.5% agarose gel and viewed under ultraviolet light prior to restriction digest use.

RFLP - The ITS region was surveyed using restriction enzymes *Cfo*I (Promega), *Bse*DI (MBI Fermentas), and *Xho*I (New England BioLabs) according to manufacturers' instructions. Restriction sites for these enzymes were previously identified as variable in the Southeastern United States (Lickey 2002). Restriction digests included 7.75 μL sterile water, 2 μL 10X buffer, 0.25 μL restriction enzyme, and 10 μL PCR reaction. The digests were incubated at 37 C for 24 hours. Restriction fragments were separated on 1X TBE 1.5% agarose gels stained with ethidium bromide and viewed under ultraviolet light.

Restriction fragment data were collected on each sample based on presence or absence of the restriction site. Homozygous collections were recorded as a 1 (presence) or a 0 (absence) while those collections exhibiting heterozygosity for the restriction site were recorded as 1, 0 (presence and absence).

18S ribosomal RNA fragment - A portion of the nuclear ribosomal 18S subunit was PCR-amplified using the forward primer SR1c and reverse primer NS6 (Hibbett 1996). The remaining PCR components were the same as those listed for ISSR analysis. The PCR protocol was: initial melting cycle of 94 C for 4 minutes, 35 repetitions of a three step amplification cycle of 94 C for 30 seconds, 60 C for 30 seconds, and 72 C for 2 minutes, ending with an extension cycle of 72 C for 1 minute. The 18S fragments were separated on a 1X TBE 1.5 % agarose gel stained with ethidium bromide and visualized under

ultraviolet light. Both λ *Hind* III and ϕX 174 *Hae* III (Promega) were used as molecular size standards on each gel to encompass the broad molecular size range of the fragments.

Numerical analysis - Data were analyzed using the NTSYS-pc (Rohlf 2000) program. The Neighbor Joining (NJ) analysis was generated within this program as well as the Principle Coordinates Analysis (PCA). After generating a compatible file, data from NTSYS-pc were converted for use by the Phylip program (Felsenstein 1989). A Dollo parsimony analysis was generated from this program as a comparison to the PCA results. The remaining numerical analyses (Hardy-Weinberg equilibrium, migration values, and inbreeding coefficients) were calculated manually using the following equations:
Hardy Weinberg Equilibrium for four alleles where p, q, r, and s are haplotypes based on RFLP data: $(p+q+r+s)^2=(p^2+2pq+2pr+2ps+q^2+2qr+2qs+r^2+2rs+s^2)$

Migration: $F_{st}=1/(4Nm+1)$
Where N equals population size, m equals migration rate between populations and F_{st} equals the amount of inbreeding due to subpopulation division (fixation index).

Inbreeding Coefficient: $F_{is}=(H_s-H_i)/H_s$
Where H_s equals the expected heterozygosity of an individual in a randomly mating population (2pq), H_i equals the heterozygosity of an individual in a subpopulation, and F_{is} equals the amount of individual inbreeding due to non-random mating within subpopulations.

Only one representative value for the duplicate samples (basidiomata identified as identical through ISSR analysis) was used when calculating the above figures. A program available via the internet, GenePop (Raymond and Rousset 1996), was also used to determine linkage disequilibrium and to calculate inbreeding. This program was also capable of calculating a chi square on Hardy-Weinberg equilibrium data, but this feature was used only to support calculations produced by manual means.

CHAPTER THREE

RESULTS

RFLP patterns - Results of digestion of the ribosomal ITS region with restriction enzymes *Cfo*I, *Bse*DI, and *Xho*I are given in Table 3.1. The presence of the *Cfo*I restriction site appears to be consistently high in all three populations while the frequencies for the restriction site of *Bse*DI are at or below 10% in each population. The *Xho*I restriction site appears to be the only enzyme with a widely variable frequency while the frequencies of *Cfo*I and *Bse*DI fluctuate only slightly between populations.

The same four haplotypes occurred within each of the three populations at Greenbrier, Cataloochee, and Sugarlands with variable frequency (Table 3.2). A chi square analysis (Table 3.3) of each population site was performed to determine: 1) if the populations were in Hardy-Weinberg (HW) equilibrium; and 2) if all of the expected haplotypes were present. Genotype frequencies were determined as indicated in Chapter 2 with haplotypes CBX = p [restriction site present for *Cfo*I (C), *Bse*DI (B) and *Xho*I (X)], CX=q, C=r,and XB=s.

As indicated by Table 3.3, the deviations from expected frequencies based on HW equilibrium are significant. None of the three populations are in HW equilibrium. Contributing to this finding is that for Cataloochee, only six of the ten expected genotypes were observed while at both Greenbrier and Sugarlands, only four of the ten were observed. There were generally more homozygous haplotypes observed than were expected, but this pattern was not consistent across all of the sites with the exceptions

Table 3.1 Data from restriction digests separated by population. Collections outlined with similar symbols were identified as one individual based on ISSR data. Column heading symbols signify presence of a restriction site for the following restriction enzymes: C=*Cfo*I, X=*Xho*I, and B=*Bse*DI.

Cataloochee	*C*	*X*	*B*	Greenbrier	*C*	*X*	*B*	Sugarlands	*C*	*X*	*B*
1617	1	0	0	6189	1	1	0	6199	1	0,1	0
6609	1	0	0	6190	1	1	0	6200	1	0	0
6611	0	1	1	6191	1	1	0	9350	1	1	0
6612	0,1	1	0,1	6192	1	0,1	0	9351	1	1	0
6614	1	0	0	6193	1	0,1	0,1	9352	1	0,1	0
6615	1	1	0	6194*	1	1	0	9253	1	1	0
6616	1	1	0	6195*	1	1	0	9374	1	0	0
6617	1	1	0	6196*	1	1	0	9375	1	1	0
9238	1	0,1	0	6197	1	1	0	9376	1	0,1	0
9239	1	0,1	0	6198	0,1	1	0,1	9377	1	1	0
9240	1	1	0	9354	1	1	0	9379	1	1	0
9243	1	0,1	0	9355	1	1	0	9386	1	0	0
9244	1	0,1	0	9356	1	0,1	0	9387	0	0,1	1
9245	1	1	0	9357	1	0,1	0	9388	1	1	0
9246	1	1	0	9358	1	0,1	0,1	9389	1	0,1	0
9277	1	0,1	0	9359*	1	1	0	9390	1	1	0
9278	0,1	1	1	9360*	1	1	0	9391	1	1	0
9279	1	0	0	9361*	1	1	0	9392	1	1	0
9281	1	0,1	0	9362	1	1	0	9393	1	0,1	0
9395	1	1	0	9363*	1	1	0	9394	1	0,1	0
9396	1	1	0	9364*	1	1	0	9863	1	1	0
9397	1	0,1	0	9365*	1	1	0	9864	1	1	0
9398	1	0	0	9366	1	1	0	9865	1	0,1	0
9399	1	0	0	9367*	1	1	0				
9400	1	0	0	9368	1	1	0				
				9369	1	1	0				
				9370	1	1	0				
				9371	1	1	0				
				9372#	1	1	0				
				9373#	1	1	0				
				9380	1	0,1	0				
				9381	1	1	0				
				9382	0,1	1	0,1				
				9383	1	1	0				
				9384	0,1	1	0,1				
				9385	0,1	1	0,1				
Frequency	0.92	0.58	0.10		0.93	0.90	0.10		0.95	0.70	0.05

Table 3.2 Collections and respective haplotypes based on restriction enzyme analysis. Collections in red or teal represent the same individuals. Collections in red were located on the 9.5 m log and collections in teal were on the 7 m log. Column headings signify various combinations of restriction sites producing haplotypes where C=*Cfo*I, X=*Xho*I, and B=*Bse*DI.

Cataloochee	CXB	CX	C	XB	Greenbrier	CXB	CX	C	XB	Sugarlands	CXB	CX	C	XB
1617			+		6189		+			6199		+	+	
6609			+		6190		+			6200			+	
6611				+	6191		+			9350		+		
6612		+		+	6192		+	+		9351		+		
6614			+		6193	+		+		9352		+	+	
6615		+			6194					9253		+		
6616		+			6195					9374			+	
6617		+			6196					9375		+		
9238		+	+		6197		+			9376		+	+	
9239		+	+		6198		+		+	9377		+		
9240		+			9354		+			9379		+		
9243		+	+		9355		+			9386			+	
9244		+	+		9356		+	+		9387				+
9245		+			9357		+	+		9388		+		
9246		+			9358	+		+		9389		+		
9277		+	+		9359					9390		+		
9278	+			+	9360					9391		+		
9279			+		9361					9392		+		
9281		+	+		9362		+			9393				
9395		+			9363					9394		+	+	
9396		+			9364					9863		+		
9397		+	+		9365					9864		+		
9398			+		9366					9865		+	+	
9399			+		9367									
9400			+		9368		+							
					9369		+							
					9370		+							
					9371		+							
					9372		+							
					9373									
					9380		+	+						
					9381		+							
					9382		+		+					
					9383		+							
					9384		+		+					
					9385		+		+					
totals	0.02%	0.48%	0.42%	0.08%		0.03%	0.79%	0.10%	0.07%		0.00%	0.70%	0.25%	0.05%

Table 3.3 Chi square analysis of Hardy-Weinberg equilibrium. All significant values (P) are 0.05.

Cataloochee				
Genotypes	**Haplotype Frequency**	**Observed**	**Expected**	**X^2**
p^2	0.0004	0	0.0100	0.0100
2pq	0.0192	0	0.4800	0.4800
2pr	0.0168	0	0.4200	0.4200
2ps	0.0032	1	0.0800	10.5800
q^2	0.2304	8	5.7600	0.8711
2qr	0.4032	7	10.0800	0.9411
2qs	0.0768	1	1.9200	0.4408
r^2	0.1764	7	4.4100	1.5211
2rs	0.0672	0	1.6800	1.6800
s^2	0.0064	1	0.1600	4.4100
				21.3542

Greenbrier				
Genotypes	**Haplotype Frequency**	**Observed**	**Expected**	**X^2**
p^2	0.0009	0	0.0261	0.0261
2pq	0.0474	0	1.3746	1.3746
2pr	0.0060	2	0.1740	19.1625
2ps	0.0042	0	0.1218	0.1218
q^2	0.6241	19	18.0989	0.0449
2qr	0.1580	4	4.5820	0.0739
2qs	0.1106	4	3.2074	0.1959
r^2	0.0100	0	0.2900	0.2900
2rs	0.0140	0	0.4060	0.4060
s^2	0.0049	0	0.1421	0.1421
				21.8378

Sugarlands				
Genotypes	**Haplotype Frequency**	**Observed**	**Expected**	**X^2**
p^2	0.0000	0	0.0000	0
2pq	0.0000	0	0.0000	0
2pr	0.0000	0	0.0000	0
2ps	0.0000	0	0.0000	0
q^2	0.4900	13	10.7800	0.4572
2qr	0.3500	5	7.7000	0.9468
2qs	0.0700	0	1.5400	1.5400
r^2	0.0625	3	1.3750	1.9205
2rs	0.0250	0	0.5500	0.5500
s^2	0.0025	1	0.0550	16.2368
				21.6512

being at the Greenbrier location in haplotypes p^2, r^2, and s^2 and in the p^2 haplotype at Cataloochee. A haplotype "tree" (Fig. 3.1) was used to identify whether these collections clustered together by locality and the relationships between different haplotypes. As indicated by Figure 3.1, one haplotype appears in only two of the three locations; all the other haplotypes are frequent and present at each collection site. Each haplotype differs by a single character with the exception of XB-CX and CXB-C which differ by two characters.

ISSR results - Inter-simple sequence repeat analysis was conducted on each of the 84 collections to determine the size of individuals and interpopulation gene flow. Of the 31 primers originally tested, primers Manny and Mao proved to be the most consistent for amplifying the DNA and produced the most variation between individual collections (Figs. 3.2 and 3.3).

Data sets were generated that compared all individuals amplified by a single primer. The two data sets for each primer were combined to form a single table (Table 3.4) and this was used to generate a similarity distance matrix followed by a NJ analysis (Fig. 3.4). The NJ analysis indicates that there are no distinct clusters in which all the taxa originate from a single location, but some slight grouping based on location is evident. A dollo parsimony analysis was also performed with these data and showed a similar distribution to that of Neighbor Joining and was therefore not included in this compiliation. The frequencies of each character were calculated according to population (Table 3.5). Several unique alleles were identified, most of which were from Greenbrier.

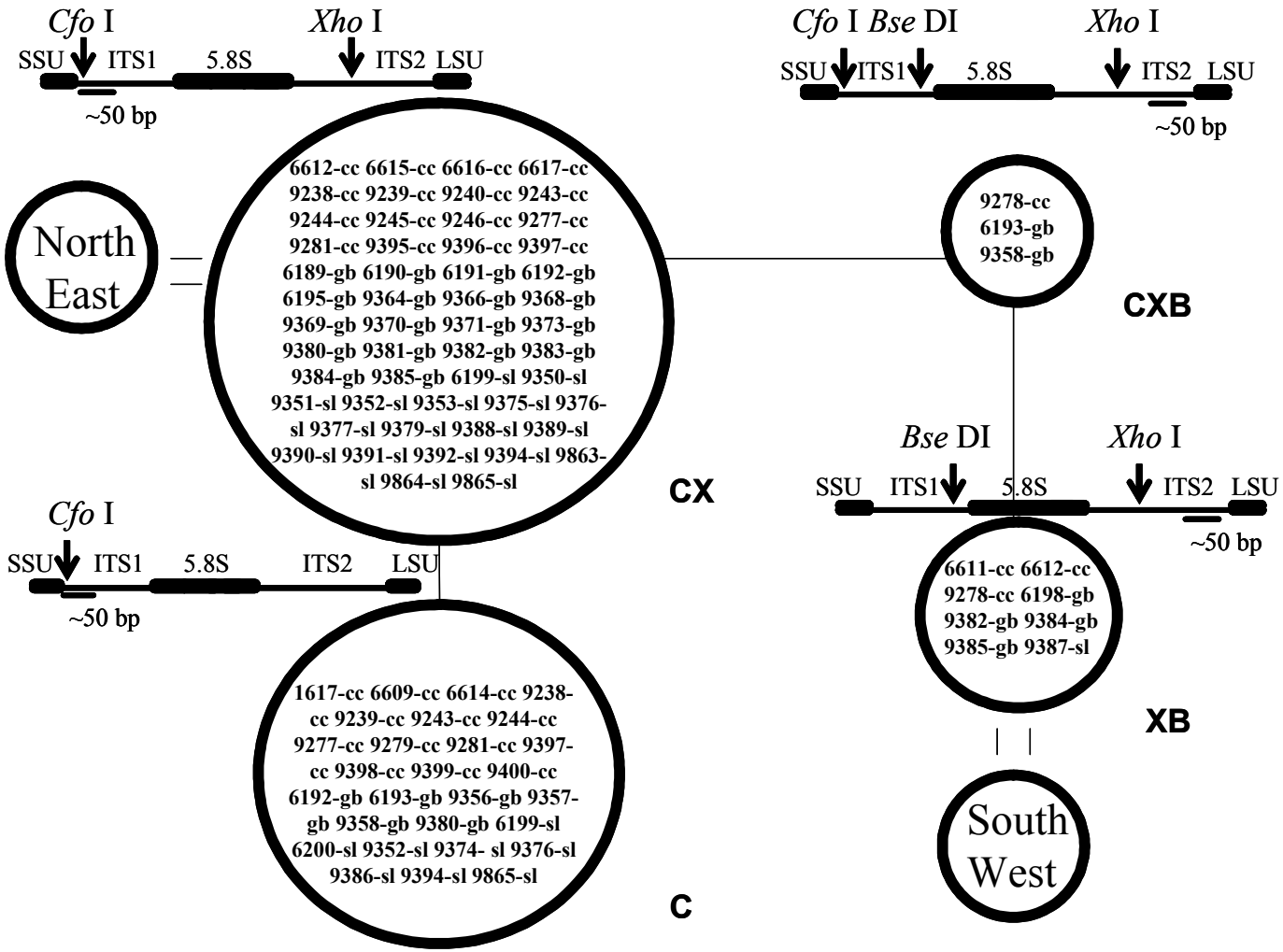


Figure 3.1 Haplotype network of all *A. pyxidatus* collections anchored with North American haplotypes produced from Lickey (2002).

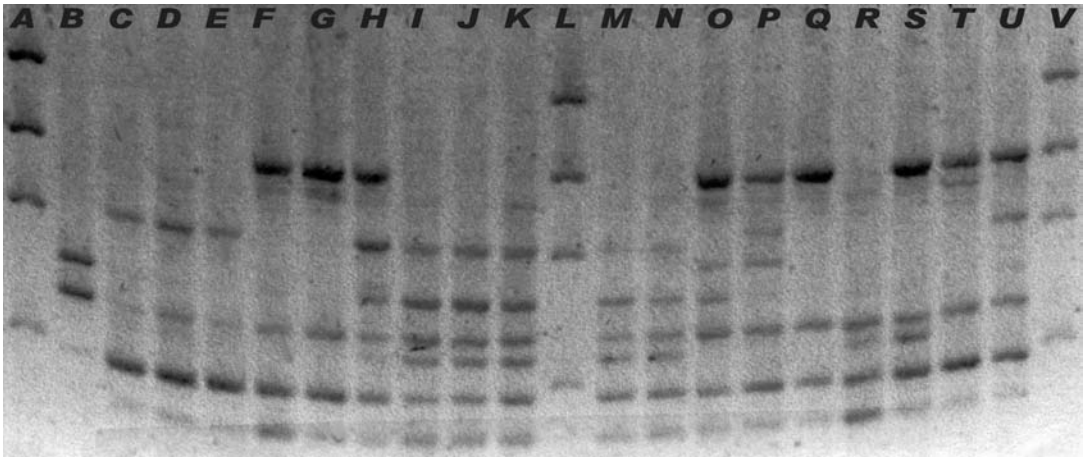


Figure 3.2 Variability in PCR amplification among *A. pyxidatus* collections primed with ISSR primer Manny. Lanes are: A, Phi X *Hae* III size marker; B, 9352-sl; C, 6189-gb; D, 6190-gb; E, 6191-gb; F, 6197-gb; G, 6198-gb; H, 9354-gb; I, 9369-gb; J, 9370-gb; K, 9371-gb; L, Phi X *Hae* III size marker; M, 9372-gb; N, 9373-gb; O, 9380-gb; P, 9381-gb; Q, 9382-gb; R, 9383-gb; S, 9383-gb; S, 9384-gb; T, 9385-gb; U, 9395-cc; and V, Phi X *Hae* III size marker.

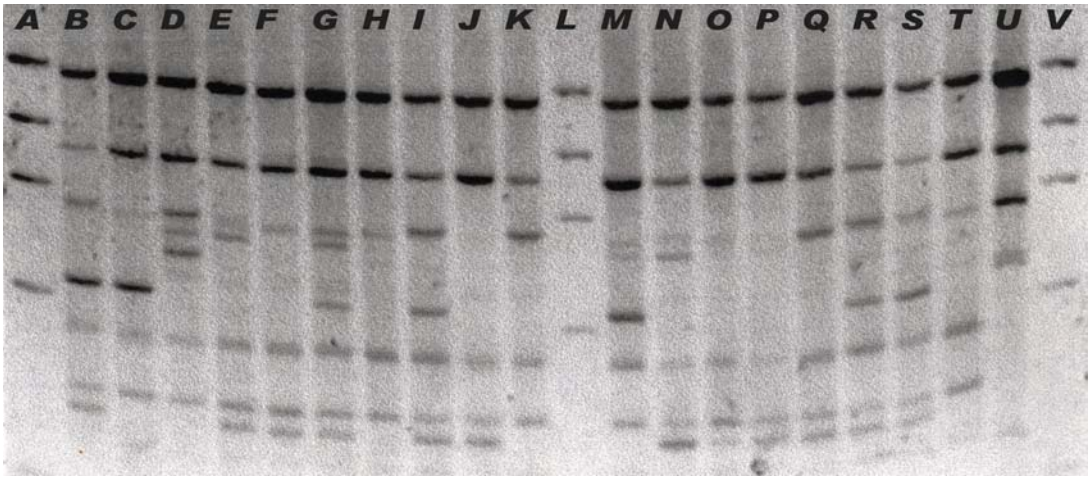


Figure 3.3 Variability in PCR amplification among *A. pyxidatus* collections primed with ISSR primer Mao. Lanes are: A, Phi X *Hae* III size marker; B, 6199-sl; C, 6200-sl; D, 9350-sl; E, 9351-sl; F, 9352-sl; G, 9374-sl; H, 9375-sl; I, 9376-sl; J, 9379-sl; K, 9386-sl; L, Phi X *Hae* III size marker; M, 9387-sl; N, 9388-sl; O, 9389-sl; P, 9390-sl; Q, 9392-sl; R, 9393-sl; S, 9394-sl; T, 9253-sl; U, 9367-sl; and V, Phi X *Hae* III size marker.

Table 3.4 ISSR data collected for 84 collections of *A. pyxidatus*. Bands 1-15 are based on amplification using Mao primer and bands 16-26 were amplified with Manny primer.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1617-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
6189-gb	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0	1	1	0
6190-gb	1	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0	1	1	0
6191-gb	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0
6192-gb	1	1	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0
6193-gb	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0
6194-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
6195-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
6196-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
6197-gb	1	1	0	0	1	0	1	0	0	1	1	1	0	0	0	1	1	0	0	0	0	1	0	1	1	0
6198-gb	1	1	0	0	1	0	1	0	0	1	1	1	1	0	0	1	1	0	0	0	0	1	0	1	1	0
6199-sl	1	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0
6200-sl	1	1	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
6609-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
6611-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0
6612-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
6614-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
6615-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
6616-cc	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0
6617-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
9238-cc	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
9239-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	1	0	0
9240-cc	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
9243-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1
9244-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
9245-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0
9246-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9253-sl	1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0	1	0	1	1	0
9277-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0
9278-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
9279-cc	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
9281-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0
9350-sl	1	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
9351-sl	1	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0
9352-sl	1	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0
9354-gb	1	1	0	0	0	1	0	1	0	0	1	1	0	0	0	1	0	0	1	0	1	1	0	1	1	0
9355-gb	1	1	0	0	1	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
9356-gb	1	1	1	0	1	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0
9357-gb	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0
9358-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	0	0
9359-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9360-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9361-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9362-gb	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9363-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9364-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9365-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0

Table 3.4 continued

9366-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
9367-gb	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9368-gb	1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
9369-gb	1	1	0	0	0	1	0	0	0	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	1	0
9370-gb	1	1	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	1	1	1	1	0
9371-gb	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	0	1	0	1	1	1	1	1	0
9372-gb	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	1	1	1	1	1	0
9373-gb	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	1	1	1	1	1	0
9374-sl	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	1	1	0	0
9375-sl	1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
9376-sl	1	1	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
9377-sl	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9379-sl	1	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	1	1	1	1	0	0
9380-gb	1	1	0	0	1	0	1	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	0	1	1	0
9381-gb	1	1	1	0	1	1	0	1	0	0	1	0	1	0	0	1	1	1	0	1	0	1	0	1	1	0
9382-gb	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	1	0	0	0	0	1	0	1	1	0
9383-gb	1	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	1	0	0	0	0	1	1	1	1	0
9384-gb	1	1	0	0	1	1	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	1	1	1	1	0
9385-gb	1	1	0	0	1	1	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	1	0	1	1	0
9386-sl	1	1	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
9387-sl	1	1	1	0	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
9388-sl	1	1	1	0	1	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	1	0	1	1	0
9389-sl	1	1	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	1	0
9390-sl	1	1	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	1	0
9391-sl	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0
9392-sl	1	1	0	0	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
9393-sl	1	1	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
9394-sl	1	1	0	0	1	1	0	1	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	0	0
9395-cc	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	0
9396-cc	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
9397-cc	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0
9398-cc	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0
9399-cc	1	1	1	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0
9400-cc	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0
9863-sl	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
9864-sl	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
9865-sl	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0

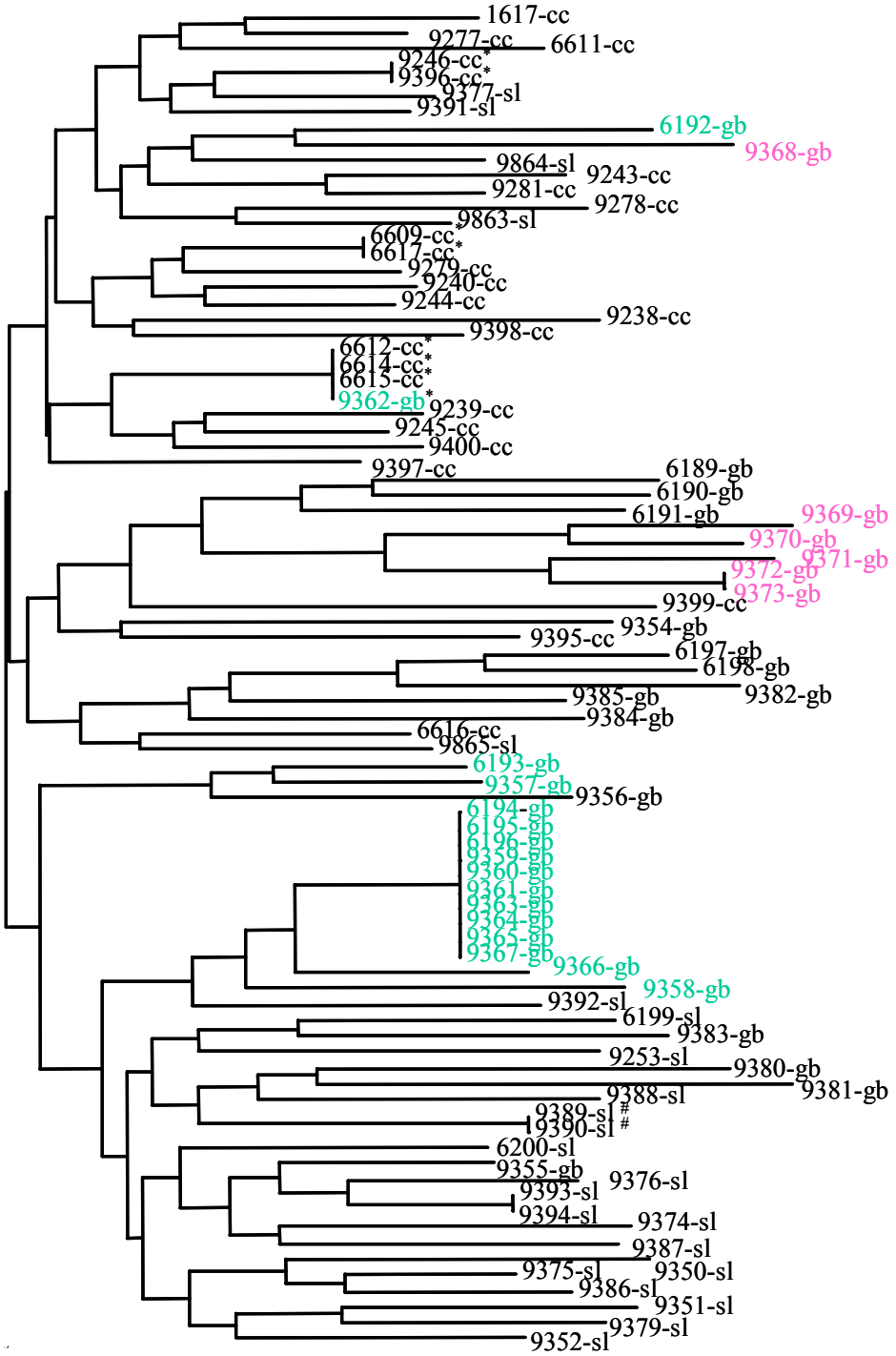


Figure 3.4 Neighbor Joining analysis of collections. Collection numbers highlighted in pink were collected from 7 meter log and blue highlighted collections were made from 9.5 meter log. Field number appendages signify collection site where cc=Cataloochee, gb=Greenbrier, and sl=Sugarlands. Collections marked with a * were distinguished from other collections by 18S data and those marked with a # were separated based on a RFLP analysis.

Table 3.5 Character frequencies of each band identified through ISSR analysis. Highlighted rows identify unique alleles found in only one of the three locations.

Band	Greenbrier	Sugarlands	Cataloochee
1	0.85	1.00	1.00
2	0.15	1.00	0.96
3	0.15	0.17	0.04
4	0.00	0.04	0.00
5	0.62	0.61	0.64
6	0.50	0.35	0.20
7	0.35	0.13	0.04
8	0.38	0.74	0.00
9	0.04	0.00	0.00
10	0.46	0.00	0.12
11	0.38	0.74	0.00
12	0.27	0.00	0.00
13	0.58	0.48	0.00
14	0.04	0.00	0.00
15	0.04	0.00	0.00
16	0.62	0.35	0.60
17	0.35	0.04	0.00
18	0.04	0.00	0.00
19	0.31	0.04	0.24
20	0.08	0.00	0.00
21	0.42	0.17	0.36
22	0.96	0.87	0.88
23	0.23	0.26	0.12
24	0.77	1.00	0.92
25	0.62	0.35	0.24
26	0.00	0.00	0.08

A Principle Coordinates Analysis (PCA) was conducted using the same ISSR data. Two vectors were extracted from the analysis and a two-dimensional representation of the results was plotted (Fig. 3.5). Collections at the Greenbrier site are the most diverse. The Sugarlands and Cataloochee samples cluster together more tightly and group within the range of the Greenbrier collections.

To address the size of an individual, NJ analysis indicated whether samples were identical to one another by placing them at the end of the same branch. The analysis suggested that several of the collections from different substrates were identical. With only one exception, further examination of the putatively identical collections using 18S and RFLP patterns showed that these collections represented different individuals. These results were then correlated with the diagram of each log. Of the six collections made on the 7 meter log, five collections were genetically unique. On the 9.5 meter log, seven of the 16 collections were genetically unique. When these collections were coupled with their map position (Fig. 3.6), collections representing a single individual logically involved several basidiomes near one another. The PCA confirmed these results by generating identical Eigen vectors (Table 3.6) for collections with the same ISSR patterns. Although the limitation of the graphical representation of this analysis does not show several collections that are superimposed at the same position, Table 3.6 lists the extracted vectors used to construct the chart and indicates which samples generated identical plot points.

18S Group I intron insertion results - In addition to the RFLP patterns, 18S patterns (Fig. 3.7) of all the collections were generated to either support or refute results from previous

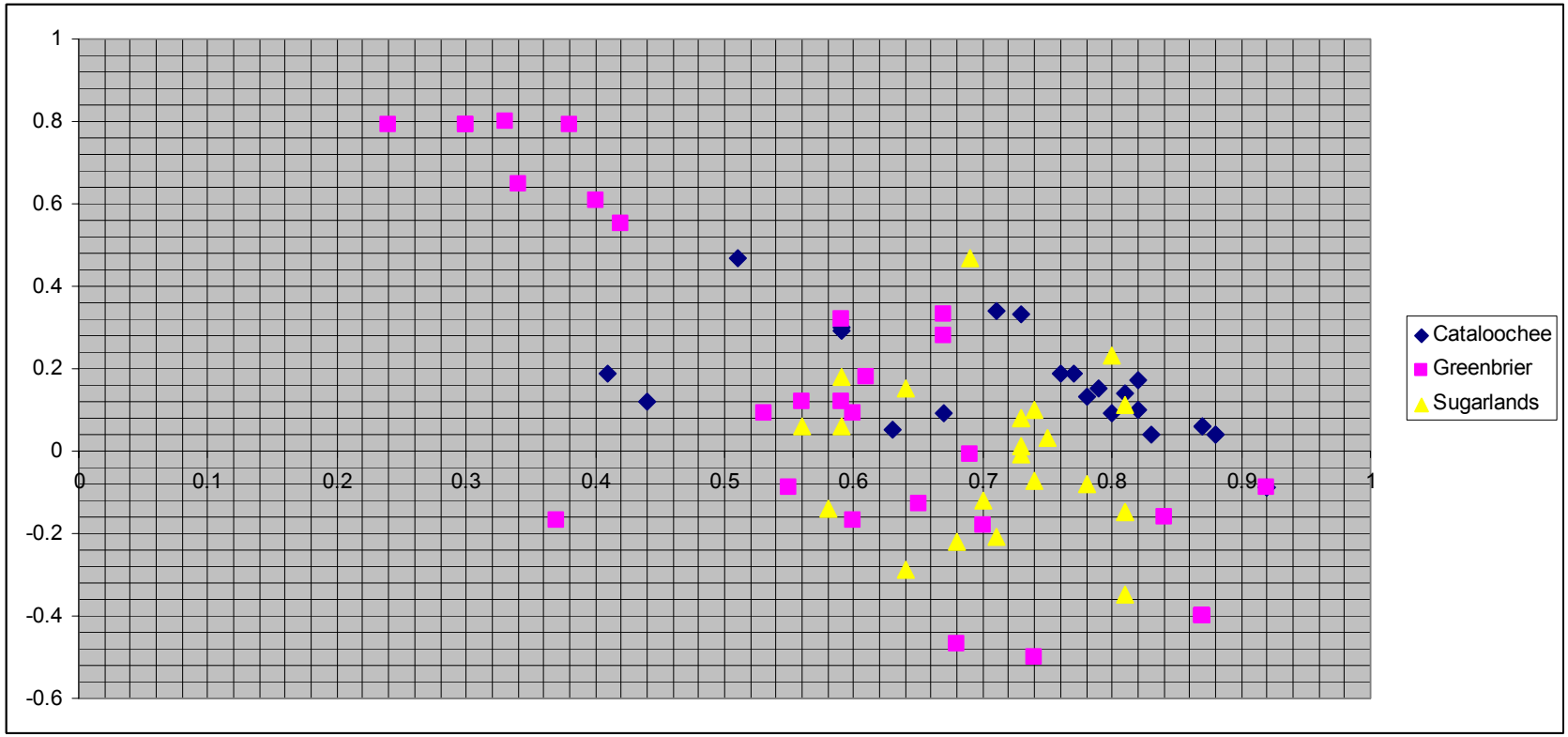


Figure 3.5 Principle Coordinates Analysis presented two-dimensionally.

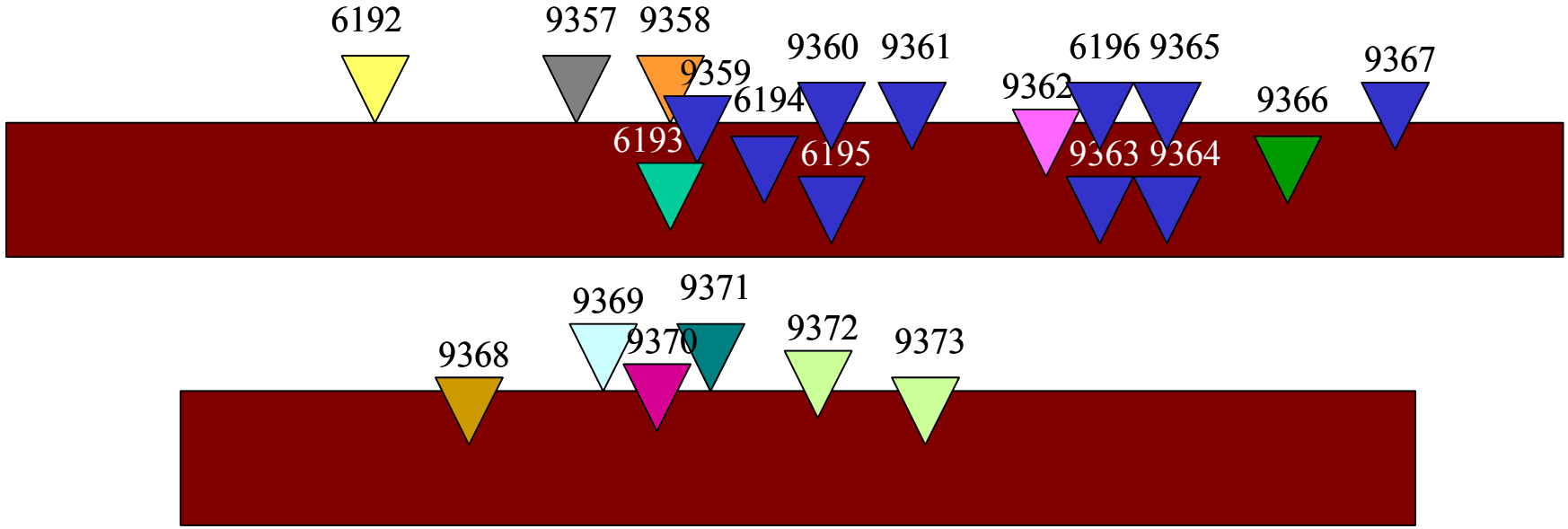


Figure 3.6 Top: Map of basidiomes collected on 9.5 meter log at Greenbrier. Below: Map of basidiomes collected on 7 meter log at Greenbrier. Similar colors indicate genetically identical basiomata.

Table 3.6 Eigen vector weightings for each collection used to produce the two-dimensional plot in Fig. 3.5. Collections outlined in bold are the same individual.

Cataloochee		
1617-cc	0.67	0.09
6609-cc	0.88	0.04
6611-cc	0.63	0.05
6612-cc	0.92	-0.09
6614-cc	0.92	-0.09
6615-cc	0.92	-0.09
6616-cc	0.77	0.19
6617-cc	0.88	0.04
9238-cc	0.44	0.12
9239-cc	0.78	0.13
9240-cc	0.76	0.19
9243-cc	0.59	0.3
9244-cc	0.81	0.14
9245-cc	0.83	0.04
9246-cc	0.87	0.06
9277-cc	0.79	0.15
9278-cc	0.51	0.47
9279-cc	0.82	0.1
9281-cc	0.71	0.34
9395-cc	0.59	0.29
9396-cc	0.87	0.06
9397-cc	0.82	0.17
9398-cc	0.73	0.33
9399-cc	0.41	0.19
9400-cc	0.8	0.09

Greenbrier		
6189-gb	0.34	0.65
6190-gb	0.42	0.55
6191-gb	0.4	0.61
6192-gb	0.56	0.12
6193-gb	0.69	-0.01
6194-gb	0.87	-0.4
6195-gb	0.87	-0.4
6196-gb	0.87	-0.4
6197-gb	0.6	0.09
6198-gb	0.59	0.12
9354-gb	0.59	0.32
9355-gb	0.84	-0.16
9356-gb	0.6	-0.17
9357-gb	0.7	-0.18
9358-gb	0.68	-0.47
9359-gb	0.87	-0.4
9360-gb	0.87	-0.4
9361-gb	0.87	-0.4
9362-gb	0.92	-0.09
9363-gb	0.87	-0.4
9364-gb	0.87	-0.4
9365-gb	0.87	-0.4
9366-gb	0.74	-0.5
9367-gb	0.87	-0.4
9368-gb	0.37	-0.17
9369-gb	0.33	0.8
9370-gb	0.38	0.79
9371-gb	0.24	0.79
9372-gb	0.3	0.79
9373-gb	0.3	0.79
9380-gb	0.65	-0.13
9381-gb	0.55	-0.09
9382-gb	0.53	0.09
9383-gb	0.61	0.18
9384-gb	0.67	0.33
9385-gb	0.67	0.28

Sugarlands		
6199-sl	0.59	0.06
6200-sl	0.78	-0.08
9253-sl	0.64	0.15
9350-sl	0.58	-0.14
9351-sl	0.56	0.06
9352-sl	0.73	-0.01
9374-sl	0.7	-0.12
9375-sl	0.74	-0.07
9376-sl	0.68	-0.22
9377-sl	0.81	0.11
9379-sl	0.59	0.18
9386-sl	0.71	-0.21
9387-sl	0.64	-0.29
9388-sl	0.73	0.01
9389-sl	0.73	0.08
9390-sl	0.73	0.08
9391-sl	0.8	0.23
9392-sl	0.81	-0.35
9393-sl	0.81	-0.15
9394-sl	0.81	-0.15
9863-sl	0.69	0.47
9864-sl	0.75	0.03
9865-sl	0.74	0.1



Figure 3.7 Variability in PCR amplification among *A. pyxidatus* collections primed with forward primer SR1c and reverse primer NS6. Lanes are: A, Phi X *Hae* III size marker; B, 1616-cc (contaminant); C, 1617-cc; D, 6609-cc; E, 6611-cc; F, 6612-cc; G, 6614-cc; H, 6615-cc; I, 6616-cc; J, 6617-cc; K, Phi X *Hae* III size marker; L, 9239-cc; M, 9240-cc; N, 9243-cc; O, 9244-cc; P, 9245-cc; Q, 9246-cc; R, 9277-cc; S, 9278-cc; T, 9279-cc; U, 9281-cc; and V, Phi X *Hae* III size marker.

molecular techniques that suggested more than one collection was part of a single individual. The 18S patterns were variable and were able to differentiate ambiguous results generated from ISSR analysis. The variability within this second insert both in size and in heterogeneity within the ribosomal repeat was likely the cause of the complicated and individually unique banding patterns. The DNA fragments produced broad and smeared bands that could not be reliably scored as a shared apomorhic character between individuals.

Map of individuals - The position of collections were measured and mapped on the 9.5 m and 7 m logs (Fig. 3.7).Molecular analysis based on ISSR data indicates that collections 6194, 6195, 6196, 9359, 9360, 9361, 9363, 9364, 9365, and 9367 comprise a single individual; 9372 and 9373 are identical; and 9393 and 9394 are identical. Samples 9393 and 9394 were collected from Sugarlands on separate substrates and the remaining samples were collected from Greenbrier. Greenbrier collections 9372 and 9373 were found on the 7 meter log and the remaining collections were found on the 9.5 meter log.

Inbreeding analysis - When this value was calculated for all three restriction sites, the following values were obtained:

*Cfo*I:	.401
*Xho*I:	.2414
*Bse*DI:	.4575

The computer program, Genepop (Raymond and Rousset 1996), calculated the amount of inbreeding based on population, locus, and both population and locus combined. The following values were obtained for all three alleles together:

Results by	**F_{st}**
*Cfo*I locus	0.0677
*Xho*I locus	0.1904
*Bse*DI locus	0.0074*
Cataloochee population	0.0022*
Greenbrier population	1
Sugarlands population	0.0078*
all populations and loci	0.000153*

* significant at α=0.05

The same program was used to calculate linkage disequilibrium. *Cfo*I and *Bse*DI did not segregate independently (P = 0.01) as is expected of loci that are only about 100 bp apart at the beginning of ITS1. *Xho* I, however, segregated independently from both *Cfo*I and *Bse*DI. For this reason, inbreeding coefficients were calculated using only *Cfo*I paired with *Xho*I and *Bse*DI paired with *Xho*I. The calculated inbreeding coefficients were still significant, suggesting non-random mating.

Results by	**F_{st}**
*Xho*I and *Cfo*I loci	0.008719*
*Xho*I and *Bse*DI loci	0.00893*

* significant at α=0.05

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

Resolution of an individual - Darwin wrote in 1869 that the location of a species border was often delineated by the presence of a related species. Could this idea extend to individuals? According to the circumscription of an individual in this study, a fungal individual may consist of one or more basidiomata and associated mycelium. If all genetic markers are identical for two or more basidiomata, each was considered part of the same individual. Previous studies have indicated that the size of a fungal individual has a broad range depending on the species and the data. Fungal size may also be related to species lifestyle (saprobic, mycorrhizal, or pathogen) and successional preference (Bruns 1991). A study done by Kay and Vilgalys (1992), using *Pleurotus ostreatus,* indicated that several separate individuals could occupy the same substrate while Smith *et al* (1992), determined that over 30 acres of northern Michigan was occupied by a 1500 year-old *Armillaria gallica*.

Based on the results from this study, an individual of *A. pyxidatus* is similar in size to that of *Pleurotus* and several related individuals can occupy a single log. In contrast to the initial description by Dodd (1972) in which he stated that the mycelial pad of *A. pyxidatus* reaches a diameter of 0.2 m, the smallest individual to produce more than one fruiting structure in this study spanned a distance of 0.2 m while the largest multiple fruiting individual measured 1.9 m. This appears to be consistent with observations by Hughes (pers. comm.) who found genetically identical fruitbodies on adjacent logs within a 10 m^2 region of Wisconsin by using mitochondrial DNA as a molecular marker. In this study, only one such case was found in which two collections from Sugarlands repeatedly

produced identical genetic markers despite their location on separate logs. The field notes do not discuss how distant the collection sites were, but both samples were collected on the same day and the collection numbers are sequential, so it can be assumed that they were close to each other based on our patterns of collection.

Artomyces pyxidatus is a saprobe and restricted by the size of its substrate. Spores are randomly dispersed by air currents or other unknown mechanisms to the surrounding environment in which some fraction of those spores fall on suitable substrate. Since only one clone was found to occupy separate logs in this study, it is reasonable to advocate that *A. pyxidatus* spores do not travel far from their original parent. Although the Hughes study (pers. comm.) suggested that this may be a more frequent occurrence than the data from this study, it may have been possible to distinguish between the contiguous fruiting individuals in the Hughes study with the use of additional molecular techniques. Very little is known about spore dispersal mechanisms in some fungi, but it is widely accepted that oceans are a significant geographic barrier to gene flow between populations located on different continents (James *et al* 1999, Methven *et al* 2000, Hughes *et al* 1999, Vilgalys and Sun 1994). Although the mountains in this study are substantially less prohibitive, spores might be exposed to differences in temperature and atmospheric pressure to survive dispersal over the peaks of the Smokies.

Random breeding of populations - A tetrapolar breeding system favors outbreeding due to the multiple alleles present for the two mating factors. Hardy-Weinberg estimates for each of the three populations (Table 3.2) show that none of the populations are in

equilibrium. The critical Chi square value is 16.92 (P = 0.05) and in all populations the calculated value was above 21. When the observed and expected values of homozygotes and heterozygotes were compared, there were, in almost every case, an excess number of observed homozygotes. The mating system of these populations, therefore, would appear to be that of a predominantly inbreeding system and the calculated inbreeding values (page 31) indicate that *A. pyxidatus* inbreeds more than expected for a tetrapolar basidiomycete. Since random breeding is a condition that must be satisfied to attain Hardy-Weinberg equilibrium, violation of this due to inbreeding could cause the significantly deviant values calculated from each population.

Inbreeding in these populations is probably due to a combination of several factors. These populations appear to be relatively small and isolated with a limited ability for long-distance spore dispersal. These factors together with a limited availability of suitable substrates create favorable conditions for inbreeding. With a small population, gene variety is limited and favors random genetic drift. Without access to outside populations with potentially dissimilar genetic material, the gene pool stays relatively unchanged, with occasional mutations providing the only new sources for genetic variety. The saprobic lifestyle of *A. pyxidatus* appears to restrict the fungus to a particular log on which spores apparently fall and anastomose with neighboring spores that were likely produced by a fruitbody on the same or adjacent log.

Linkage disequilibrium – Another factor that may cause deviant HW calculations is linkage between the three restriction sites. Each of the three sites is located within the same segment of DNA. The site for enzyme *Bse*DI is located between the other two sites

approximately 100 base pairs from the *Cfo*I site and 350 base pairs from the *Xho*I site. Linkage between two factors violates one of the assumptions of HW equilibrium and the close proximity of the restriction sites for *Bse*DI and *Cfo*I highlighted the need to investigate the statistical significance of this issue. When calculations were performed to assess the relevance of linkage, the *Bse*DI and *Cfo*I sites were significantly linked. Neither of these sites was significantly linked to the *Xho*I restriction site. Inbreeding was calculated using only unlinked sites (*Xho*I with either *Cfo*I or *Bse*DI), however, and significant amounts of inbreeding were still present (see page 36).

Population dynamics – The three cove hardwood populations appear to be subpopulations of a single large population. Analyses based on data collected from ISSR and RFLP techniques show that while there is some genetic divergence based on subpopulation, there is also an extensive amount of genetic similarity among individuals from the three collection sites. The data generated from RFLP analysis indicate that restriction site frequencies for *Cfo*I and *Bse*DI are generally consistent across all three of the subpopulations, but the frequency of *Xho*I varies from site to site. If the subpopulations were isolated and evolving, and given sufficient time, drift and inbreeding would result in gene frequency changes and fixation of alleles. The consistency of frequencies for *Cfo*I and *Bse*DI indicates that: 1) gene flow is occurring between the subpopulations; 2) the ITS region is highly conserved, which prevents the rapid mutation of restriction sites; or 3) the three subpopulations have not been separated long enough to permit measurable genetic change.

Is gene flow currently occurring? Geographic barriers such as oceans and mountain ranges have been an effective barrier to genetic interaction for many fungi (Methven *et al* 2000, Hughes *et al* 1999, Vilgalys and Sun 1994, Kay and Vilgalys 1992, Ed Grand unpublished data). A new species may eventually develop when these barriers cause the isolation of allopatric populations. The presence of a mountain ridge between Cataloochee and the other two sites would be a geographic obstacle selecting against free genetic exchange. For gene flow to be occurring among the three populations, spores of *A. pyxidatus* must be able to survive at altitudes higher than those of the collection sites. Not only must *A. pyxidatus* survive the elevation and temperature differences inherent to mountain ranges, but the ranges themselves must supply a habitat suitable for the nutritional preferences of the fungus. As the elevation increases in the Smoky Mountains, various conifer trees (spruce, fir, pine) and trees on which A. pyxidatus is not commonly found (mountain ash, cherry, birch, etc.) become the dominant wood material. The *Liriodendron*, *Acer*, and *Quercus* preferred by *A. pyxidatus* are rare to non-existent and may not be plentiful enough to sustain sympatric populations. Although it is possible for fungal spores to disperse over long distances (Hallenberg and Küffer 2001, James and Vilgalys 2001, Rishbeth 1959), it is the probability of those spores to encounter suitable niches with the appropriate nutrition that determine if a fungus will persist (James *et al* 1999). The environment of the high altitude ridges is probably not suitable habitat for *A. pyxidatus* and therefore may prevent the sampled study sites from exchanging genes freely, however, passes or "gaps" are as low as 2500 feet in the Smokies.

An alternate route for gene exchange might be through interconnected patches of forest around the periphery of the GSMNP. The existence of hardwoods in the valleys of

the mountains and adjacent lands might have enabled populations to exchange genes without going over the ridges but by going around them instead. These areas, however, would have been the first to be lumbered during deforestation of the Smokies which might have reduced this type of opportunity.

The second possibility for gene exchange is that the ITS region is so highly conserved that the detection of genetic variability is limited due to lack of significant long-term mutation. The ISSR analysis, however, indicates that there is some genetic separation of the three locations. These marker are more variable within populations than the ITS region and would reveal genetic mutation at a much quicker rate. The variable frequency of ISSR loci would therefore argue against the notion that free gene exchange must be inferred due to ITS genetic conservation as would the finding of unique, population-specific ISSR alleles in these three populations.

A third possible explanation for the consistent RFLP frequencies is that the three sites sampled have not been isolated from each other long enough to have evolved genetic differences. The NJ analysis contradicts this idea because it shows some separation of the collection sites. A refugium of cove hardwood forests is theorized to have been present along major rivers in the southern region of North America approximately 18,000 years ago (Delcourt and Delcourt 1993). The refugia flora spread north as the last glaciation period ended and reached what is now the GSMNP from 2000 to 8000 BP depending on the species (Delcourt and Delcourt 1993). The encroachment of the hardwood regions would have taken several thousand years. This may mean that *A. pyxidatus* is a relatively recent (*c*. 9000 ya) arrival to the region. The three sites sampled in this study have a history of human activity that includes logging and

colonization. The original forests of the three regions were virtually destroyed within the last century and the subsequent conservation efforts made each location ideal for the secondary successor plants like *Liriodendron*, *Acer*, and *Quercus* which are good substrates for *A. pyxidatus*. It is difficult to say whether *A. pyxidatus* was present prior to these events and survived in small restricted populations surrounded by farmland or whether it was common in the old growth forest. It is now thriving in each regenerating cove hardwood area. Even if *A. pyxidatus* infiltrated the Smoky Mountains after the conclusion of the last glaciation and was present prior to the habitat destruction, it still may not be old enough to allow for the development of genetically delineated populations based on ITS data. This would explain why some of the data generated from the molecular techniques in this study maintain genetic frequencies among all three populations (RFLP analysis) while others show mixed results of both separation and interaction (ISSR data).

Both NJ and PCA analysis were generated based on the ISSR data. The NJ tree shows a slight clustering of collections based on location, but still contains some mixture of collections from other locations within each clade. When the PCA vectors are graphed two-dimensionally, the resulting chart indicates that Greenbrier has the most variable genetic content because the data coordinates span the largest surface area. Contained within this same general range are the data points for all the collections made in both Sugarlands and Cataloochee. There is no separate clustering based on location. Collections from Sugarlands and Cataloochee do not have the broad genetic range that those from Greenbrier do which indicates that there is less genetic diversity at the former two sites. It is evident, therefore, that although some subpopulations are less diverse, the

collection sites are not genetically differentiated from one another so that it is detectable using this analysis. The combination of these two analyses using the same data produce mixed results with one (PCA) indicating almost no genetic differentiation based on collection site and the other (NJ) showing incomplete but identifiable differentiation of the three subpopulations.

It is unknown why Greenbrier has more genetic diversity than the other two sites. One possibility involves the amount of human activity in the area. The specific collection location at Greenbrier was near a picnic area. It is possible, although perhaps not very probable, that the influx of people and animals to this picnic region supplied the Greenbrier population with a variety of spores. If, however, one applies this same theory to the other two collection sites and considers the specific collection locations of each, it becomes apparent that this human-mediated genetic diversity theory is equally likely in those regions as well. The Sugarlands collection area is parallel to a horse trail. The Cataloochee collection area (refers only to the 6 collections made by the author) neighbors the Beech Grove School House, an historic building that attracts many visitors. It would seem, therefore, that if human activity was the source of genetic diversity at Greenbrier, the other two sites would demonstrate this diversity as well. It should also be noted that *A. pyxidatus* does not appear to be particularly attractive to insects (Steglich and Sterner 1988). Grazing animals do not appear to find the fungus very appetizing either because none of the signs of foraging were observed on any collected fruitbodies. Most deteriorated fruitbodies appeared to be intact and parasitized by other microorganisms.

A second possible reason for the genetic disparity of Greenbrier involves its geographic location relative to the other two sites. A map of the GSMNP (Fig. 4.1) reveals that Greenbrier is situated between the other two sites. This locality could either be: 1) a juncture for the influx of spores from other regions; or 2) the source of genetic diversity that generated the less-diversified populations in Sugarlands and Cataloochee. Given the previously mentioned hypothesis that *A. pyxidatus* spores are unable to traverse over the mountains due to habitat depletion at high altitudes, it is unlikely that Greenbrier is a site at which migrant spores gather. The fact that the Neighbor-Joining analysis (Fig. 3.5) presents a tree with Greenbrier collections mixed with predominantly Cataloochee and Sugarlands clades suggests that the genetic migration direction is the opposite and travels from Greenbrier to other neighboring sites. The Greenbrier population also produced higher frequencies of 16 out of 26 ISSR characters. The Greenbrier site could have been founded long ago and, over time, produced other populations at the fringes of the range that, due to their location or eventual separation from the parent population, became established with less genetic variability. Gottlieb (1973) as interpreted by Cole and Kuchenreuther (2001), predicted that:

> "in progenitor-derivative taxa 1) the pair would exhibit a high degree of genetic similarity 2) the progenitor would contain more genetic variation than the derivative, 3) the progenitor would possess a number of alleles not present in the derivative, and 4) the derivative would possess few or no unique alleles."

Gottlieb's principles could apply here. If the word "populations" were substituted for "taxa," and Greenbrier was considered the progenitor to the derived populations of



Figure 4.1 Map of GSMNP. Collection sites are indicated by a red star.

Cataloochee and Sugarlands, one could correlate the results of this study to these tenets. First, collections from Greenbrier show a high degree of similarity to collections from the other two sites as presented by the NJ analysis in which collections were distributed among the groups more widely and were either sister or interior to clusters of the other populations. The PCA also showed this wide genetic distribution that overlapped the pattern of the other two sites. Additionally, all four of the haplotypes generated from RFLP data were present in Greenbrier as well as the other two sites (with only one exception). Second, the Greenbrier site supported more genetic variation than the other sites as evidenced by the PCA genetic distribution. Thirdly, six of the eight unique alleles found among all of the populations were identified from Greenbrier. Finally, one of each of the two remaining unique alleles was found in Cataloochee and Sugarlands.

Haplotype distribution – Haplotype clustering showed that with only one exception at Sugarlands, all four of the same haplotypes were present at each site. Sugarlands was also the site with the lowest population sample, however, so it was possible that this missing haplotype might have been recovered if more fruitbodies had been collected. The presence of the same four haplotypes suggests that they either predated the establishment of the populations or that they were maintained through interbreeding. Data presented by Lickey (2002) showed a broad range of two of these same four haplotypes. Given the previously discussed reasons that interbreeding between the populations is limited, the evidence from these two studies supports the idea that the haplotypes were established prior to Park invasion.

The two remaining haplotypes found in this study appear to be unique derivations of the other main haplotypes. One haplotype may have resulted from a mutation that altered the restriction site for *Xho*I so that the enzyme no longer recognized the sequence. The other haplotype, however, probably gained a restriction site from a hybridization event between the two main haplotypes. A diagram illustrating the collection locations of various haplotypes (Fig. 4.2) indicates that the GSMNP may be part of a hybrid zone for *A. pyxidatus*.

Overall, this study was able to show that an *A. pyxidatus* individual can reach a size of 1.9 meters, produce multiple fruitbodies, and coexist with other individuals on the same log. A large population undergoing free gene exchange appears to have once existed in the GSMNP and is now producing isolated subpopulations with an apparent center of genetic diversity in the vicinity of Greenbrier, Tennessee. Based on the results of haplotype analysis, this population is located within a possible hybrid zone of two more widely distributed main haplotypes.

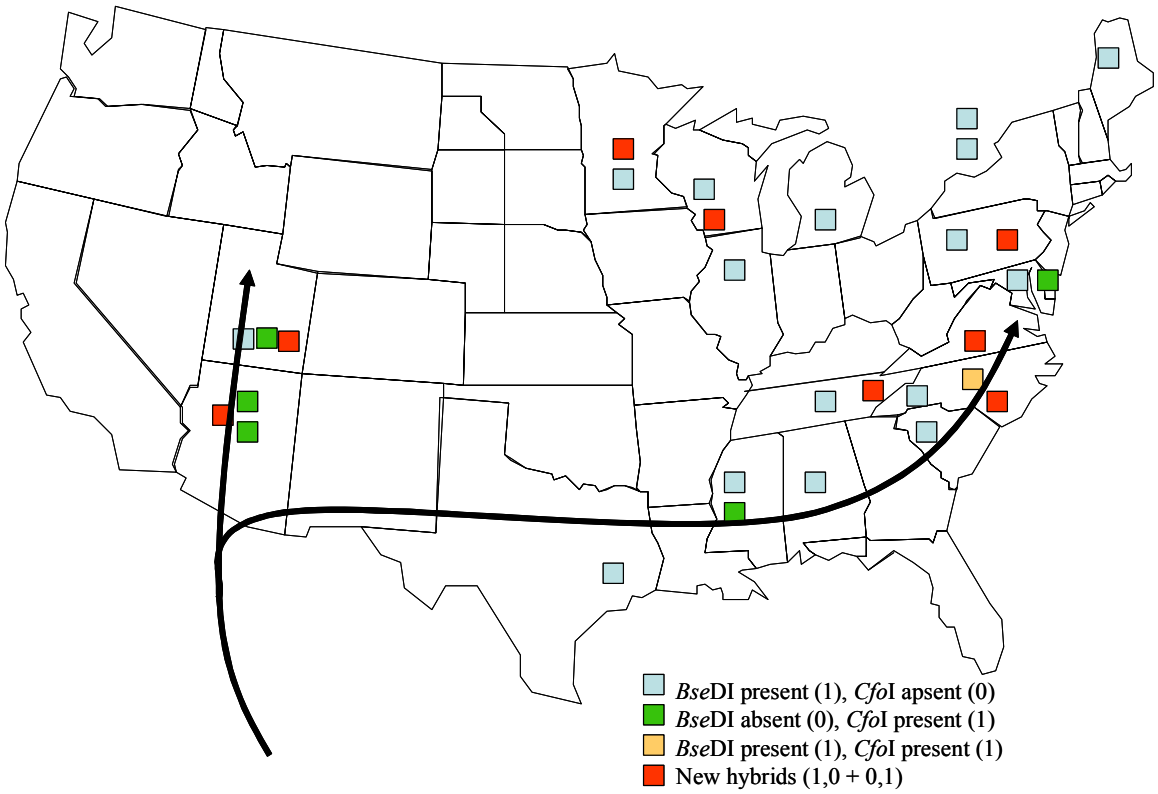


Figure 4.2 Haplotype distribution of *A. pyxidatus* within the United States extrapolated from data in Lickey (2002).

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APPENDIX

Media preparation:

Malt Extract Agar (MEA)

Malt Extract (DIFCO)	12g
Agar (DIFCO)	20g
Distilled Water	1L

Potato Dextrose broth (PD)

PD broth (DIFCO)	24g
Distilled Water	1L

Agarose gel (1.5%):

FMC-LE agarose	1.5 g
1X TBE buffer	100 ml
Ethidium bromide	5 µl

Phi X *Hae* digest

Phi X Hae digest (Promega)	1000 µg/mL	20 µL
TE buffer		800 µL
10X Tracking Dye		100 µL
		1000 µL

Mix and dispense in 5 microfuge tubes, 200 µL per tube. Freeze in -20 C.

VITA

Shannon Marie Wallace Tieken was born in Bloomington, Indiana on March 8th, 1977. She attended public grade school and high school in Spencer, IN graduating in 1995 as class valedictorian. In an effort to flee from her blink-and-you-miss-it rural Indiana town, she moved south to Evansville, Indiana to major in biology at the University of Southern Indiana. After graduating in 1999 with a Bachelors degree, her dreams of making it big in the field of science led her to Raleigh, North Carolina where she worked for a government agency as a glorified librarian and was trained on how to cut up rodents. Realizing that there had to be a better route to fame in the science industry, she applied and was accepted by the Botany department at the University of Tennessee, Knoxville where she studied the size and population interactions of the fungus *Artomyces pyxidatus* in the Great Smoky Mountains National Park.