MICROEVOLUTIONARY STUDIES IN *MARASMIELLUS PRAEACUTUS* AND *COLLYBIA SUBNUDA*, TWO LITTER-DECOMPOSING BASIDIOMYCETES.

by

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Dissertation submitted to the faculty

of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biology

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Microevolutionary studies in *Marasmiellus praeacutus* and *Collybia subnuda*, two litter-decomposing basidiomycetes.

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(Abstract)

The distributions of mating alleles in local populations of the litter-decomposing agarics *Marasmiellus praeacutus* and *Collybia subnuda* were determined by mating crosses. The tetrapolar *M. praeacutus* has an unexpectedly low mating allele diversity at both the local and the regional level. This is probably due to a combination of factors which results in limited spore dispersal. The pattern of mating allele distribution among closely adjacent genets suggests that di-mon crossing (the Buller phenomenon) may contribute to the population structure of this species. *Collybia subnuda* has a mating allele diversity estimated at 45 for the species, with a 95% confidence interval of 19 to 187. On a local scale, closely adjacent genets of *C. subnuda* did not share mating alleles, indicating that *C. subnuda* is an outcrossing species. Two partially intersterile groups were identified within the *C. subnuda* morphospecies. They were not differentiated morphologically, geographically, or by the DNA sequence of the intergenic transcribed spacer (ITS) region of the ribosomal RNA-encoding gene family.

Intersterility group (ISG) 1 is usually found on oak leaf litter, and ISG 2 is usually found associated with oak wood. Collection records, mating crosses, and spore-catching experiments indicate that the two ISGs are distributed sympatrically throughout the sampled range. Both ISGs produce binucleate spores in low frequency, and thus have the potential for secondary homothallism. Spore-catching experiments indicate that the spore rain of C. subnuda varies greatly over space and time. Spore viability studies show that C. subnuda spores have a limited viability. The implications of these observations for the population structure and speciation in C. subnuda are discussed.

ACKNOWLEDGEMENTS

This work is dedicated to Dr. Orson K. Miller, Jr., and to his wife, Hope, whose support and guidance throughout my graduate studies were invaluable. I greatly appreciate the independence to pursue my own lines of investigation under Dr. Miller's supervision. Dr. Miller's commitment to his graduate students goes far beyond mycological pedagogy, and the education I received from him will serve me for a lifetime.

The members of my committee provided invaluable support at critical junctures of this study. To Drs. Gary Griffin, Khidir Hilu, Ronald Petersen from the University of Tennessee, Bruce Turner, and David West, I extend my warmest thanks.

Dr. Scott Gordon and Coleman McCleneghan of the University of Tennessee were very helpful in guiding my approach to genetics experiments. Dr. Roy Halling of the New York Botanical Gardens graciously confirmed my identifications of Collybia species and was very encouraging and supportive of this study. Dr. Rytas Vilgalys helped me understand phylogenetic species concepts in Collybia and provided moral and material support throughout this study. Miguel Volovsek at the Department of Botany, Duke University, provided important technical and conceptual

guidance in the use of the automated sequencer.

Dr. Charles Rutherford and the many postdocs, graduate students, and technicians who have inhabited his lab over the past 5 years provided continuous technical and moral support. Brian Williamson, in particular, was always willing to discuss and defend the arcane mysteries of molecular biology.

I cannot adequately express my thanks for the moral, intellectual, and emotional support of my fellow graduate students in the Mycology Lab of Virginia Tech. To Kathy Jacobsen and Greg Eaton, I extend my heartfelt thanks for helping out and putting up with me. To Cathy L. Cripps, who started this unusual and occasionally bizarre adventure at the same time as I, how can I possibly thank you for all your help? Who would have thought that two people so different from each other would get along so well? It would have been hell without you, Cath!

I thank my fiancée Caroline, for providing light when the darkness threatens to overwhelm, for helping me understand the important things, for her good humor, and for her love and companionship.

Financial support for this study was provided by the Sigma Xi Society (GIAR grant #9203), the Highlands Biological Station, the Horton Center Foundation, and the Department of Biology at Virginia Tech. Logistical support

was provided by the Virginia Museum of Natural History.

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CHAPTER I: DISTRIBUTION AND DIVERSITY OF MATING ALLELES IN *COLLYBIA SUBNUDA* AND *MARASMIELLUS PRAEACUTUS*

INTRODUCTION

Investigations into the population biology and micro-evolution of the fungi have been hampered by the lack of understanding of fungal individualism. In contrast to most higher life forms, the body of an individual fungus is not readily apparent. Consequently, only during the past two decades has the concept of fungal individualism been readily accepted by mycologists (see Rayner, 1991). Prior to this, the accepted paradigm of fungal population structure considered the vegetative thallus of a fungus to exist as a "genetic mosaic," in which nuclei of all different genotypes intermingled within the same mycelium. Now, it is commonly accepted that fungi exist as genetically distinct individuals, or "genets," which occupy discrete and frequently visibly delineated volumes of resource substrate.

The development of an acceptance of fungal individualism by the mycological community has been reviewed previously (Murphy, 1992). With the discovery and utilization of somatic incompatibility tests to determine the distribution of individuals in the field, fungal population biology experienced a significant boost. Table 1 summarizes most of the reports which have utilized somatic

incompatibility testing to determine genet sizes. It is immediately apparent from this table that the taxa investigated are primarily tree pathogens and wood decay fungi, and that very few studies have investigated fungi which occupy other ecological roles, such as mycorrhizal fungi or litter decomposers. Furthermore, the data indicate a huge range in observed genet sizes even within the tree-pathogenic ecotype. A considerable amount of research is required, then, simply to outline the ranges of fungal genet sizes within and between a variety of ecological types.

Previous studies on the mycoflora of two forest types on a southern Appalachian mountain focused on the occurrence of two dominant, litter decomposing fungi, Marasmiellus praeacutus (Ellis) Halling and Collybia subnuda (Ellis ex Pk.) Gilliam (Murphy, 1992; Murphy and Miller, 1993). These species were chosen for population-level studies because of their ecological importance as litter decomposers, and the ease with which tissue and single-spore isolates could be cultured from them. Individual sporocarps were mapped on permanent plots (hereafter referred to as "the Brush Mountain plots") and tissue isolates were used in somatic incompatibility tests, which showed that genets of both species appeared to be small (less than approximately 5 m diam).

Of course, identifying genet size is only the initial

phase in the development of an understanding of a fungal species' population biology, but without prior knowledge of a species's population structure, sampling is problematic. The eventual goal of a population study might be to compare the evolutionary divergence of different populations. However, inadvertent biased sampling of a population may occur from resampling the same genet or by sampling populations with non-random allelic distributions. Once genet size is determined, the next step in a fungal population study must be to investigate the genetic relatedness of individuals within a population.

Again, however, mycology has been hampered by the paradoxical simplicity of the fungal mycelium. Despite the complex physiology of fungi, most species offer only a single character which is known to be inherited in a Mendelian manner and which can be relatively easily scored. This character is the mating genotype. Many workers have used the distribution of mating alleles in a natural population to make inferences concerning local population structure (Raper, 1966; Burnett, 1965; Table 2).

Raper (1966) provides a thorough treatment of fungal mating genetics. In most basidiomycetes, mating compatibility within intersterility groups is under the control of genes located at one or two loci, referred to as the A or B loci. It is now known that both of these loci

Table 1: Presumptive clonal size in fungi as determined by somatic incompatibility tests: reports from the literature.

Ecology	Fungus	Clonal size[1]	Reference
Lignicolous			
	Armillaria luteobubalina	580 m, or 10 km?	Kile,1983
	Armillaria mellea, s.l.	450 m	Shaw and Roth, 1976
	Armillaria mellea, s.l.	400-450 m	Anderson, et al., 1979
	Armillaria bulbosa	15 ha[2]	Smith, et al., 1992
	Armillaria mellea	7/8 mi	Adams, 1974
	Coriolus versicolor	many/stump	Todd and Rayner, 1978
	Echinodontium tinctorium	1/tree	Wilson, 1991
	Ganoderma lucidum	≥1/tree	Adaskaveg and Gilbertson, 1987
	Ganoderma tsugae	≥1/tree	"
	Heterobasidion annosum	1-30m, 1-13 trees	Stenlid, 1985
	Peniophora rufa	many/limb	Chamuris and Falk, 1987
	Phaeolus schweinitzii	1/tree	Barrett and Uscuplic, 1971
	Phellinus weirii	All trees w/i 50 m diam.	Childs, 1963
	Phellinus weirii	1 km	Dickman and Cook, 1989
	Piptoporus betulinus	many/tree	Adams, et al. 1981
	Pleurotus ostreatus	several /log	Kay and Vilgalys, 1992
	Poria carnegiea	several/saguaro	Lindsey and Gilbertson, 1977
	Tricholomopsis platyphylla	150 m	Thompson and Rayner, 1982
Litter decomposer			
	Collybia subnuda	2.3m	Murphy and Miller, 1993
	Marasmiellus praeacutus	4.6m	"
	Marasmiellus praeacutus	17.5m	Murphy (this study)
	Marasmius androsaceus	3.5m	Holmer and Stenlid, 1991
	Mycena galopus	2.5m	Frankland, 1984
Mycorrhizal			
	Laccaria bicolor	12.5m	Baar, et al. 1994
	Laccaria bicolor	10-15m	de la Bastide, et al., 1994
	Suillus bovinus	30 m	Dahlberg and Stenlid, 1990
	Suillus granulatus	<50m	Jacobsen, et al., 1992
	Suillus luteus	@8m	Fries, 1987
Turf inhabitant			
	Marasmius oreades	1/fairy ring[3]	Mallet and Harrison, 1988

[1] Refers to the maximum distance observed between two somatically compatible samples.

[2] Clonal size in this study was determined by molecular methods but is included here for the sake of comparison.

[3] Size not stated.

Table 2. Mating allele distributions within biological species: reports from the literature.

Ecology Fungus	# samples[1]	summary[2]	Reference
Lignicolous, tetrapolar			
Armillaria mellea	10	T:14,15	Korhonen (1978)
Armillaria mellea "Gr. 3"	6	T:9or10,9or10	Ullrich and Anderson (1978)
Armillaria mellea	3	T:5,5	Anderson, et al. (1979)
Armillaria luteobubalina	9	T:11,10	Kile (1983)
Crucibulum vulgare	?	T:3(5),11(15)	[4]Fries (1936)
Cyathus striatus	?	T:4(5),5(5)	[4]Fries (1936), (1940)
Piptoporus betulinus	N/A	T:see note[3]	Adams, et al. (1981)
Pleurotus ostreatus	8	T:13,13	Kay and Vilgalys (1992)
Polyporus abietinus	?	T:23(100),26(200)	Fries and Jonasson (1941)
Polyporus obtusus	24	T:39(112),39(112)	Eggertson (1953)
Podoscypha ravenelii	prob. 3	T:5,4	Welden and Bennet (1973)
Schizophyllum commune	many	T:96(339),56(64)	Raper, et al. (1958)
Schizophyllum commune	12	T:6,6	Brasier (1970)
Lignicolous, bipolar			
Auricularia auricula	5	B:10(large)	[4]Barnett (1937)
Coprinus comatus	6	B:9(30)	[4]Saunders (unpub)
Fomes cajanderi	18	B:33(21-54)	Neuhauser and Gilbertson (1971)
Fomes subroseus	5	B:9(50)	[4]Mounce and Macrae (1937)
Heterobasidion annosum	9	B:10	Stenlid (1985)
Heterobasidion annosum	53	B:40	Chase and Ullrich (1983)
Polyporus betulinus	101	B:28(30)	Saunders (1956); Burnett, (unpub)
Poria carnegiea	18	B:29(90)	Lindsey and Gilbertson (1977)
Sistotrema brinkmanii			Ullrich and Raper (1974)
Group I	11	B:21(58-?)	
Group II	31	B:55(276->10[4])	
Group III	25	B:31(39-200)	
Group IV	6	B:11(15-?)	
Sparassis radicata	12	B:17	Martin and Gilbertson (1978)
Coprophilous			
Coprinus fimetarius	?	T:27,27	[4]Brunswik (1924)
Coprinus macrorhizus	?	T:20(240),20(240)	[4]Kimura (1952)
Mycorrhizal			
none			
Humicolous			
Coprinus lagopus	?	T:14(large),14(large)	[4]Hanna (1925)
Turf inhabitant			
Marasmius oreades	16	B: 8	Mallett and Harrison (1988)

[1] Samples are defined here as "clones" or "genotypes," and were interpreted from the literature. Data from replicate samples of the same clone or genotype were pooled.

[2] T= tetrapolar, B=bipolar. Numbers indicate observed allele diversity. Numbers in parentheses are estimates. For tetrapolar species, numbers preceding commas indicate A locus alleles, and numbers following commas indicate B locus alleles.

[3] Adams et al. (1981) report shared alleles among clones occupying the same tree, but do not quantify their results.

[4] In: Burnett, (1965).

are composed of several closely linked genes, some homologous, others non-homologous and termed "ideomorphs" (Kües and Casselton, 1992). The complex structure of the mating loci has resulted in a certain amount of inconsistency in terminology among workers in fungal genetics; Raper (1958) referred to the alternate forms of the mating loci as "incompatibility factors," Whitehouse (1949) called them "allelomorphs," and Chase and Ullrich (1990) and others have referred to them as "alleles." While acknowledging the complex, multi-genic nature of the mating loci, I will in this study refer to the observed alternate forms of these loci as alleles, and designate individual allelic combinations as "mating allele genotypes."

The unusual bipolar (one locus determining mating compatibility) and tetrapolar (two loci) heterothallism exhibited by many basidiomycetes is frequently presented in introductory mycology textbooks (i.e., Kendrick, 1985; Webster, 1980) as a system which promotes outcrossing. The implicit assumption is that all basidiomycetes are, in fact, outcrossing, and adjacent genets are not closely related. This, however, is not necessarily valid. Spores produced by fungi with tetrapolar or bipolar mating systems are, respectively, 25% or 50% self-compatible, and so the possibility exists that selfcrossing, rather than outcrossing, occurs regularly in species which are unable to

effect long-range spore dispersal. Field observations of heavy spore deposits immediately below sporocarps (pers. obs.) provide evidence that mushrooms may not always be outcrossing, particularly given the 25 - 50% mating intercompatibility among spores produced by a single genet. In some studies (i.e., Kay and Vilgalys, 1992; Lindsey and Gilbertson, 1977) the occurrence of shared mating alleles within a substrate unit (such as a log) indicate non-random mating allele distribution. This distribution was probably caused by short range spore dispersal from an established genet. However, like the somatic incompatibility studies, nearly all of the studies investigating mating allele distribution in fungi have investigated wood-decomposing fungi. These fungi usually produce their sporocarps high in the air column. Since the elevation of the point of spore release may greatly affect spore dispersal (Ingold, 1971), litter-decomposing species which produce sporocarps close to the ground may have limited spore dispersal relative to wood-decomposing species which produce their sporocarps on tall plant hosts. Restricted spore dispersal, if it occurs, may have profound consequences for the population structure and evolution of fungi.

The purpose of this portion of the study was to determine the diversity and distribution of mating alleles in M. praeacutus and C. subnuda, including samples from on

and off the Brush Mountain plots. Other methods may also be appropriate for the investigation of the relatedness of adjacent individuals in a population of fungi. These include biochemical and molecular approaches, such as isozyme and DNA-based studies, which are expensive and may not provide the required genetic resolution. Alternatively, mating tests are relatively simple to perform and are inexpensive. While these tests are not suitable for measuring the relatedness of different individuals, they are able to detect parent-offspring relationships. In addition, properly administered mating tests (Boidin, 1986) provide a suitable method for testing the outcrossing model of fungal mating systems. Furthermore, having litter-decomposing fungi represented in the growing database on mating allele frequency and diversity was considered valuable.

Nevertheless, mating tests to determine mating allele diversity and distribution can be labor intensive, which probably explains why they have not been widely applied. A single mating test involves the pairing of two monokaryons on an agar plate, incubation, and examination of the resulting mycelium microscopically for the formation of clamp connections. Since pairings are usually made in all possible combinations, the amount of work required increases substantially with increased sample sizes. The most comprehensive research on mating allele distribution and

diversity involves the tetrapolar Schizophyllum commune Fr. (Raper, 1962; Raper, et al., 1958; Table 2). Studies of S. commune used macroscopic differences in the morphology of shared A and shared B hemicompatible reactions to determine mating allele genotypes in this tetrapolar fungus. In Schizophyllum, a cross between two single-spore isolates (SSIs) with a common A allele yields a distinctive "flat" colony morphology, whereas common B alleles yield a "barrage" reaction. Thus, not only can the reaction be scored without microscopy, but a researcher needs to use only a single pair of compatible SSIs (hereafter referred to as a "set" of SSIs) to represent each collection. The convenient reaction types of S. commune enabled researchers to use sample sizes which have not been approached by workers using other species.

Unfortunately, preliminary tests with M. praeacutus showed no difference in colony morphology between common A or common B reactions. Therefore, a new approach to the investigation of tetrapolar fungi which lack morphological characters useful for distinguishing common-A versus common-B matings is proposed and tested. These approaches are appropriate for tetrapolar basidiomycetes which meet the following criteria (next page):

1. Spores which germinate and grow in culture.
2. The primary mycelium is uninucleate, unclamped, and homokaryotic; the secondary mycelium is binucleate, clamped, and heterokaryotic.
3. The fungus is not amphithallic.
4. Nuclear migration and clamp formation occur readily in crosses between compatible monokaryons.

Determining mating allele genotypes in a tetrapolar fungus is problematic, because an incompatible cross can be caused by mating allele identity at either one or both mating loci. A cross between the two sets from one collection and two sets from another collection would yield complete and unambiguous results, but the labor involved would be enormous (Fig. 1).

The approach described here is based on a theoretical analysis of all possible genotypic combinations involving crosses of both sets from two collections (a 4 X 4 matrix). It was observed from this analysis that in some genotypic combinations, a unique result is obtained when only one pair of sets was crossed. In these cases, unambiguous genotypic identification could be made with only 4 crosses (a 2 X 2 matrix) rather than 16 crosses (a 4 X 4 matrix). For instance, if the two collections have completely different alleles at both the A and B loci, 4 of 4 crosses (100%) will

produce clamp connections; no other combination of shared and unshared alleles gives this result. Other combinations give results with two or three possible interpretations. These can always be resolved by introducing the other set of one of the collections to the crosses. The maximum number of crosses is thus eight, still considerably fewer than the 16 required of a full 4 X 4 matrix. A decision-making flow chart and the rationale for its design are included in Appendix 1. A similar experimental design was developed independently and used successfully by Kay (1991) for the wood-rotting oyster mushroom Pleurotus ostreatus.

METHODS

General

On-plot collections of C. subnuda and M. praeacutus were made periodically throughout the growing season. Off-plot collections were made in similar habitats when weather conditions indicated favorable collecting. Collections were wrapped in waxed paper or stored in closed plastic boxes and transported promptly to a laboratory facility for fresh descriptions, culture isolation, and drying. All collections have either been entered into the Virginia Tech Mycological Herbarium or are kept by the author and are available for examination upon request.

Each collection was assigned a collector's number and

the tissue isolates were given the same number. Single spore isolates of a particular collection were assigned the same integer with a unique decimal number added to it (for instance, collection JFM 621 has an associated tissue isolate JFM 621 and a series of SSIs JFM 621.01-621.12). The collections have been entered into the Virginia Tech Mycological Herbarium and assigned VTMH numbers. The isolates have been entered into the Virginia Tech Culture Collection (VTCC) and assigned VTCC numbers. Each database (JFM, VTMH, and VTCC) is organized by DBASE software (Borland International). Despite the proliferation of different numbers for the same genetic material, each database contains a field for "collector's number." This feature allows the databases to be linked and searched independently or together. In this report, all collections and cultures will be referred to by collector's number, unless otherwise stated.

The methods used for tissue and single-spore isolation, di-mon crosses and mating crosses were previously described (Murphy, 1992; Murphy and Miller, 1993). The nuclear condition of hyphal cells from selected crosses and spores was determined by mounting samples in DAPI (4'-6-diamidino-2-phenylindole, 1 mg/mL) and by observing nuclei with epifluorescence microscopy (Petersen and Halling, 1993; Williamson and Fennel, 1975).

Collybia subnuda

Personal observations and herbarium records indicate that the fruiting season of C. subnuda is May through October. Vilgalys (pers. comm.) reports that C. subnuda fruiting begins in autumn and continues through November in the Piedmont region of North Carolina. Typically, C. subnuda fruits after at least two days of warm, humid weather and frequent showers. Infrequent showers or even downpours will not stimulate fruiting unless the forest detritus is soaked and atmospheric humidity levels remain high. Thus, collections were made opportunistically during the fruiting season of C. subnuda, when warm, wet conditions prevailed.

Collections of C. subnuda were made from additional regions to supplement the already extensive collections on the Brush Mt. plots (Murphy, 1992). According to herbarium records, the distribution of C. subnuda is from New York westward through Ontario, Michigan and Wisconsin, and southward through Illinois, Indiana, Ohio, West Virginia, New Jersey (type locality), Virginia, Tennessee, North Carolina, and South Carolina. Limited resources prohibited sampling of all the known range or outside the range, but collections were made in New York, Ohio, West Virginia, Virginia, North Carolina, and South Carolina. Based on genet size observed previously (Murphy, 1992; Murphy and

Miller, 1993), repeated sampling of the same genet was avoided by limiting the minimum distance between collections to approximately 10 m.

Intracollection mating crosses (self-crosses) of C. subnuda involved at least six SSIs; if necessary, additional SSIs were included until two compatible isolates were found. These were then used as tester strains in subsequent intercollection crosses. In this study, a "set" is defined as two intercompatible SSIs from a single collection. In C. subnuda, each SSI in a set contained one of the two mating alleles possessed by the parent mycelium; a set, therefore, contained both mating alleles present in the parent mycelium and represented it in subsequent intercollection crosses.

Intercollection crosses of C. subnuda were performed by crossing SSIs of sets from different collection in all possible combinations. The formation of clamp connections in hyphae resulting from a cross was considered indicative of intercompatibility and therefore non-identity of the mated SSIs' mating alleles. Incompatible matings lacked clamp connections and indicate identity of one or two mating alleles. For example, any two collections of C. subnuda can share 0, 1, or 2 mating alleles. A cross between the SSI sets derived from the two collections will yield, respectively, 4/4, 3/4, or 2/4 compatible reactions. To test this conjecture, a multi-isolate intercollection cross

was made which included several SSIs of both mating types.

Marasmiellus praeacutus

Self-crosses of M. praeacutus involved eight SSIs initially. If only three of the four mating types were recovered, additional SSIs were added to the self-cross until the fourth was found. In this manner, two sets of SSIs were obtained from each M. praeacutus collection. Both isolates of each set are compatible with each other but incompatible with isolates from the other set.

Intercollection crosses between single sets of SSIs from the Brush Mt. plots were made according to the flow-chart presented in Appendix 1. An additional eight collections from regions off the Brush Mt. plot were added to the interset crosses to test if local and regional mating allele diversities were different. Single-spore isolates from off-plot collections were first crossed with each other to determine if they shared any mating alleles. They were then crossed with selected SSIs from plot 2 to determine if alleles were shared between on- and off-plot collections. Once the off-plot collections had been screened against the selected isolates from plot 2 and against each other in 2 X 2 crosses, mating allele genotypes were deduced from the resulting pattern of intercompatibility.

RESULTS

Collybia subnuda

Figure 2 shows the locations of the *C. subnuda* collections made on the Brush Mountain plots and included in this study. Most of these collections are represented by tissue and single spore isolates. Somatic incompatibility tests performed previously (Murphy, 1992) determined the genetic identity of these isolates. In most cases, only single samples of each genet (i.e., one collection) are represented in these crosses, but occasionally sets obtained from somatically compatible isolates were used to test the consistency of the methods used in this study and the previous study.

Complete sets of SSIs were obtained from 12 collections representing 10 genets (5 on each of two plots). Many collections were made which were not used in this study, because SSIs from them were not obtained. It should be noted that the voucher specimens of these collections may be useful in future, molecular-based investigations of the population biology of *C. subnuda*.

Interset mating crosses could usually be scored after 7 days. By this time, clamp connections formed by compatible matings were seen throughout the mycelium on the plate, not just at the contact zone. All crosses were examined microscopically, but it was observed that incompatible

crosses formed a dense wall of pigmented mycelium at the contact point of the two cultures. This mating incompatibility reaction was identical in appearance to the somatic incompatibility reactions previously reported (Murphy, 1992; Murphy and Miller, 1993). The dikaryotic condition of clamped mycelia and the monokaryotic condition of unclamped mycelia were confirmed by DAPI staining of the nuclei.

The data presented in Figure 3 indicate that different SSIs from the same collection give consistent and predictable reactions when crossed with each other. Results from the C. subnuda interset crosses are given in Figure 4. Sets obtained from somatically compatible collections always gave identical results, indicating that somatic and mating compatibility testing are consistent. On plot 3, none of the different genets shared mating alleles, indicating that no parent/offspring relationships exist among these samples. On plot 1, one pair of genets shared a single allele; the rest of the genets on the plot had unique genotypes. Three alleles were common to both plots.

Of a total of 20 possible mating alleles from the 10 genets tested, 16 were found. Using the method given by Stevens (1941), these data allow a species estimate of 45 mating alleles in C. subnuda, with a 95% confidence interval of 19-187. Because the estimate for C. subnuda is based on

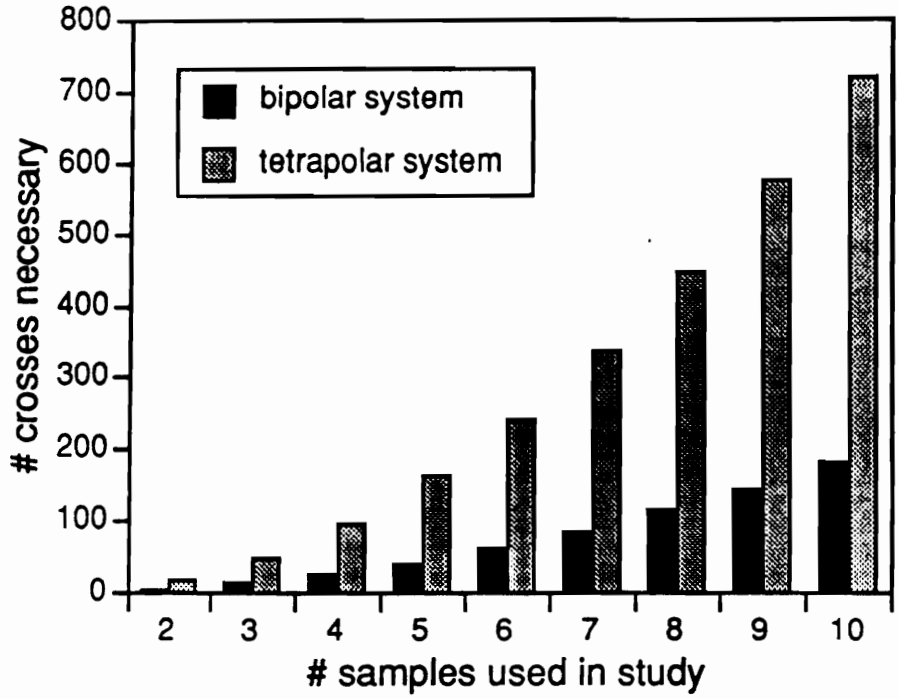

Figure 1. Relative effort required to determine mating allele genotype by intercollection pairing for bipolar and tetrapolar species.

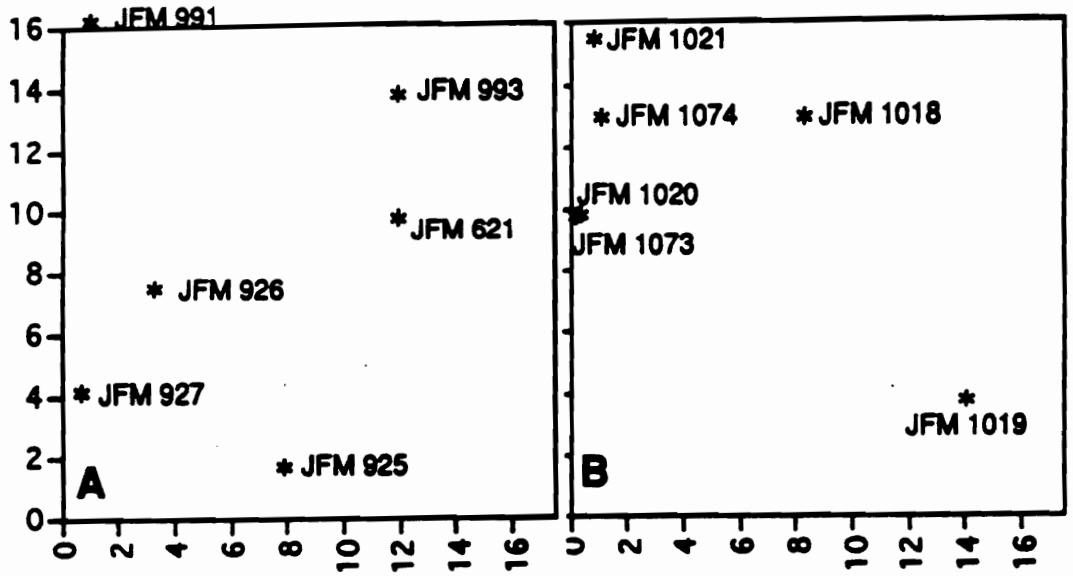

Figure 2. Distribution of Collybia subnuda collections on the Brush Mt. plots. Increments are in meters. A) Plot 1. B) Plot 3. For ease of viewing, many recorded occurrences of C. subnuda are not shown. For the location of these collections, and for precise location and design of the plots, see Murphy, 1992.

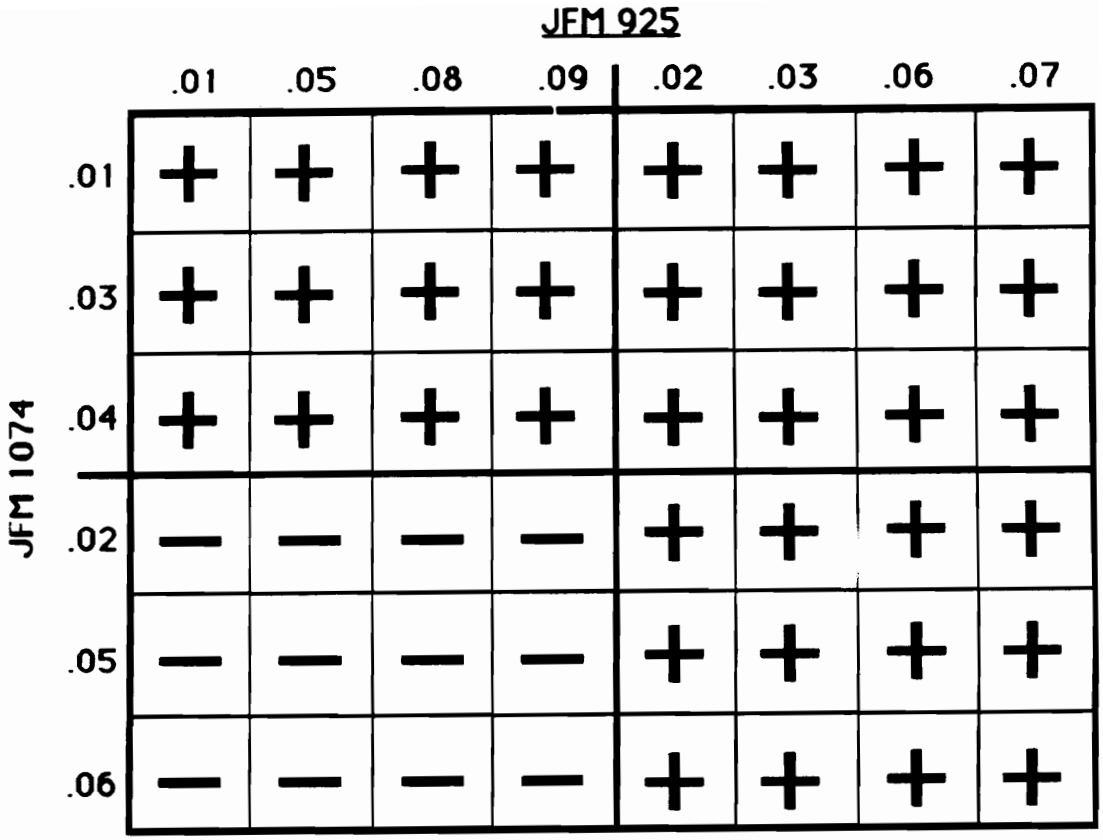

Figure 3. Multi-isolate intercollection mating cross between two Collybia subnuda isolates which share one mating allele.

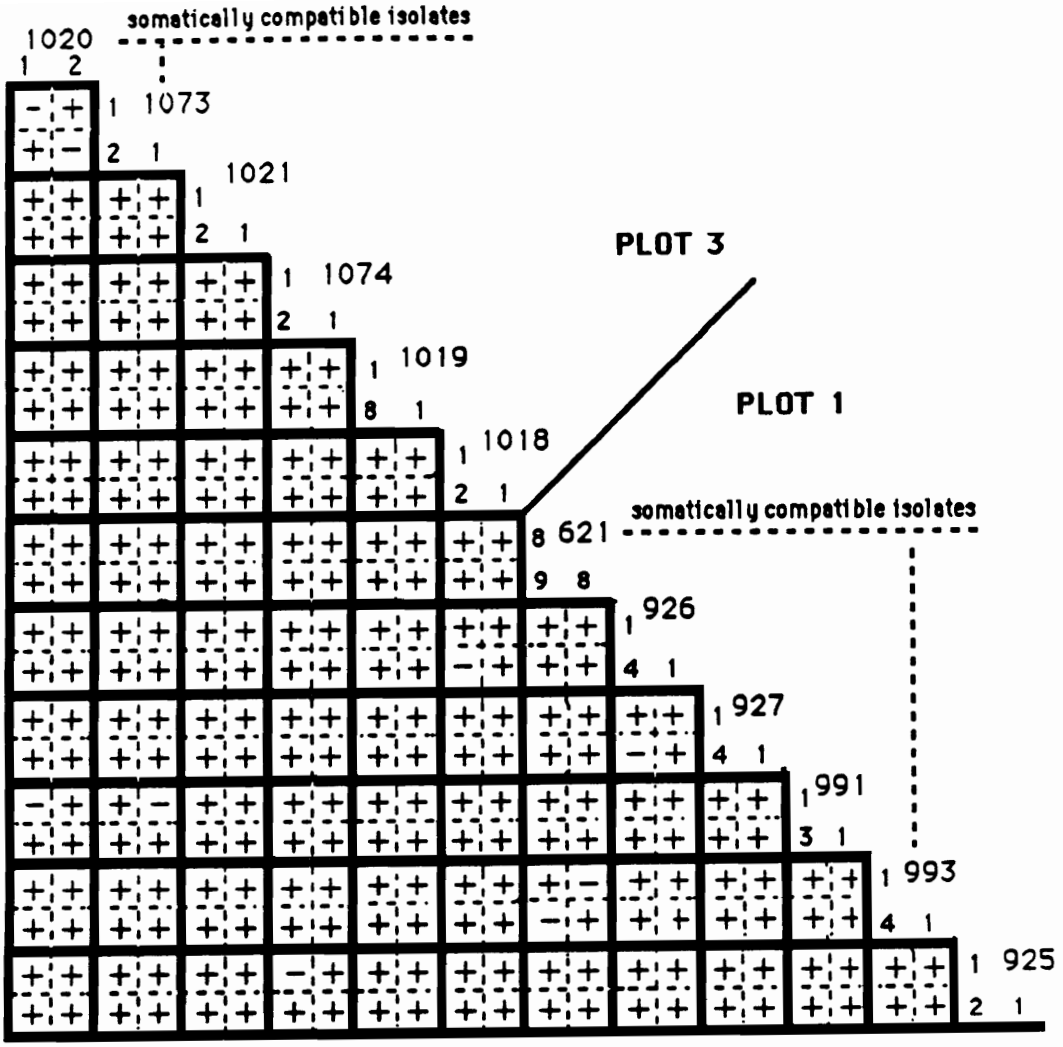

Figure 4. Collybia subnuda intercollection mating cross results, Brush Mt. plots. (+) indicates clamp connection formation; (-) indicates no clamp connection formation.

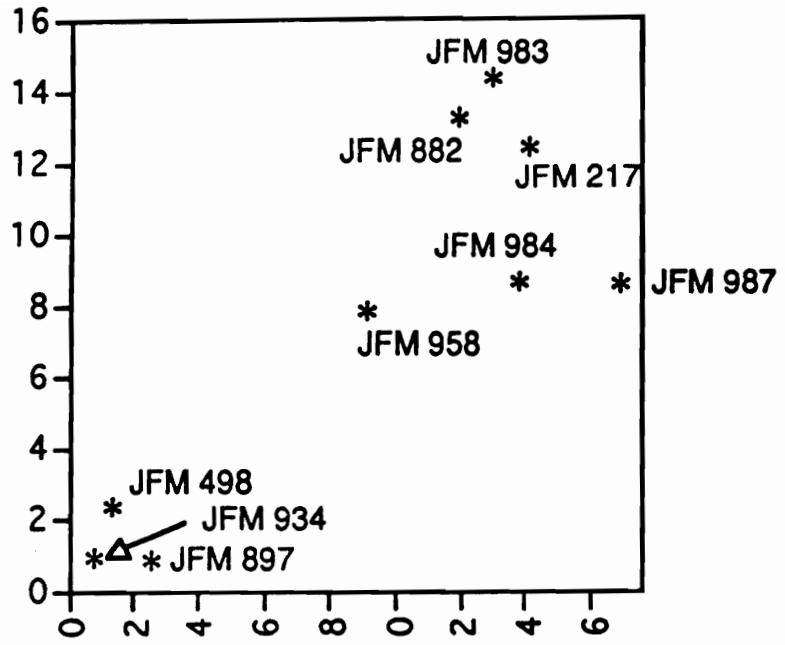

Figure 5. Location of collections of Marasmiellus praeacutus on the Brush Mt. plot. See Fig. 2 for additonal notes.

a sample of only 10 genets, an effort was made to increase its accuracy by adding additional off-plot samples to the study. Surprisingly, however, C. subnuda isolates from areas less than a kilometer away were often wholly incompatible with the Brush Mountain isolates. Further investigation led to the discovery that C. subnuda consists of at least two intersterile groups (ISGs). This finding, discussed in Chapter 2, overshadowed the importance of increasing sample size to acquire a more accurate estimate of mating allele diversity, and the original estimate remains unmodified.

Marasmiellus praeacutus

Although M. praeacutus was very common on the frequently sampled Pinus pungens-dominated Brush Mountain plots (Fig. 5), collecting under Pinus elsewhere was not as rewarding as collecting under hemlock (Tsuga canadensis), probably because the mesic habitat preferred by hemlock also favored fruiting of associated litter decomposers. Further collecting is required, however, before any generalizations can be made about the substrate preferences of M. praeacutus.

Clamp connections on compatible matings were abundant after approximately seven days. Nuclear staining by DAPI confirmed that clamped hyphae were dikaryotic and unclamped

hyphae were monokaryotic. Twelve sets of SSIs were used in the Brush Mt. interset crosses, several of which originated from somatically compatible isolates. In most cases, genotypes could be determined by examining the combinations of compatible and incompatible results in the original matrix (Fig. 6). In some cases, additional crosses were included to resolve the different possible interpretations of the original matrix (Fig. 7). The allelic designations were assigned in the chronological order of their determination.

Every interset mating cross between sets derived from somatically compatible isolates gave results which supported the genetic identity of the isolates (data not shown). In one case, interset mating results showed that two collections separated by approximately 17 m had identical mating alleles. Dikaryotic tissue isolates of these collections were available and were crossed in a somatic compatibility test. A compatible reaction resulted, indicating genetic identity of two collections. This result indicates that the size and shape of genets of M. praeacutus may be more complex than previously thought.

In all, sets of SSIs from six different genets from the Brush Mt. plot were crossed. The location of the genets and their mating allele genotypes are given in Fig. 8. Many of the alleles were shared between different genets, and some

genets shared more than one allele. Some allele combinations occurred more than once on different genets throughout the plot. For instance, the A_1B_3 combination was found in five of the six genets sampled, including two somatically compatible collections separated by 17 m.

As indicated by the pattern of compatible and incompatible crosses, mating alleles were shared among the off-plot collections (Fig. 9) as well as between the off-plot and Brush Mt. collections (Fig. 10). Mating allele genotypes for all collections included in the intercollection crosses are given in tables 3 and 4. Four A alleles and one B allele were shared between the Brush Mt. and the off-plot collections. Most notably, allele B_7 was found on Brush Mt., Poverty Hollow (approximately 10 km from the plot), and in the Mt. Rogers National Recreation Area (approximately 100 km from the plot). Among the off-plot collections, four A alleles and three B alleles were shared. Allele A_8, for instance, was found on Brush Mt. (not on the plot), in the Mt. Rogers National Recreation Area, and at Oconee State Park, South Carolina. Eleven A alleles and 12 B alleles were identified in the eight off-plot collections. From these and the Brush Mt. plot data, estimates for the total mating allele diversity of M. praeacutus were derived (Stevens, 1941; Table 5).

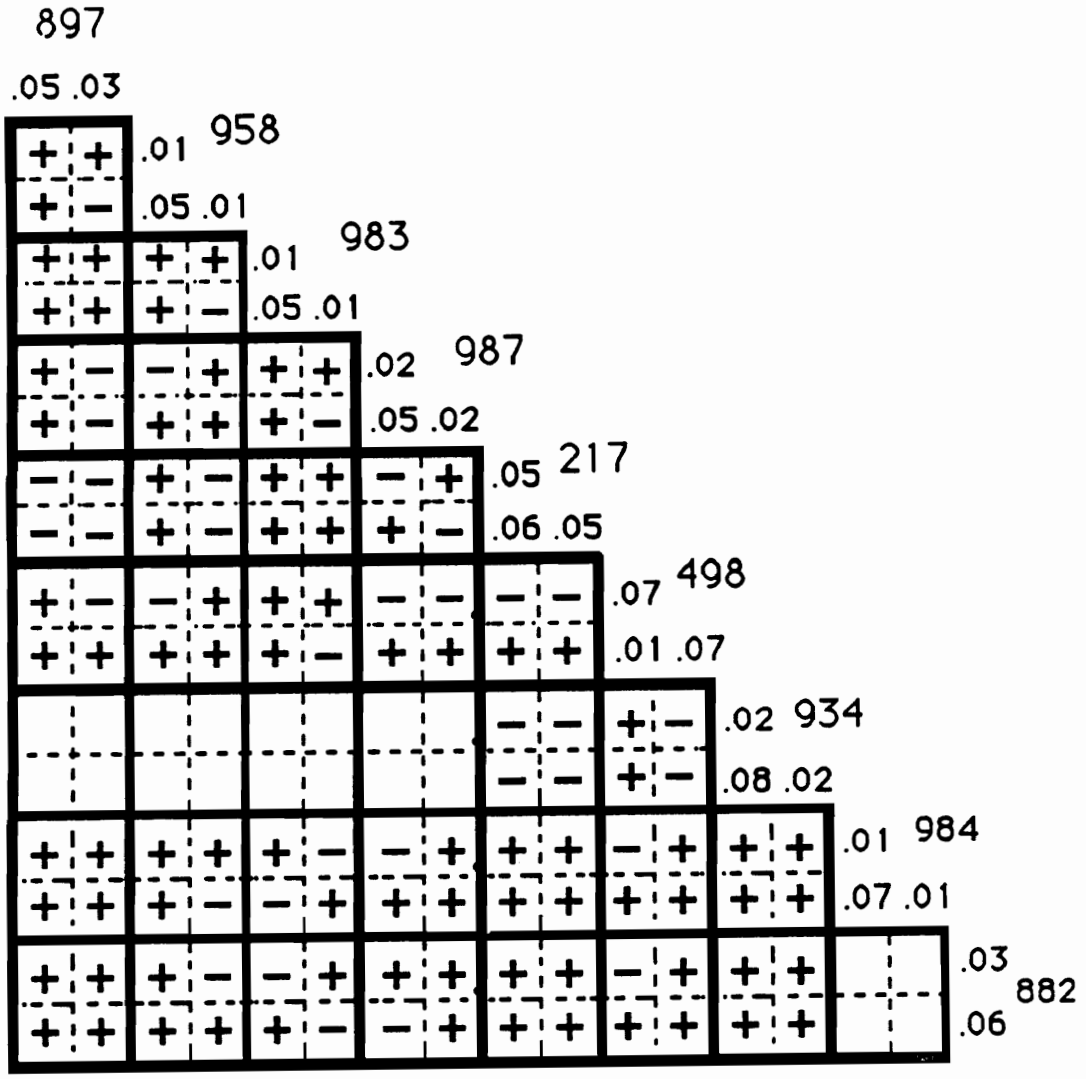

Figure 6. Intercollection mating cross between Marasmiellus praeacutus collections on the Brush Mt. plot. (+) indicates clamp connection formation; (-) indicates no clamp connection formation. Integers indicate JFM collections. Collections JFM 217 and 897 were somatically compatible. Empty boxes indicate cross not performed.

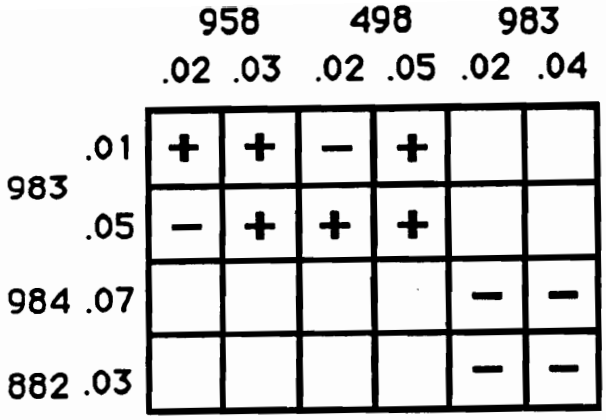

Figure 7. Additional crosses necessary to resolve mating allele genotypes of Marasmiellus praeacutus on plot 2.

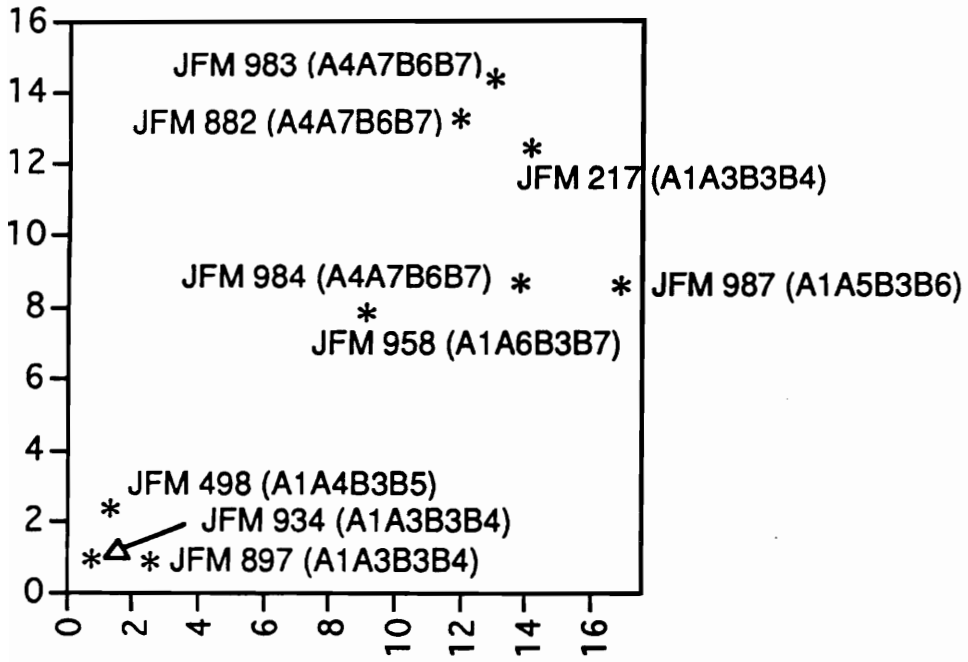

Figure 8. Location of collections of M. praeacutus on the Brush Mt. plot, with mating allele genotypes noted. JFM 934, 897, and 217 are somatically compatible. Tissue isolates were not available to test JFM 983, 882, and 984, although their genotypes certainly suggest they are clonal.

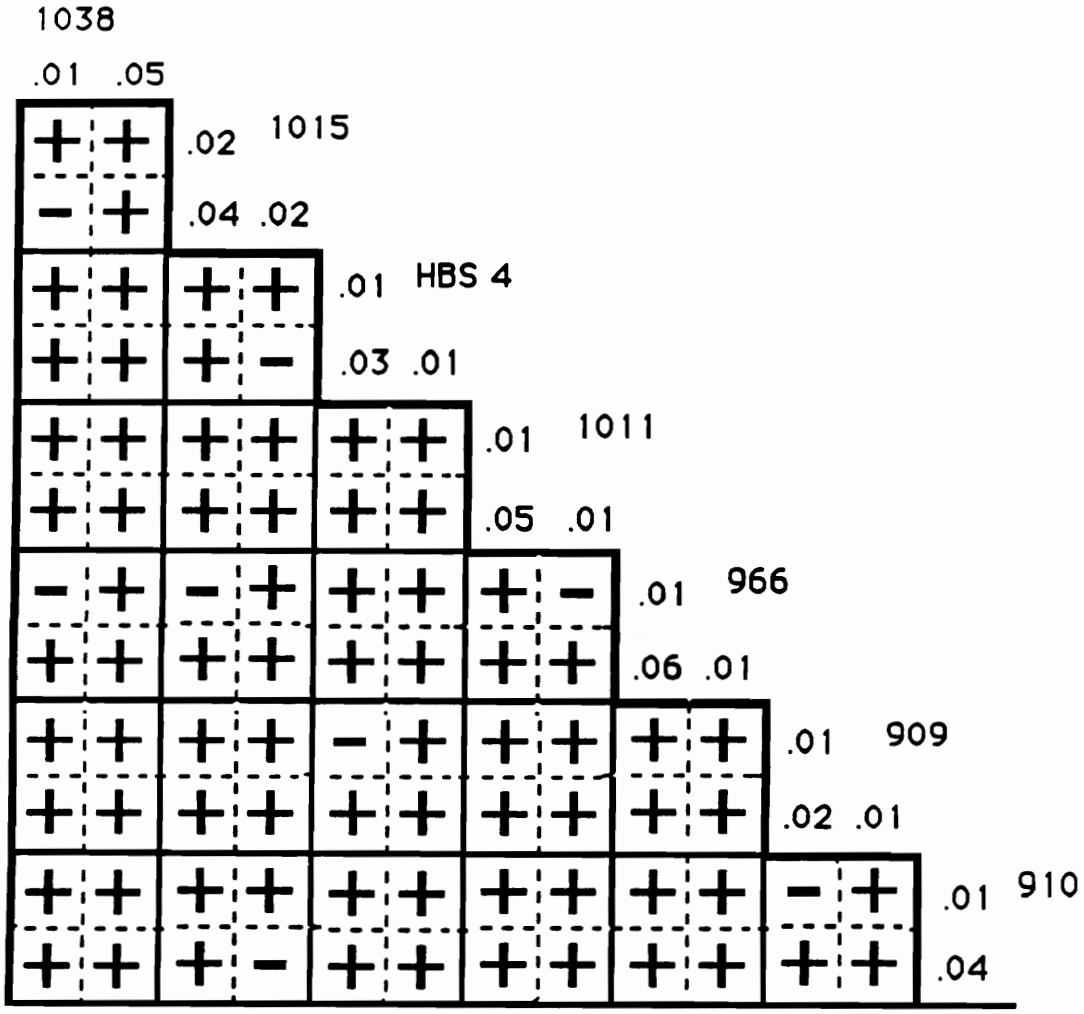

Figure 9. Intercollection crosses between off-plot Marasmiellus praeacutus collections. (+) indicates clamp connection formation; (-) indicates no clamp connection formation.

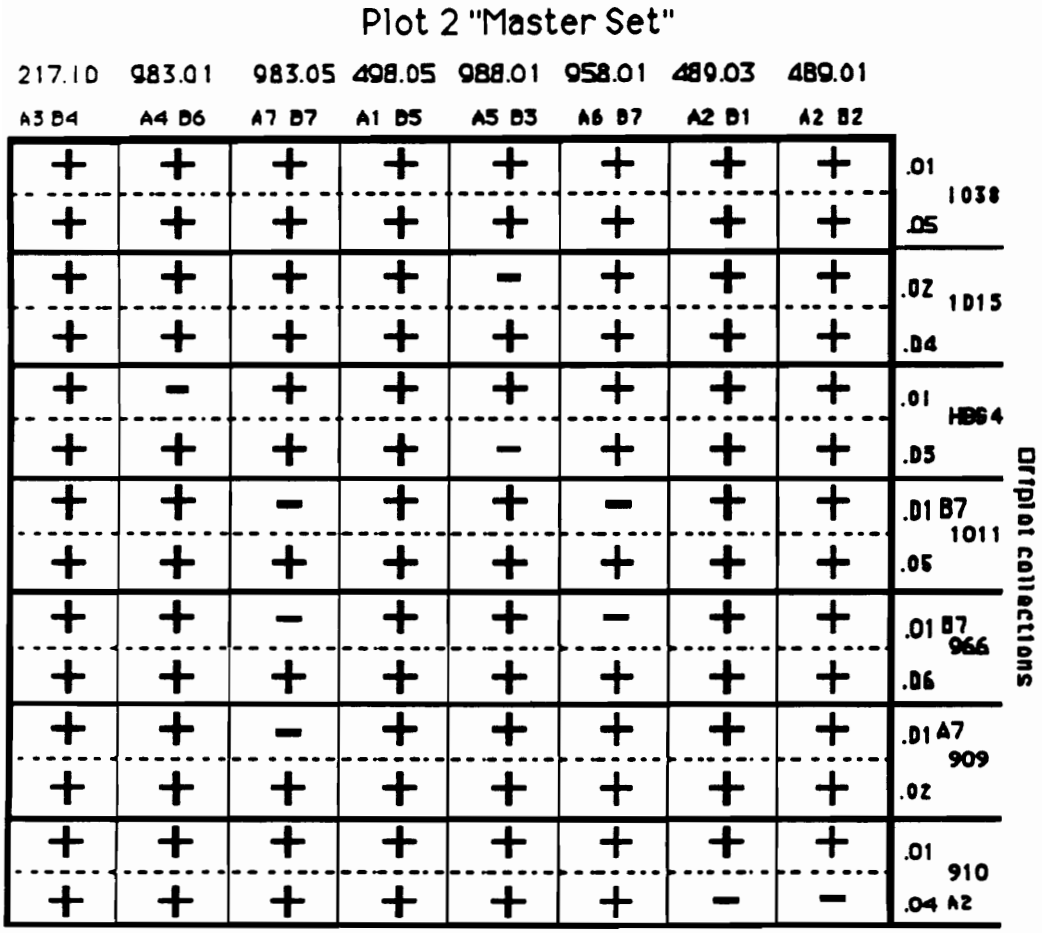

Figure 10. Marasmiellus praeacutus intercollection crosses between selected isolates from plot 2 and off-plot collections. (+) indicates clamp connection formation; (-) indicates no clamp connection formation.

Table 3. Mating allele genotypes of Marasmiellus praeacutus genets on Brush Mountain plot 2.

Collection	ssi #	Genotype
JFM 217[1]		$A_1A_3\ B_3B_4$
	.05	A_1B_4
	.06	A_3B_3
JFM 498[1]		$A_1A_4\ B_3B_5$
	.01	A_4B_5
	.07	A_1B_3
JFM 882		$A_4A_7\ B_6B_7$
	.03	A_7B_7
	.06	A_4B_6
JFM 897		$A_1A_3B_3B_4$
	.03	A_1B_3
	.05	A_3B_4
JFM 934[2]		$A_1A_3B_3B_4$
JFM 958		$A_1A_6\ B_3B_7$
	.05	A_1B_3
	.01	A_6B_7
JFM 987		$A_1A_5\ B_3B_6$
	.02	A_5B_3
	.05	A_1B_6
JFM 983		$A_4A_7\ B_6B_7$
	.01	$A_4\ B_6$
	.05	$A_7\ B_7$
JFM 984		$A_4A_7\ B_6B_7$
	.01	A_4B_6
	.07	A_7B_7

[1] Determined previously (Murphy, 1992)
[2] Genotypes of the SSIs of this isolate are not available.

Table 4. Mating allele genotypes of off-plot collections of *Marasmiellus praeacutus*.

Collection	ssi #	genotype	locale
JFM 489		A_1A_2 B_1B_2	Poverty Hollow, Montgomery Co, VA
	.01	A_2B_2	
	.03	A_2B_1	
JFM 909		A_7A_9 B_8B_{11}	Along Blue Ridge Parkway near Montebello,
	.01	A_7B_{11}	Rockbridge Co., VA
	.02	A_9B_8	
JFM 910		$A_2A_9B_8B_{10}$	Along Blue Ridge Parkway near Montebello,
	.01	A_9B_8	Rockbridge Co., VA
	.04	A_2B_{10}	
JFM 966		$A_8A_{13}B_7B_{16}$	Along Lewis Fork Creek, Mt. Rogers Nat. Rec. Area, Grayson Co., VA
	.01	A_8B_7	
	.06	$A_{13}B_{16}$	
JFM 1011		$A_{12}A_{14}$ B_7B_{15}	Poverty Hollow, Montgomery Co, VA
	.01	$A_{14}B_7$	
	.05	A_{12} B_{15}	
JFM 1015		A_5A_8 $B_{10}B_{13}$	Plot 4, Brush Mt., Montgomery Co, VA
	.02	A_5B_{10}	
	.04	A_8B_{13}	
JFM 1038		A_8A_{10} B_9B_{12}	Oconee St. Pk., Oconee Co., SC
	.01	A_8B_9	
	.05	$A_{10}B_{12}$	
HBS04		$A5A_{11}$ $B_{11}B_{14}$	Highlands, Macon Co., NC
	.01	$A_{11}B_{14}$	
	.03	A_5B_{11}	

Table 5. Estimated mating allele diversity and 95% confidence intervals at the A and B mating loci in M. praeacutus.[1]

	A locus			B locus		
	Estimate	Upper	Lower	Estimate	Upper	Lower
Off-plot data only	19	31	5	28	89	6
Pooled data	16	25	10	20	34	12

[1] Estimates and 95% confidence intervals were calculated according to Stevens (1941).

DISCUSSION

Collybia subnuda

The similarity in appearance of mating and somatic incompatibility zones suggests a common physiological and, perhaps, genetic basis for the reactions. Additional research on this area is badly needed.

The observed high allelic diversity of *C. subnuda* indicates that adjacent genets are probably not siblings and that *C. subnuda* is most likely a regularly outcrossing species. None of the sampled genet pairs had a direct parent/offspring relationship. While it remains possible that the tested genets were 2 generations removed from each other, this hypothesis seems unlikely. It was remarkable that more mating alleles (2 or 3) were shared between plots (several km apart) than within plots; this observation suggests that the outcrossing model applies to *C. subnuda*. The number of alleles estimated from the data for the entire species is 45, which corresponds well with other reports (see Whitehouse, 1949; Burnett, 1965; Table 2). For population-level sampling, then, it would not be inappropriate to sample closely adjacent genets.

The discovery of intersterility groups within *C. subnuda* is particularly significant, and will be discussed in more detail in Chapter 2.

Marasmiellus praeacutus

In contrast to C. subnuda, M. praeacutus had an unexpectedly low allele diversity and a high number of allelic repeats, at both local and regional levels. Although tissue isolates of appropriate collections were not dedikaryotized and tested for nuclear mating allele genotypes, the pattern of distribution of the A_1B_3 allelic combination on the Brush Mt. plot suggests that di-mon crossing has occurred (Buller, 1931). The unexpected frequent occurrence of shared alleles among different genets on the plot indicate either low allelic diversity in the species as a whole or local, non-random distribution of mating alleles.

The mating allele estimates for M. praeacutus are lower than expected for tetrapolar fungi (Raper, et al, 1958; Whitehouse, 1949; Table 2). The algorithms used for these estimates assume random distributions and equal frequencies of mating alleles; clearly this may not be the case in M. praeacutus, and these estimates should be judged accordingly. However, even if the possible effect of non-random allele distribution is eliminated by omitting the plot 2 data from the analysis, the calculated upper limits remain considerably lower than the 100 alleles typical of tetrapolar basidiomycetes. It appears than an unknown mechanism or mechanisms are operating to limit the mating

allele diversity in M. praeacutus.

Without additional experiments, the explanation for the low mating allele diversity in M. praeacutus will remain a matter for conjecture. The following observations may be relevant to future studies of this species. Marasmiellus praeacutus sporocarps range from 10-30 mm tall (Halling, 1987, and pers. obs.), which is often insufficient to raise the pileus above the litter layer (pers. obs.); consequently, spore dispersal may be limited. Spore dispersal may also be affected by limited spore viability. The viability of spores of M. praeacutus was not investigated in this study; however, the results of the study of C. subnuda spore viability (see Chapter 3) may be generally applicable to other species of fungi (such as M. praeacutus) with thin-walled, hyaline spores. Short-range spore dispersal and limited spore viability may result in decreased colonization potential, decreased levels of gene flow between separate habitats, and a consequent increase in the importance of founder events in the evolutionary history of this organism.

A low level of mating allele diversity has also been observed in the very distantly related bird's-nest fungi (Fries, 1943). In some sampled geographical regions, only two alleles per mating locus, the lowest possible number, were found. Further sampling led to the finding that

within-region diversity was always low, but between-region diversity was higher. In these fungi, peridioles containing large numbers of spores are dispersed by the splashing of raindrops. The dispersal distance cannot be very great, and upon germination of the many spores within the peridiole, self-crossing undoubtably occurs.

Similar habitat and stature types occur in many diminutive, litter-decomposing fungi, particularly marasmioid and mycenoid species, and it is possible that population structures similar to that of M. praeacutus will be found when these are investigated.

An important consequence of these findings concerns the methodology of population sampling. One of the dangers of sampling a fungal population is accidentally resampling the same genet, or closely related group of genets, and unintentionally obtaining a biased sample. The higher fungi have a wide variety of genet sizes, from the huge Armillaria genet reported by Smith, et al. (1992), to the much smaller genets of C. subnuda and M. praeacutus. An adequate strategy for population sampling must therefore include determination of genet sizes and even genet shapes. In this study, mating tests led to the discovery of somatically compatible isolates of M. praeacutus from collections 17 m distant, a new record for the species. The vegetative hyphae of these collections may or may not be physically

separated. If this sort of genet structure is common in M. praeacutus, it is possible that genets may grow and become fragmented over time. A consequence of this phenomenon would be a population structure consisting of a mosaic of small, closely related genets and ramets scattered across the landscape. Population structures such as this have been suggested by the studies of the litter decomposer Marasmius androsaceus (Holmer and Stenlid, 1991) and the root-rotting fungus Phellinus weirii (Murr.) Gilb. (Dickman and Cook, 1989), although P. weirii genet sizes are considerably larger than the litter decomposers studied to date. The mosaic structure of fungal populations may be common in fungi occupying relatively continuous substrates, such as soil, roots, or litter, and may contrast with the population structure of fungi occupying discretely defined substrates, such as above-ground wood or dung. The outline and structure of fungal populations, therefore, may vary considerably between species and ecological habits, and sampling strategies must take this into account.

In addition, it is now clear that one cannot assume that an agaric species is outcrossing without considerable risk. The higher fungi exhibit population structures which range from entirely inbreeding (i.e., the birds-nest fungus studied by Fries, 1943) to mostly outcrossing. While population structure can be inferred from the

characteristics of any particular species (i.e., sporocarp size, spore morphology, sporocarp substrate, etc), it is strongly suggested that it be determined empirically.

CHAPTER 2: INTERSTERILITYGROUPS IN *COLLYBIA SUBNUDA*

INTRODUCTION

General

The unusual intersterile crosses between collections noted in Ch. 1 demanded a closer examination. Accordingly, experiments were designed to investigate the degree of intersterility between various isolates of *C. subnuda*, their geographic distribution, and their morphological, ecological, and molecular differentiation. In addition to preparing the way for further population-level studies, it was hoped that this investigation would refine the current *C. subnuda* species concept.

Intersterility in fungi is not uncommon (Boidin, 1986), and mycologists generally agree that the development of intersterility barriers is an early event in the process of speciation. The development of an intersterility barrier, however, is neither a necessary nor sufficient grounds for delimiting species in the fungi. Vilgalys (1991), for instance, describes how morphological variation and the development of intersterility barriers are unlinked phenomena in the development of species. He proposes a phylogenetic species concept "which provides information about allopatric mating groups based on combined data from mating compatibility, morphology, and macromolecules." Disagreement exists, however, concerning the interpretation

of sympatric, morphologically identical but mating-incompatible sibling species. This chapter addresses the arguments of sympatric versus allopatric speciation in fungi, and the potential of either process to cause the current distribution of C. subnuda intersterility groups.

One caution must be made routinely when using intercompatibility tests for the investigation of speciation; plasmogamy is not necessarily indicative of interfertility. Ideally, one could induce dikaryons synthesized from mating crosses to form sporocarps and test their spores for viability, but inducing sporocarp formation is rarely possible and unfortunately is not yet in C. subnuda. Because plasmogamy is at least a strong indicator of a close relationship, it will be referred to as "intercompatibility" in this study.

Boidin (1986) cautioned against premature judgement of intersterility. He described some of the possible explanations of observed intersterility which are not due to incompatibility barriers. These possibilities include i) astatocoenocytic hyphae (a mycelium with clamps only in aerial hyphae), ii) shared mating alleles between the isolates crossed, iii) aberrant or senescent isolates and iv) inadequate incubation time. Collybia subnuda does not have astatocoenocytic hyphae (pers. obs.). Incompatibility resulting from shared mating alleles is unlikely considering

the large number of mating alleles estimated in C. subnuda (see Ch. 1). Nevertheless, to eliminate this possibility two intercompatible isolates from each collection were used in intersterility group mating crosses. Using more than two isolates per collection is not practical for large numbers of intercollection crosses, but in a limited experiment seven isolates from each of two different collections were crossed in all possible combinations as a check against aberrant isolates. Lastly, all crosses giving negative results were re-incubated for an extended period before reexamination.

Molecular studies

Williams, et al. (1990) describe a method they dubbed random amplified polymorphic DNA (RAPD) for the analysis of molecular evolution at the inter- and infraspecific level. Extensive efforts to apply the RAPD method in this study met with non-reproducible results. Consequently, an alternative approach was taken.

Garber, et al., (1988) described the structure of the rRNA genes in Cochliobolus and summarized reports in other higher fungi. In the higher fungi which have been studied, the rRNA genes are organized into tandemly repeating units of 60-130 copies. Each unit is composed of the 17s, 5.8s, and 25s ribosomal genes. The 5s ribosomal gene may or may

not be part of the tandem repeats. Intergenic transcribed spacer regions separate the 17s and 5.8s genes (ITS1) and the 5.8s and 25s genes (ITS2). Because the genic regions are highly conserved, and the ITS regions are less conserved, the rRNA genes have been particularly useful in fungal molecular systematics (Bruns, et al., 1991). Garber, et al. (1988) found the rRNA genes to be homogenous within individuals, which suggested that these loci are under the influence of concerted evolution. Yao (1992), however, presents evidence that in Peronosclerospora, rRNA genes may be heterogenous within individuals, since amplification of this region resulted in more than one product, each of which hybridized to rDNA probes. The heterogeneity of the rDNA genes in Peronosclerospora may be exceptional, however, since this situation has not been reported in other fungal taxa.

Restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR)-amplified ITS region has been used to distinguish infraspecific strains of Cryptococcus spp. (Vilgalys and Hester, 1990), Pythium (Chen, 1992), and Rhizoctonia solani (Liu and Sinclair, 1992; Liu, et al., 1993). Sequence data provide a higher resolution of molecular variation than RFLP data. Species for which sequence data of the ITS region have been useful include Laccaria bicolor (Gardes, et al., 1991) and

Heterobasidion annosum (Kasuga, et al., 1993). Accordingly, the DNA sequences of samples of each C. subnuda ISG were analyzed and compared.

METHODS

Mating studies

Mating crosses were made as described in Chapter 1 except as follows. Often, five crosses were performed on one 100 X 15 mm agar plate. This method introduces the danger of confounding results when the mycelium from one mating cross contacts the mycelium from another, so care was taken to examine the crosses before this occurred. Crosses were incubated at 25° C, for it was found that the fungus grew faster and clamp connections formed more rapidly at this temperature. Occasionally, agar plates containing crosses were placed at 5° C to slow growth until they could be examined. Microscopic examination of the crosses on the agar was made directly, without slide preparation, thereby saving a considerable amount of time and effort. Crosses were examined for clamp connections after approximately seven days, since clamps were rare in younger crosses but abundant throughout mycelia after approximately a week. In selected crosses, the nuclear condition was determined by epifluorescence microscopy and DAPI staining.

The extent of inter-incompatibility between the two C.

subnuda groups was assayed in two experiments. The first involved crosses among seven SSIs from each of two collections previously shown to be incompatible (JFM 621 and JFM 898, Fig. 11). The second experiment involved crosses among 13 collections (for collection numbers, see Fig. 12). In this experiment, six SSIs from each collection were initially self-crossed, and additional SSIs were added until both mating types were recovered. Two isolates of compatible mating type were chosen from each collection and used as representative tester isolates for intersterility testing. All possible combinations of tester isolates of the 13 collections were crossed on individual 60 mm agar plates. Intersterility crosses were examined microscopically after approximately one week. If no clamps were observed, the crosses were incubated an additional seven weeks, at which time macroscopic notes were taken on culture morphology and microscopic examination for clamps was made throughout the plates.

Following the intersterility tests, one isolate from each of two intersterility groups (ISGs) was chosen as a representative tester isolate. The tester isolate from ISG 1 originated from collection JFM 621. The mating system of C. subnuda was first described from this collection (Murphy, 1992). The tester isolate from ISG 2 originated from JFM 898. This collection was made in Poverty Hollow, VA,

several kilometers from JFM 621 and near the plots used by Jacobsen, et al. (1992). Intercollection crosses with this isolate provided the first clue that C. subnuda was composed of two ISGs. These isolates have been deposited at the American Type Culture Collection (Rockville, MD).

Additional collections were identified to ISG by crossing one SSI with both of the ISG tester isolates as described previously. The possibility exists that the SSI used in a cross may belong to the same ISG but share a mating allele with the tester isolate. The expected observation in this case would be an unknown isolate which is incompatible with both tester isolates. When an unknown isolate tested negative with both ISG tester isolates, a second cross was made between the unknown and with the alternate, mating allele complementary testers, and the voucher specimen for the unknown was reexamined.

Biogeography and ecology

Collecting locations were expanded to encompass more of the known range of C. subnuda, which extends from southeastern Canada and the U.S. Midwest south through the Appalachians to South Carolina and Tennessee (see Halling, 1987; and material examined by the author). The mycelium below the sporocarps was destructively sampled (and subsequently replaced), and the nature of the colonized

substrate was classified as either leafy detritus alone, woody alone, or a combination of leafy and woody detritus. The term "woody" here includes tree stems with bark intact.

Effect of substrate on ISG phenotype

To test whether substrate could effect switching of isolates from one ISG to the other, compatible tester isolates from both ISGs were cross-inoculated onto wood and leaf substrates. Oak sawdust and hand-crushed oak leaves, collected in October 1992 and maintained at -20° C, were used. Fifteen grams of substrate were placed in 500 ml ehrlenmeyer flasks, covered with 250 ml double-distilled water, and autoclaved for 45 min. After cooling, the standing water was discarded and each flask was inoculated with five, 1 cm disks of mycelium-covered agar. The substrates were inoculated for three wk at 25° C in the dark. The fungi were reisolated onto MEA and crossed in all possible combinations as described previously.

Morphological studies

Detailed macroscopic notes on sporocarps and vegetative substrate were taken at the time of collection, including observation of sporocarp colors according to Kornerup and Wanscher (1967). Microscopic examinations were made of dried material rehydrated with 95% ethanol, which was

allowed to evaporate. This procedure was followed by the addition of 3% KOH and congo red. Close attention was paid to the characteristics of the pileipellis and the hymenium. Observations of pleurocystidia and cheilocystidia were made by separating the gill edge from the gill face with a razor blade and examining each separately. Microscopic observations were made with a calibrated Leitz binocular microscope, and drawings were made with a Leitz drawing tube. In contrast to collections obtained from herbaria, collections made by the author could be identified to ISG if SSIs were obtained from them.

Collybia subnuda has often been misidentified in North America as Collybia peronata, a European species (Gilliam, 1976; Halling, 1983). In fact, Ellis referred to what would become the holotype as "Marasmius peronatus subnudus." Guzman has two Mexican collections deposited at DAOM (Holmgren, et al., 1990) and designated C. peronata. These collections were of particular interest, since the occurrence of C. peronata in the New World would be notable. Alternatively, if these were misidentified collections of C. subnuda, proper identification would considerably extend its known southern range. Accordingly, the Guzman collections were requested and examined.

Spore drops on aluminum foil were obtained from 11 collections and maintained at 4 C. Future investigations

should probably maintain spores at -20 C (R. Vilgalys, pers. comm.; see also chapter 3), since spore viability tends to decrease rapidly with time. Suspensions of spores were made in 3% KOH, and length and width measurements were taken from random samples of 15 spores from each collection. The resulting data were analyzed statistically using NCSS ver. 5X software.

Molecular studies

Mycelia for DNA isolation were grown in aerated liquid culture as follows. Sterile malt-extract broth (1.5%, 1.4 L) in 2 L Ehrlenmeyer flasks were inoculated with cubed agar cultures and maintained at room temperature. Air was filtered through 0.2 μ filters (Gelman Sciences) and bubbled through the liquid culture at a rate sufficient to keep the inocula suspended. Mycelia were harvested after approximately 2 weeks incubation, rinsed briefly in tap water, frozen in liquid nitrogen, lyophilized, ground in liquid nitrogen with a mortar and pestle, and stored at -20C prior to DNA extraction. Small-volume DNA isolation was performed using either a chloroform/phenol (Lee and Taylor, 1990) or a CTAB-based (Gardes and Bruns, 1993) extraction method. An unidentified compound, probably composed of polysaccharide, co-precipitated with the DNA but did not interfere with subsequent DNA amplification and sequencing.

Ribonucleic acids were removed by treatment with 100 μg/mL RNA-ase. DNA was stored in 100 μL TE (10mM Tris-Cl, 1 mM EDTA, pH 7.4) at -20° C. Quantification of the DNA was made with a Hoeffer Scientific TKO fluorometer, and concentrations ranged between 1 and 60 ng/μL.

Primers used in amplification of the ITS region were ITS1 and ITS4 (White, et al., 1990). Reaction volumes were 25 μL and contained 50 mM KCl, 10 mM tris-HCl, 1.5 mM $MgCl_2$, 100 μM each dNTP, 1 U Taq DNA polymerase (Perkin Elmer Cetus), 200 μM each primer, and 5 μL of a 1:100 dilution of DNA in water. Amplification was performed on a Perkin-Elmer Cetus 480 thermocycler using the following cycling conditions: 1 cycle of 94 C for 3 m; 35 cycles of 94 C for 1 m, 50 C for 30 s, and 72 C for 1 min; 1 cycle of 72 C for 8 min; soak at 4 C. Amplified products were precipitated by the addition of 1/2 volume 4M NH_4-acetate and 2 volumes isopropanol. The precipitate was washed once with 80% ethanol, dried by lyophilization, and resuspended in 25 μL H_2O. Amplification products were visualized following electrophoresis with DNA size and concentration standards on 1% agarose gels.

Each DNA sample was sequenced using the 5.8s and 5.8sr primers developed by the Duke University Mycological Systematics Lab (Durham, NC), in addition to the ITS1 and ITS4 primers referred to above. Sequencing was accomplished

with an automated sequencer (Applied Biosystems ABI 373) using the parameters recommended by the manufacturer. This system utilizes di-deoxy chain terminating nucleotides labelled with fluorescent markers. The DNA fragments generated by the PCR-mediated cycling reaction are filtered through Sephadex (which removes unused nucleotides and ions), precipitated, dried by lyophilization, resuspended in 3 μL formamide treated with a de-ionizing resin (BED resin, Bio-Rad), heated briefly at 94 C and snap-cooled on ice. The resulting mixture of denatured DNA fragments, each end-labelled with a fluorescent di-deoxynucleotide, was separated by electrophoresis on a polyacrylamide gel and recorded automatically. The consensus sequence for each sample was determined by visual comparisons of the aligned chromatograms of replicate sequences generated by the different primers, using software provided by Applied Biosystems.

Material examined:

Collybia biformis (Pk.) Singer

USA; TENNESSEE Cocke Co., Great Smoky Mts. Nat. Park, Big Creek Ranger Station, 22 July 1989, RP 3833 (coll. R. Bhandary), as C. subnuda (**TENN** 48608).

Collybia cf. cylindrospora Kauffman

USA; FLORIDA Gainesville 9 Aug 1985, on leaf litter,

SAR 5143, as C. subnuda (**DAOM** 194869).

Collybia dichrous (Berk & Curt.) Gilliam

CANADA; ONTARIO - Pinery Prov. Park, 6 Aug 1983, on rotting log in rich hardwood forest, C. Campbell 27, as C. subnuda (**DAOM** 189189).

USA; PUERTO RICO Univ. P. R. Mayaguez Exp. Station, on slopes of Toro Negro, 27 May 1992, on stick, RP 4463, as C. subnuda (**TENN** 50955).

Collybia peronata (Bolt. ex Fr.) Singer

CZECHOSLOVAKIA; SO. MORAVIA, Zlobicales, 20 June 1975, on decaying wood in hardwood forest, McKnight 15389, (**BPI** 802455).

MEXICO; VERACRUZ Llano Grande de Las Vigas, 5 Sept 1970, on wood of Pinus, Guzman 2269 (Coll. F. Ventura), (**BPI** 802456). Municipio de Las Vigas, 24 Sept., 1971, on wood of Pinus, Guzman 4322 (Coll. F. Ventura), (**BPI** 802457).

SCOTLAND; Fifth & Kuiness Co., Auchmuir Braes Plantation, 5 Sept., 1978, amidst hardwoods (Fagus, Fraxinus), OKM 17677, (**VPI** 2223).

Collybia subnuda (Ellis ex Pk.) Gilliam

CANADA; ONTARIO - St. Lawrence Is. Nat. Park., Thwartway Island, 12 Sept. 1975, on pine needles, SAR 1988, (**DAOM** 153876). Pinery Prov. Park, 22 Aug. 1983, in hardwood duff, GT 830822/03, (**DAOM** 190051). Algonquin Park, Bern Lake, 7 July 1982, in hardwood duff in mixed woods, GT

820707/02, (**DAOM** 190247). Petawawa, 9 Oct. 1986, on needles and leaves, SAR 5897, (**DAOM** 196400).

USA; INDIANA Franklin Co., Brigham's Ridge near Metamora, 7 Aug. 1977, on hardwood litter, Cooke 54691, (**DAOM** 193744).

NEW JERSEY Newfield, Sept. to Oct. 1882, N. Amer. Fungi 909, among fallen leaves in woods, Ellis, holotype (**NY**).

NEW YORK Dutchess Co., Poughkeeepsie, near Hornbeck Ridge, 10 July 1994, on woody detritus in deciduous forest, JFM 1480, (**VPI**). Ulster Co., Mohonk Forest, 10 July 1994, mostly on woody detritus, also on leaves, in deciduous forest, JFM 1482, (**VPI**).

NORTH CAROLINA Highlands, 13 July 1972, on fallen leaves in deciduous woods, R. L. Hesler, (**TENN** 24748). Macon Co., 7 Aug. 1993, on hardwood leaf litter (Quercus prinus and Hamamelis virginiana L.) with one sporocarp on small stick, JFM 1280 (Coll. R. Petersen), (**VPI**). Macon Co., Standing Indian Campground, 7 Aug. 1993, on stick and adjacent leaves, JFM 1285, (**VPI**). Macon Co., Overflow Cr., 8 Aug. 1993, on woody detritus, JFM 1287, (**VPI**). Macon Co., Blue Valley, 14 June 1989, on wood, RP 1804, (**TENN** 48444). Macon Co., Bull Pen Rd., 21 June 1989, on wood, RP 1810, (**TENN** 48590). Macon Co., Coweeta Hydrologic Lab, Otto, 22 July 1989, on twigs, RP 1818, (**TENN** 48353). Durham, 29 July

1994, on sweetgum litter and pine straw, RV 94/155, (**HN** 2218).

OHIO Old Man's Cave State Park, 30 June 1976, on wood (hardwood?), Cooke 52254, (**DAOM** 193742). Old Man's Cave State Park, 30 June 1976, on stick, Cooke 52261, (**DAOM** 193743). Old Man's Cave State Park, 30 June 1976, on stick, Cooke 52256, (**DAOM** 193745).

PENNSYLVANIA Jennerstown, Camp Sequonta, 17 Sept. 1993, on litter under hardwood, OKM 25851 (**VPI**). Jennerstown, Camp Sequonta, 17 Sept. 1993, on litter under hardwood, JFM 1429 (=OKM 25851), (**VPI**). Jennerstown, Camp Sequonta, 17 Sept. 1993, JFM 1431 (Coll. OKM), (**VPI**).

SOUTH CAROLINA Sumter Nat. Forest, Oconee Co., Oconee St. Pk., 7 Aug. 1993, on fallen branch, JFM 1268, (**VPI**).

TENNESSEE Great Smoky Mts. Nat. Park, Cade's Cove, 25 July 1972, decaying, fallen limbs, L. R. Hesler, (**TENN** 37259).

VIRGINIA Jefferson Nat. For., Montgomery Co., Poverty Hollow, 19 May 1992, on bark of Quercus sp., JFM 898, (**VPI**). Jefferson Nat. For., Montgomery Co., Brush Mt., 27 June 1992, on hardwood leaf litter, JFM 926, (**VPI**). Jefferson Nat. For., Montgomery Co., Brush Mt., 27 June 1992, on hardwood leaf litter, JFM 927, (**VPI**). Jefferson Nat. For., Montgomery Co., Brush Mt., 14 Aug. 1992, on hardwood leaf litter, JFM 991, (**VPI**). Jefferson Nat. For., Montgomery

Co., Brush Mt., 14 Aug. 1992, on hardwood leaf litter, JFM 993, (**VPI**). Jefferson Nat. For., Montgomery Co., Brush Mt., 16 Aug., 1992, on hardwood leaf litter, JFM 1018, (**VPI**). Giles Co., Salt Pond Mountain, Horton Center, 16 Aug. 1992, on hardwood leaf litter, JFM 1026, (**VPI**). Jefferson Nat. For., Montgomery Co., Poverty Hollow., 12 July 1993, on bark of Quercus sp., JFM 1222, (**VPI**). Giles Co., Mountain Lake Biol. Station, 12 July, 1993, on hardwood leaf litter, JFM 1223, (**VPI**). Giles Co., Salt Pond Mountain, Horton Center, 15 July 1993, on rotten stick and adjacent leafy detritus, JFM 1226, (**VPI**). Jefferson Nat. For., Poverty Hollow, Montgomery Co., 15 July 1993, on large fallen limb, JFM 1230, (**VPI**). Jefferson Nat. For., Montgomery Co., Brush Mt., 15 July 1993, on hardwood leaf litter, JFM 1234, (**VPI**). Giles Co., Salt Pond Mountain, Horton Center, 15 July 1993, on buried stick and adjacent leaf litter, JFM 1235, (**VPI**). Jefferson Nat. For., Montgomery Co., Poverty Hollow, 31 July 1994, on hardwood leaf litter, JFM 1582, (**VPI**).

WEST VIRGINIA Monongahela Nat. For., Pocahontas Co., Cranberry Glades, 17 July 1994, on hardwood leaf litter, JFM 1500, (**VPI**).

RESULTS

Mating studies

Collections JFM 621 (ISG 1) and JFM 898 (ISG 2) were chosen for a multi-isolate mating cross. Seven single-spore isolates from JFM 621 were crossed with each of seven single-spore isolates of JFM 898. Both mating types were represented in each group of isolates. All the resulting 49 crosses were incompatible with the exception of five of the crosses involving JFM 621.03 (Fig. 11), and incompatibility zones formed in most of the incompatible crosses. The pattern of intercompatibility was not consistent with the mating-type genotypes of the isolates, indicating another genetic mechanism controlling intercompatibility.

In two of the crosses involving JFM 621.03, a pigmented zone formed at the junction of the two mycelia. Clamps were observed only on one side of this zone, indicating unilateral nuclear migration from one isolate into the other. In the remaining three compatible crosses, no incompatibility zone formed, and clamps were scattered throughout the mycelium. The mycelium in these crosses appeared unusually heterogeneous and was slow-growing, suggesting the presence of some form of physiological disturbance. Transfers from these five compatible crosses were made, four of which resulted in the formation of clamped mycelia and one which gave unclamped mycelia.

Nuclear staining with DAPI confirmed the di- and monokaryotic nature of these mycelia, respectively.

Thirteen collections were included in the initial intersterility crosses (Table 6). Two distinct groups were identified from these crosses (fig 12). Within each group, intercompatibility was complete, as gauged by clamp connection formation after approximately one week. No shared mating alleles were observed among collections within each group. The two groups were intersterile, and clamp formation was never observed after one week incubation. After eight weeks incubation, however, clamp formation was observed in some of the crosses between the two groups. In these crosses, clamp connections were never observed throughout the mycelium, but were irregularly present in sectors of the mycelium (Fig 13). In some of these crosses, clamp formation occurred only on one side of the cross, indicating one-way nuclear migration (see Fig. 13, top two plates on the rightmost column). An incompatibility zone of densely interwoven, pigmented hyphae occurred in these as well as in the fully incompatible crosses, and could be used as a macroscopic indicator of incompatibility, although microscopic confirmation was always made. No pattern could be discerned from the distribution of the unusual crosses within the cross matrix, with the exception of JFM 1089.01, which showed some level of compatibility with all except one

of the isolates in the alternate group.

JFM 621

JFM 898 ssi's	.02	.03	.04	.05	.06	.07	.09
.03	-	+	-	-	-	-	-
.04	-	+	-	-	-	-	-
.05	-	-	-	-	-	-	-
.07	-	+	-	-	-	-	-
.08	-	+	-	-	-	-	-
.09	-	+	-	-	-	-	-
.10	-	-	-	-	-	-	-

Figure 11. Multi-isolate intercollection mating cross between ISG 1 (JFM 621) and ISG 2 (JFM 898). (-) indicates clamp connections were not formed after three weeks' incubation; (+) indicates that clamps formed somewhere on the mycelium, but sometimes these were sparse or unevenly distributed (see text). Isolates from each collection included both mating types.

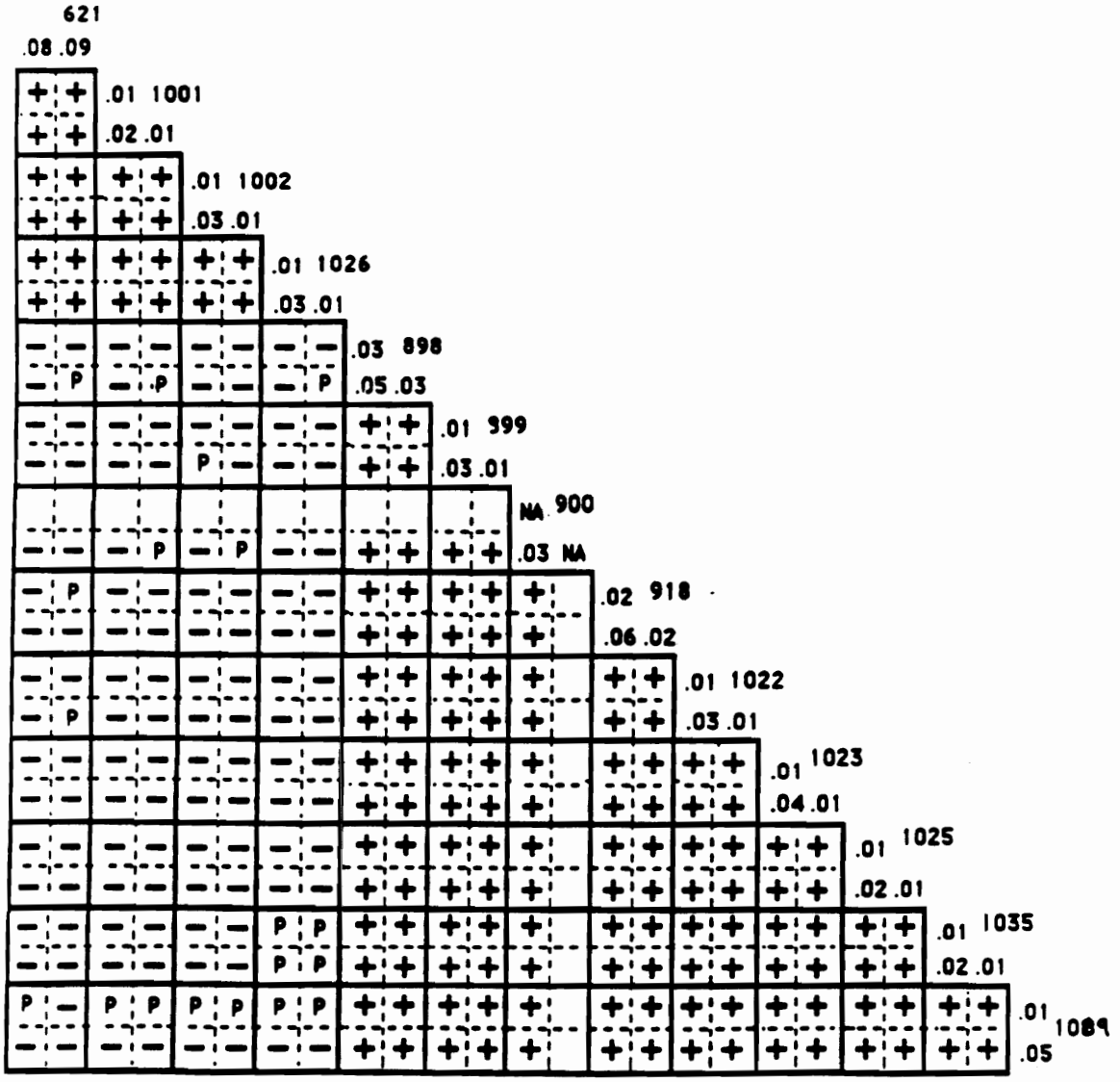

Figure 12. Intercollection crosses between Collybia subnuda tester sets. (+) indicates clamp connection formation; (-) indicates no clamp connection formation; (p) indicates irregular clamp formation (see text). Numbers refer to JFM collections. Empty boxes indicate crosses not performed.

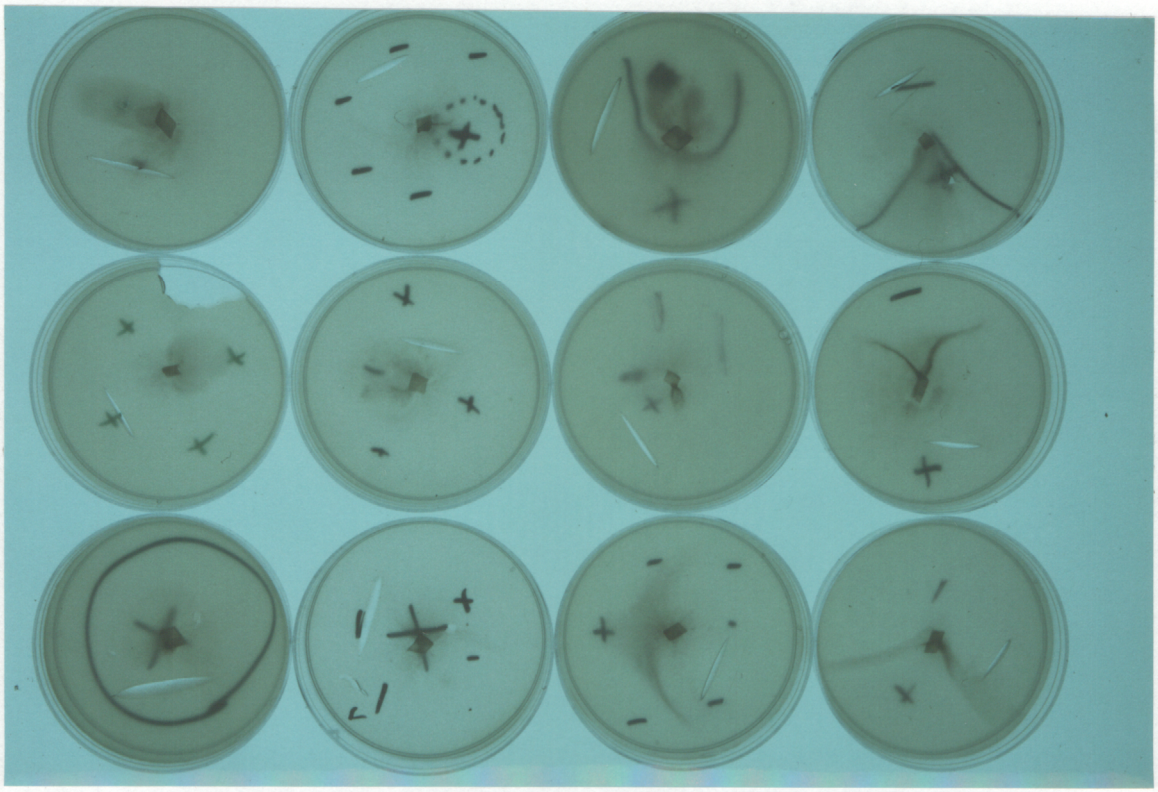

Figure 13. Compatible and irregular crosses between Collybia subnuda ISG 1 and ISG 2.

Table 6: Origin of the isolates included in the C. subnuda intersterility tests.

JFM collection #	Location
621	Brush Mt. Plots
898	Poverty Hollow, Montgomery Co., VA
899	Poverty Hollow, Montgomery Co., VA
900	Poverty Hollow, Montgomery Co., VA
918	Poverty Hollow, Montgomery Co., VA
1001	Mt. Lake, Salt Pond Mt., Giles Co., VA
1002	Mt. Lake, Salt Pond Mt., Giles Co., VA
1022	Horton Center, Giles Co., VA
1023	Horton Center, Giles Co, VA
1025	Horton Center, Giles Co., VA
1026	Horton Center, Giles Co., VA
1035	Oconee St. Pk., Oconee Co., SC
1089	Blue Ridge Parkway near Asheville, Transylvania Co., VA

Biogeography and ecology

A total of 78 collections, in addition to those made on the Brush Mt. plots, were made of C. subnuda and identified to ISG (Table 7). Intersterility testing was rapid and yielded unambiguous results after seven days incubation. No additional ISGs were observed. No SSI from any collection crossed with both of the ISG tester strains within the 1-2 week incubation period. These results further support the hypothesis that there is a strong barrier to crossing between the two groups. In a few cases, an unknown isolate tested negative with both tester isolates. These isolates were especially interesting, because the possibility exists that more than two ISGs of C. subnuda exist. However, reexamination of the vouchers determined that the collections involved in these crosses had been misidentified, and were usually C. biformis or C. spongiosa.

The biogeographic distribution of the C. subnuda ISGs is given in Figure 14. All collections made on the Brush Mt. plots belong to ISG 1. In addition to these, 28 collections from other regions were identified to ISG 1. Most of these collections were in Virginia, but one collection of ISG 1 was made in NY (JFM 1482, in Ulster Co.) and two were made in North Carolina (RHP 1818 and JFM 1285, both in Macon Co.). Fifty collections were identified to ISG 2. Many of these were made in the southern Appalachians

of Virginia, North and South Carolina. Nevertheless, a single collection identified to ISG 2 was made in NY (Dutchess Co.), two were made in Ohio, and one in Pennsylvania.

Close observation of the mycelium giving rise to the sporocarps showed that there is a close association between ISG and substrate (Fig 15). Collections belonging to ISG 1 are usually associated with a leafy substrate. Woody detritus was never the sole substrate of ISG 1 collections, although woody substrates were occasionally mixed with leaves in the mycelial mat below the sporocarps. Bleaching of the cortex and wood of these substrata indicated that the mycelium was using bark, wood and leaves as a nutritional source. In contrast, ISG 2 collections were usually associated with some form of woody substrate and occasionally were associated only with woody detritus. The substrate required by ISG 2 appeared to be primarily bark and cortex, since sporocarps of ISG 2 were never collected from decorticated wood, and were occasionally observed on bark pieces alone. It appears, therefore, that there is a link between ISG and ecology in C. subnuda.

Effect of substrate on ISG

Mating tests among tester strains of both ISGs cross-inoculated onto woody and leafy substrates show that

substrate does not affect ISG mating reactions (Fig. 16).

Morphological studies

The holotype of C. subnuda was examined to ensure that collections in this study were properly identified. In addition, identification of collections of both ISGs as C. subnuda were confirmed by Dr. Roy Halling of the New York Botanical Garden (pers. comm). My observations of the holotype differed from the description by Halling (1983) only by the observed presence of pleurocystidia and infrequent bisterigmate basidia (Fig. 17). The pleurocystidia originate in the lamellar trama and rarely extend beyond the level of the basidial sterigmata, but were clearly seen in good cross sections and in crush mounts (gill edge removed to avoid confusion with cheilocystidia).

Spores from fresh pilei of collections JFM 1580 and JFM 1581 contained a small proportion of binucleate spores, but a third collection (JFM 1582) contained strictly uninucleate spores. The same results were obtained from spores obtained after drying and rehydrating the pilei.

Examination of specimens from 6 eastern cryptogamic herbaria confirmed the northern distribution of C. subnuda reported by Halling (1983) and extended its observed southern distribution (Fig. 17). Reports of C. subnuda from Florida, Puerto Rico, and Mexico were not supported by the

examination of voucher specimens from these locations.

Voucher specimens from collections identified by mating tests to ISG were examined to determine if morphological differentiation has accompanied divergence of the intersterility groups. Intraspecific variation has already been observed by this author and others. For instance, one of M. Gilliam's annotations identifies a VTMH collection as "Collybia subnuda, a variable fungus which needs more work." The observed variable macromorphological characters include cap color, color of stipe ground tissue, and sporocarp size. Variable microscopic characters are presence or absence of pleurocystidia and spore size. Neither of these characters was associated with intersterility group, and therefore neither can be used reliably to identify sporocarps to ISG. The length and width of spores from spore deposits were measured (n=15 from each collection) and analyzed statistically for differences. Statistically significant differences ($p<.05$) were found among mean spore lengths and widths, but the differences were as much within than between intersterility groups. Herbarium collections, therefore, could not be identified to ISG, and the distributions of the ISGs must be based on collections which are backed by living isolates.

The Guzman collections identified as C. peronata were

compared with separate European collections of C. peronata made by K. McKnight and by O. K. Miller, Jr. and were clearly different from them. The European collections were associated with hardwoods, were larger in stature than the Guzman collections, and had a yellow-brown stipe vesture. Microscopically, they had occasionally branched, interwoven pileipellis elements, and a spore range of 2.9-3.2 X 8.2-9.1 μm. The Guzman collections were on pine wood, were smaller in stature, had a light colored stipe vesture, an unbranched pileipellis structure, and a spore range of 3.6-4.5 X 8.6-10.5 μm.

Even without fresh characters, the Guzman collections are very similar to C. subnuda. The pileipellis of C. subnuda is quite distinctive. It is composed of parallel to slightly interwoven, clamped, unbranched hyphae with annular to spiral encrusting elements. While the older specimens of the Guzman collections lacked the encrusting elements so typical of C. subnuda, younger specimens had them, indicating that perhaps the incrustations had been washed or worn off in the older specimens. The only character separating the Guzman collections from C. subnuda is the habitat on pine wood. In this study, C. subnuda was never seen growing on pine wood. The Guzman collections may represent a habitat shift in C. subnuda, or they may represent a related but evolutionarily divergent lineage.

Molecular studies

751 bases were sequenced from the ITS region of five samples, including three samples of ISG 1 and two samples of ISG 2 (Figure 18). The sequences were similar for all samples. Three samples, including one of ISG 2 and 2 of ISG 1, had identical sequences. A third sample of ISG 1 differed from the first three by a single transition. A fourth sample (JFM 1480) differed from the others by two transitions and one insertion. The insertion creates an AVA II restriction enzyme recognition site within the ITS 1 region, which suggests that restriction analysis of amplified rRNA genes could be used to track this mutation. In general, however, the observed low level of rRNA gene sequence variation indicates that this region is nearly invariant within C. subnuda and does not provide information about the divergence of the two ISGs.

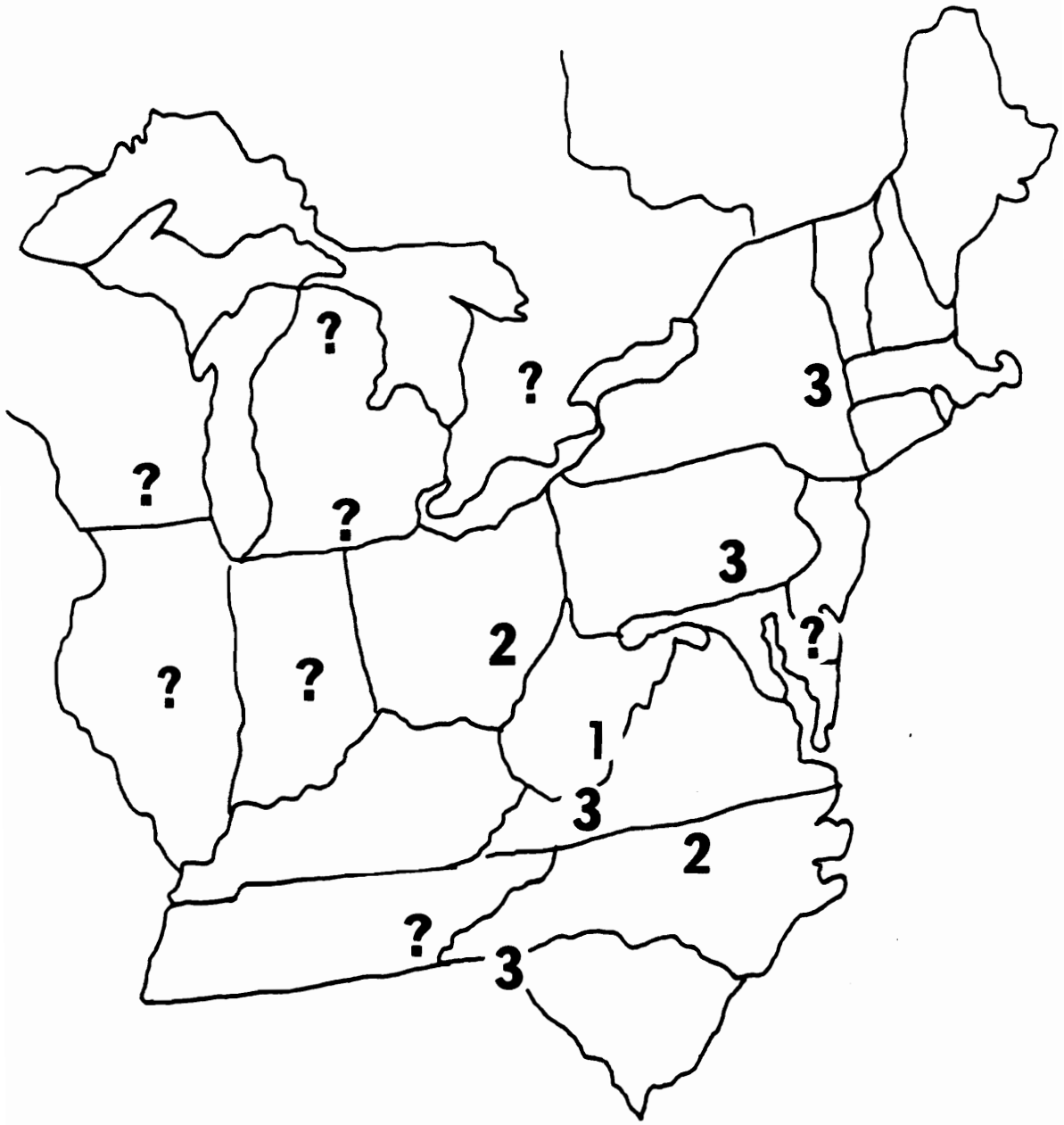

Figure 14. Distribution of Collybia subnuda intersterility groups. (1) = ISG 1; (2) = ISG 2; (3) = both ISGs collected; (?) = herbarium record, not identifiable to ISG.

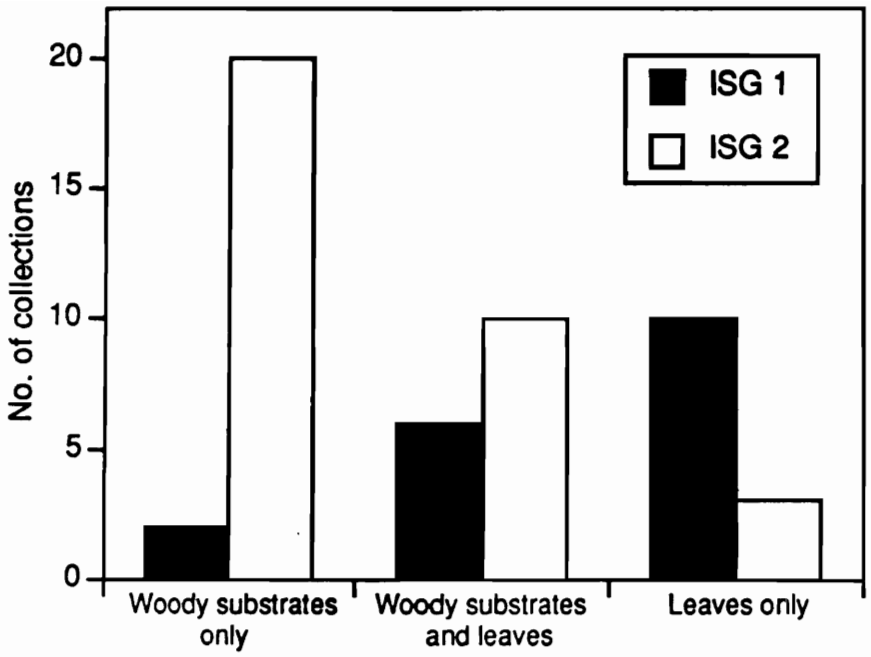

Figure 15. The observed vegetative substrate frequencies of the two Collybia subnuda intersterility groups. See text for definition of woody substrates.

621.08w							
-	621.08L						
+	+	621.09w					
+	+	-	621.09L				
-	-	-	-	898.03w			
-	-	-	-	-	898.03L		
-	-	-	-	+	+	898.05w	
-	-	-	-	+	+	-	898.05L

Figure 16. Crosses between Collybia subnuda ISG1 and ISG 2 tester strains grown on wood and leaf substrates. Intersterility group 1 is represented by isolates 621.08 and 621.09. Intersterility group 2 is represented by isolates 898.03 and 898.05. (w) indicates isolate incubated on wood. (L) indicates isolate incubated on leaves. (+) indicates clamp connection formation. (-) indicates no clamp connection formation.

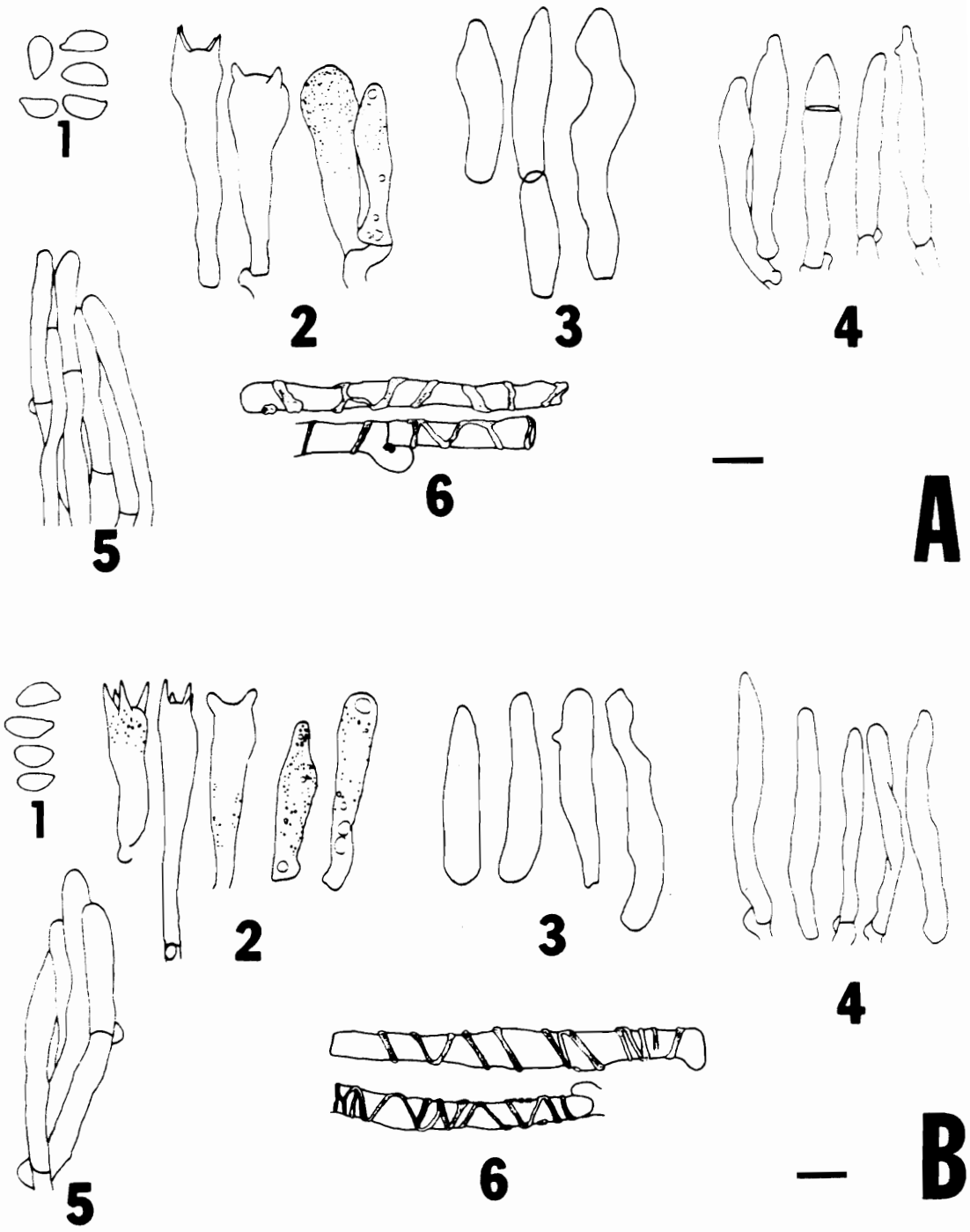

Figure 17. Micromorphological characters of Collybia subnuda. A) ISG 1. B) ISG 2. 1=spores; 2=basidia and basidioles; 3=cheilocystidia; 4=pleurocystidia; 5=caulocystidia; 6=pileipellis elements. Bar = 10 μ.

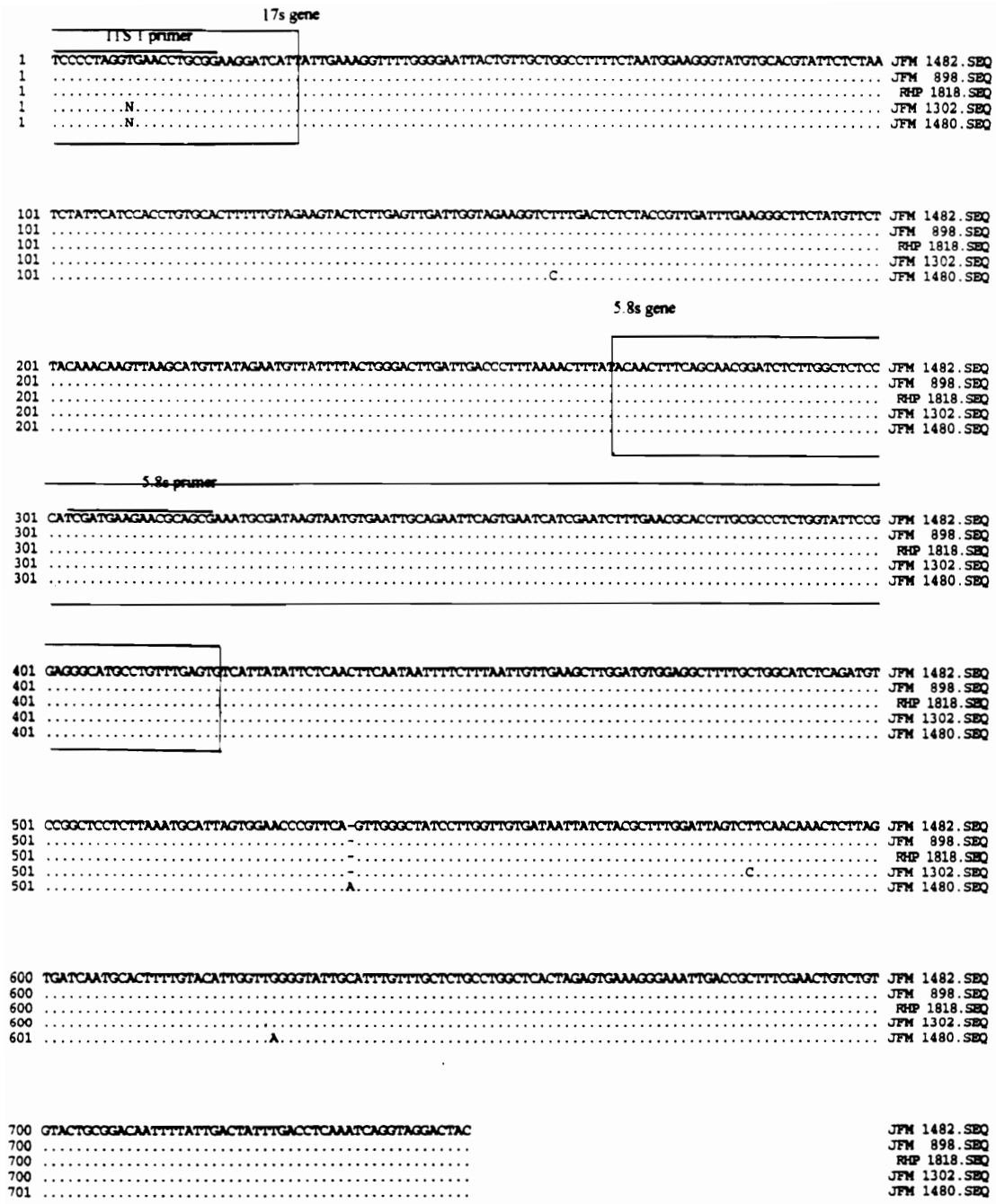

Figure 18. DNA sequence comparison of the rDNA ITS region from five collections of Collybia subnuda. The sequence for JFM 1482 was used as the reference sequence. Identical bases are indicated by a dot. Insertions are indicated by a dash. An "N" indicates an uncertain nucleotide identification. Oligonucleotide primer sites are underlined and labelled. The 17s and 5.8s genes are boxed and labelled. The 5′ end of the 28s gene was not sequenced.

Table 7: Origin of isolates used in ISG identification crosses of C. subnuda.

collector's #[1]	ISG	Locale[2]	State
1285	1	SIC	NC
1482	1	MOH	NY
1429	1	JEN	PA
1431	1	JEN	PA
621	1	BRM	VA
925	1	BRM	VA
926	1	BRM	VA
927	1	BRM	VA
993	1	BRM	VA
1018	1	BRM	VA
1020	1	BRM	VA
1074	1	BRM	VA
1234	1	BRM	VA
1026	1	HCR	VA
1225	1	HCR	VA
1226	1	HCR	VA
1228	1	HCR	VA
1416	1	HCR	VA
1001	1	POV	VA
1223	1	SPM	VA
1302	1	SPM	VA
1303	1	SPM	VA
1499	1	CRG	WV
1500	1	CRG	WV
1501	1	CRG	WV
1502	1	CRG	WV
1503	1	CRG	WV
1504	1	CRG	WV
1505	1	CRG	WV
1506	1	CRG	WV
1507	1	CRG	WV
1508	1	CRG	WV
1509	1	CRG	WV

collector's #[1]	ISG	Locale[2]	State
1338	2	ASH	NC
1089	2	BRP	NC
1091	2	BRP	NC
1324	2	BLU	NC
1289	2	SIC	NC
1327	2	VHT	NC
1287	2	WWW	GA
1288	2	WWW	GA
1290	2	WWW	GA
1291	2	WWW	GA
1297	2	WWW	GA
1480	2	HBR	NY
1524	2	WAY	OH
1536	2	WAY	OH
1430	2	JEN	PA
1035	2	OCO	SC
1267	2	OCO	SC
1268	2	OCO	SC
1269	2	OCO	SC
1270	2	OCO	SC
1271	2	OCO	SC
1272	2	OCO	SC
1273	2	OCO	SC
1274	2	OCO	SC
1022	2	HCR	VA
1023	2	HCR	VA
1025	2	HCR	VA
1229	2	HCR	VA
1235	2	HCR	VA
1417	2	HCR	VA
1418	2	HCR	VA
898	2	POV	VA
900	2	POV	VA
918	2	POV	VA
1222	2	POV	VA
1231	2	POV	VA

(continued)

collector's #[1]	ISG	Locale[2]	State
1232	2	POV	VA
1308	2	POV	VA
1309	2	POV	VA
1310	2	POV	VA
1311	2	POV	VA
1312	2	POV	VA
1313	2	POV	VA
1467	2	POV	VA
1471	2	POV	VA
1474	2	POV	VA
1580	2	POV	VA
1581	2	POV	VA
1582	2	POV	VA

[1]Collection #'s are of JFM

[2]Abbreviations:

ASH	On Blue Ridge Parkway near Asheville, Transylvania Co., NC.
BLU	Blue Valley, near Highlands, Macon Co., NC.
BRM	Brush Mt., Montgomery Co., VA.
BRP	Blue Ridge Parkway near Lexington, Amherst Co., VA.
CRG	Cranberry Glades, Pocahontas Co., WV.
HBR	Hornbeck Ridge, Dutchess Co., NY.
HCR	Horton Center, near Salt Pond Mt., Giles Co., VA.
JEN	Jennersville, Chester Co., PA.
MOH	Mohonk Forest, Ulster Co., NY.
OCO	Oconee St. Park, Oconee Co, SC.
POV	Poverty Hollow, Montgomery Co., VA.
SIC	Standing Indian Campground, Macon Co., NC.
SPM	Salt Pond Mt., Giles Co., VA.
VHT	Van Hook Trail, near Highlands, Macon Co., VA.
WAY	Wayne National Forest near Dart, Washington Co., OH.
WWW	War Woman Wildlife management area, Rabun Co., GA.

DISCUSSION

The results of the intercollection mating crosses clearly show the division of C. subnuda into two partially intersterile groups. It is uncertain how complete the barrier to gene flow is between the two groups. While a small number of the spores produced by any particular individual may be compatible with a small number of the spores from an individual of the other ISG in vitro, crossing between the two groups in nature may occur rarely or never. Dikaryotization between the two groups can occur on agar and can result in the formation of a stable dikaryon, but the conditions for in vitro dikaryotization require prolonged incubation and transfers. Given the relative facility with which within-group crossing occurs, it would seem that breeding between single-spore isolates within an intersterility group is strongly favored. Nevertheless, the potential for interbreeding exists, since the two groups are sympatric. For discussions of partial intersterility in other fungal taxa, see Boidin (1986) and Petersen (1995).

Hybridization between the two groups and consequent introgression of genetic material may explain their phenotypic and genetic similarity. Alternatively, the two groups have only recently developed intersterility, and insufficient time has elapsed for their differentiation.

Apparently, there has been sufficient time for their divergence along ecological lines, since there is a clear ecological difference between the two groups. In the majority of cases, intersterility group 1 is found on leafy detritus, and ISG 2 is found on woody detritus. This observation suggests that the genetic barrier between the two groups is practically complete, since one would expect that introgression would weaken the ability of the different groups to maintain ecological differentiation. If this is true, then molecular divergence would be expected.

However, very little molecular difference was noted at the ITS region, but preliminary evidence indicates that the ITS region may not be highly divergent in the genus Collybia (R. Vilgalys, pers. comm.). Other molecular approaches that could be used in future studies of C. subnuda ISGs include DNA sequences of the intergenic regions of the rRNA gene family and mitochondrial DNA, DNA-DNA reassociation, and a comparison of satellite DNA.

The intraspecific population structure of C. subnuda is similar to that of Heterobasidion annosum (Fr.) Bref. Three sympatric intersterility groups of H. annosum occur in Europe (Korhonen, 1978; Capretti, et al., 1990). As in C. subnuda, partial intercompatibility occurs between the H. annosum ISGs and they have different substrate preferences. Kasuga, et al. (1993) found very low rRNA gene sequence

variation between ISG "F" and ISG "S," and suggest that one evolved from the other. These observations suggest similar microevolutionary processes in C. subnuda and H. annosum.

As mentioned in the introduction, the occurrence of intersterility groups in the fungi is not uncommon, and it may or may not be coupled to morphological variation. In fact, statistically significant morphological differences may occur within an ISG; see, for instance, Vararia amphithallica Boidin, Lanquetin and Gilles (Boidin, 1986). Significant differences in mean spore size were found within the C. subnuda intersterility groups. On the other hand, morphologically identical fungi may belong to distinct incompatibility groups; see, for instance, Hyphoderma mutatum (Peck) Donk (McKeen, 1952), Fomitopsis pinicola (Sw:Fr.) Karst (Mounce and Macrae, 1938), and Marasmius spp. (Gordon, 1994; Petersen, 1995). Boidin (1986) interpreted these situations as indicative of taxa in the process of speciation, particularly if the intersterility barrier is incomplete; this is the view adopted here for C. subnuda.

SPECULATIONS ON SYMPATRIC VS. ALLOPATRIC DIVERGENCE OF THE C. SUBNUDA ISGs

Commonly accepted hypotheses for speciation include sympatric and allopatric origination. The invocation of sympatric or allopatric evolution to explain present

distributions is difficult to test, and this author hesitates to commit to any explanation for the divergence of the C. subnuda ISGs without compelling evidence. The evidence for both sympatric and allopatric origins of the ISGs is presented and discussed below.

The allopatric mode is the simpler explanation for the origin of the two intersterility groups. At some unknown time in the past, populations of a previously panmictic population became physically separated. In isolation from each other, they diverged genetically. The divergence probably preceded the development of intersterility barriers, since allopatric species often retain intercompatibility (Boidin, 1986; Vilgalys, 1991). They probably diverged at neutral, non-coding DNA regions, and possibly along ecological lines as well. If they diverged ecologically, selection through competitive interactions would result in increased ecological divergence when the physical barrier or barriers between the populations were removed, particularly if hybrids between the formerly allopatric populations were less competitive. If hybrids were less fit, then the development of intersterility barriers would be selectively advantageous, and sympatric intersterility groups (ISGs) would arise. Boidin (1986) presented a thorough discussion of allopatric speciation in the fungi.

Evidence of allopatric genetic divergence in fungi has been shown in Pleurotus (Vilgalys and Sun, 1994; Petersen, 1995), and in the Collybia dryophila (Bull.:Fr.) Kumm. complex by Vilgalys (Vilgalys, 1991). In these studies, isozymes (Petersen, 1995) and neutral, non-coding genomic regions (Vilgalys and Sun, 1994; Vilgalys, 1991) were shown to have diverged in mating compatible but geographically separate populations. As argued by Hallenberg (1991, 1992), allopatric speciation in the fungi becomes more plausible when the limits of spore dispersal are considered. According to these arguments, spore dispersal is effective primarily at a local level, with long distance dispersal limited by spore viability, the diminishing possibility of finding suitable germination substrate and a mate, and competitive interactions.

It will be shown here (Ch. 3) that C. subnuda spores have limited viability, and that there is high temporal and spatial variation in C. subnuda spore deposition. These factors may predispose a fungus to population isolation and allopatric speciation.

In addition, there may be a link between C. subnuda and Quercus which could be important in understanding the origin of the two ISGs. All collections of C. subnuda made in this study were associated with oak detritus. Several collecting trips were made to Poplar Grove of the Joyce

Kilmer Forest, a virgin cove hardwood forest in western North Carolina. Although conditions for the fruiting of C. subnuda were ideal during this period, and collections of many other litter decomposing fungi were made, C. subnuda was not observed growing in Poplar Grove, which appeared to be devoid of Quercus. Unfortunately, the surrounding hillsides where Quercus grows (G. Griffin, pers. comm.) were not explored. Vilgalys (pers. comm.) claims that C. subnuda is common amongst Pinus pungens in the Piedmont region of southeastern North America. He sent a collection reportedly found on pure Pinus detritus. However, the collection notes indicate both Pinus and Liriodendron as a substrate, and it is possible that Quercus detritus was also present. Additional study is required before it can be concluded that C. subnuda is dependent on Quercus, but the available evidence indicates that it is. While oak species in eastern North America now have broadly overlapping ranges, the distribution of oak species in southwestern North America and northern Mexico is extremely patchy (Miller and Lamb, 1985). Perhaps the ancient distribution of Quercus in eastern North America was also patchy, or maybe one or both of the C. subnuda ISGs originated in southern or southwestern regions where oak presently has a patchy distribution. The combination of limited spore dispersal, host fidelity, and fragmented host range could predispose a

species to allopatric speciation, particularly if the allopatric host is differentiating evolutionarily as well. It is conceivable that the two C. subnuda ISGs arose in this manner, and that their current distribution is secondary to a formerly allopatric distribution.

Several authors, however, have suggested that sympatric evolution may occur in the fungi (Burnett, 1983; Hallenberg and Larsson, 1992; Nakasone and Micales, 1988). These authors' arguments are not satisfactory, however, for they do not adequately propose how genetic differentiation within a sympatric population of fungi can precede genetic isolation. An overlapping distribution of two sibling species in the present cannot be concluded to have resulted from sympatric speciation since secondary contact following allopatric speciation is an equally plausible explanation.

Nevertheless, sympatric evolution might occur given the following observations:

1. The genetic foundation for intersterility between sibling species may not be very complicated. Chase and Ullrich (1990a,b) suggest that intersterility in Heterobasidion annosum is determined by five genes. While the genetic basis for intersterility may vary from species to species, the occurrence of intersterility groups in a plethora of fungal taxa strongly suggests that intersterility barriers may be fairly simple to achieve.

Intersterility may arise regularly within populations by spontaneous mutation at a constant rate, albeit low. Sympatric speciation then relies upon the survival and reproduction of such mutants, and their development into breeding populations sympatric with their progenitor.

2. Collybia subnuda produces some bi-sterigmate basidia in nearly all collections examined, and about 1% of the spores it produces are binucleate. It is likely that binucleate spores are formed by the migration of two meiotically derived nuclei from the basidium into the spore, rather than by the mitotic division of a single nucleus following its migration into the spore. This should be verified by direct observation of developing basidia and spores. If, however, the binucleate spores contain meiotically derived nuclei, then C. subnuda may possess a secondarily homothallic escape route from the intersterility mutations described above. In other words, if a mutation gave rise to a mating-incompatible individual in a population, the production of binucleate spores could allow it to reproduce.

Given these observations, the proposed mechanism for sympatric evolution is as follows. Mutant individuals rendered intersterile with their sympatric relatives can arise at regular infrequent intervals within populations of C. subnuda. These individuals would have the potential to

propagate themselves in a semi-clonal fashion via secondary homothallism. The probability of evolutionary success for these mutants is slim, since competition for identical niche resources must be great. If, however, the parental population is polymorphic for a trait, the mutant may, by chance, carry genes which make it significantly different from the parental population average (this a twist on the "founder" effect). The new mutant population, now an ISG, would be distinct from the parental ISG by mating incompatibility and its different allele frequencies. Two strong selective forces would result: 1) directional selection due to competition for similar resources, and 2) selection pressure for the maintenance and perhaps augmentation of intersterility barriers and secondary homothallism in the mutant.

The events leading up to a sympatric origin of a sibling species are unlikely to occur, but in evolutionary time many unlikely events undoubtably occur.

Only two intersterility groups in C. subnuda were identified in this study, and they appear to be sympatric throughout the sampled range. The data presented in Ch. 3 show that they are truly sympatric, as spores of both ISGs are present in the same place at the same time. However, the known range of C. subnuda is greater than the area sampled in this study, and the extreme northeastern and

northwestern parts of the range were not sampled. From the north, one particularly interesting collection from Ontario was G. Thorn 820707/02 (DAOM 190247), which formed a mature hymenium with entirely bisterigmate basidia, an unusual feature for C. subnuda. To the south, there are the Guzman collections of "C. peronata" (previously discussed) and the large region between the southern Appalachians and Mexico which has only been lightly sampled by mycologists (O. K. Miller, pers. comm). These observations suggest that important information about C. subnuda awaits discovery south of the Appalachians.

Collybia subnuda is the only bipolar fungus in the genus Collybia, which is otherwise strictly tetrapolar. The mating system of C. luxurians (section Vestipedes) and the morphologically very similar C. biformis (section Subfumosae) are also tetrapolar (Murphy, in ed.). These observations focus attention on C. subnuda as an unusual member of the genus and make the occurrence of sympatric ISGs in it all the more interesting.

CHAPTER 3: SPORE-CATCHING, SPORE VIABILITY, AND SPOROCARP REHYDRATION IN *COLLYBIA SUBNUDA*

INTRODUCTION

One objective of this chapter was to test the ability of the spore-catching method to supplement sporocarp collection in studies requiring distributional data. Two observed characteristics of *C. subnuda* made more detailed distributional information highly desirable. First, the difference in substrate utilization by the two ISGs indicated a difference in ecology. Perceived differences in local abundances of the ISGs indicated the possibility of additional ecological differences. Specifically, after several years of fieldwork and many collections from both locations, only ISG 1 was collected on Brush Mt., and, with one exception, only ISG 2 was collected in nearby Poverty Hollow, a distance of only a few kilometers. The second observation was the possible unequal abundance of both ISGs throughout the sampled range. For instance, in North Carolina, many collections of ISG 2 were made, but ISG 1 has been collected only twice, and only once by the author. Because these observations were based on collection of sporocarps, the perceived differences could be caused by incomplete sampling. Distributional data based on the collection of sporocarps is unavoidably fragmentary due to unpredictable phenology, ephemeral sporocarps, and the large

effort required for intensive sampling. Because the observed difference in ISG distribution either on a local or a regional scale may be an artifact of incomplete sampling, additional methods for determining distribution are needed.

The spore-catching method was first described by Williams, et al. (1984) and Adams, et al. (1984) and has been used by Vilgalys and Sun (1994). Monokaryons are grown on antibiotic-containing agar plates until the mycelium covers the surface. The plates are exposed to the spore rain, then sealed. After incubation, the hyphae on the plates are examined microscopically. The presence of clamp connections is presumptive evidence of dikaryotization by compatible spores that must have been present in the atmosphere during the sampling period.

Williams, et al. (1984) showed that viable spores of Coriolus versicolor (Aphyllophorales) were present in the atmosphere year round, indicating that spore dispersal could be high in this species. However, Williams, et al. (1984) also studied the spore rain of Flammulina velutipes (Agaricales, perhaps related to Collybia), and although the data presented are scanty, they indicated that the spore rain of F. velutipes is "less substantial" than that of C. versicolor. These reports indicate that spore dispersal varies from species to species, and that the spore-catching method could be a valuable method for gathering data on

spore "behavior."

The method has important requirements and makes specific assumptions. The fungus must be culturable, which is an important consideration since so many fungi, particularly mycorrhizal fungi, are not. The mycelium must be vigorous enough to cover the agar plate, since uncolonized surfaces invite contamination during the exposure period. The dikaryon must be distinguishable from the monokaryon. The fungus must be naturally resistant to contamination, since it is inevitable that spores of antibiotic-resistant contaminating fungi and bacteria will be deposited on the plate. The formation of clamp connections (or other characters indicative of a dikaryon) is assumed to be the result of dikarotization by deposited spores.

Prior observation of the growth characteristics of C. subnuda indicated that this species met all the criteria listed above and suggested that spore-catching could be useful in studies of C. subnuda. Furthermore, somatic incompatibility zones were observed in exposed, incubated spore-catching plates. Similar zones were reported by Williams, et al. (1984), who interpreted them as the product of dikaryotization events by spores which have different genotypes from each other. Following germination, plasmogamy, nuclear division and migration, each spore

converted sectors of the monokaryon into genetically different dikaryons, which created somatic incompatibility zones at the lines of contact.

This phenomenon suggested that spore-catching could be a useful tool not just for the detection of the presence or absence of spores of compatible mating type in the spore rain, but also for the quantification of that spore rain. Such an approach relies upon the following assumptions:

1. The monokaryons used do not discriminate among the spores within their intersterility groups (ISG).
2. The monokaryons discriminate perfectly between spores of different ISGs (if, as in C. subnuda, the taxon used has different ISGs).
3. Spore deposition and dikaryotization follow a Poisson distribution; that is, the deposition of a spore and its subsequent germination and dikaryotization are independent of the same events occurring in adjacent areas.
4. Spore deposition on the plate is random.

In the spore-catching portion of this study, two main experiments were performed. The purpose of the first experiment was to investigate the utility of spore-catching for determining C. subnuda ISG biogeographic distribution. The purpose of the second experiment was to investigate the

ability of the spore-catching method to quantify spatial and temporal variation in C. subnuda spore rain.

A second objective of this study was to investigate the viability of C. subnuda spores and the ability of sporocarps of C. subnuda to revive and cast spores following drying. These factors were studied because of their relevance to distribution, dispersal, and gene flow in C. subnuda.

METHODS

Spore-catching: C. subnuda regional distribution

Monokaryotic tester isolates of the two intersterility groups were inoculated onto 100 X 15 mm, 1.5% MEA plates amended with penicillin and streptomycin (30 µg/L). The plates were sealed with Parafilm, incubated at 25 C in the dark until the mycelium covered the agar surface, and stored at 5 C until needed. Each plate represents a single "spore-trap." Exposure was accomplished by opening the plates and leaving them on a horizontal surface. Control plates with lids sealed shut with parafilm were placed alongside exposed plates. After exposure, the plates were examined, debris was removed, the plates were closed, sealed with Parafilm, and marked with the date, location, and duration of exposure in addition to the ISG identifier already marked on the lid. Exposed plates were transported promptly to the lab, stored in the dark at room temperature (approx. 22 C), and examined

periodically for contamination and the development of sectors. Sector counts were made after approximately 4 weeks incubation. Preliminary experiments demonstrated that sectoring was complete at 3 weeks.

During the summer of 1993, preliminary experiments in the Brush Mt./Poverty Hollow area tested the utility of the method for determining the presence or absence of spores of both C. subnuda ISGs in the atmosphere. In these experiments, single plates of each ISG tester were exposed at various locations and times. In 1994, spore-traps were regularly exposed at collecting sites throughout the collecting season. The duration of exposure was variable, ranging from a less than an hour to overnight. Usually, two plates of each ISG were exposed at each location.

Spore-catching: spatial and temporal variation in spore rain

1. Preliminary experiment

A preliminary experiment to estimate the statistical variation in the spore-catching method was performed in June of 1994. Collecting was fair, with a few sporocarps of C. subnuda observed in the vicinity of the sampling sites, but decreasing humidity was beginning to dry out the litter layer. Five spore-traps of each ISG were exposed overnight (approximately 14 hours) near Pandapas Pond and on Brush Mt. (Fig. 19) and incubated as described above. The plates were

subsequently examined and sectors counted at 3 and 7 weeks, but no difference in the number of sectors was observed after the first count. Based on these observations the number of replicates for subsequent experiments was adjusted to 10.

2. Transect experiment

A transect with four equidistant sampling stations was established along a shoulder ridge from Poverty Creek to the summit of Brush Mountain (Fig. 19). The sampling period began on 21 July, 1994, during favorable weather conditions for the fruiting of C. subnuda. Periodic showers and warm humid weather had preceded the sampling period, the forest leaf litter was saturated, and young sporocarps of C. subnuda were observed in the vicinity of the sampling stations. All sporocarps observed in the vicinity of the sampling stations were removed. Ten sporetraps of each ISG were placed on the ground and exposed to the atmosphere. To test the change in spore rain over time, the plates at stations 1 and 4 were replaced every 4 hours over a 24 hour period. To test the change in spore rain over distance, plates were exposed at all four stations from 1400 hrs to 1800 hrs on 21 July, and from 1000 hrs to 1400 hrs on 22 July.

Spore viability and sporocarp rehydration studies

To estimate spore viability over time, a spore print from JFM 1470 was obtained by moistening a pileus with tap water and placing it covered overnight on a piece of aluminum foil. On days 0, 1, 2, and 5, wedges of the spore print were excised, and the spores were suspended by vigorous mixing for 2 min in 10 ml sterile water. The concentration of spores in the resulting suspension was calculated by hemacytometry. Serial dilutions were made and 0.1 mL of the suspension and each dilution were spread-plated onto 1.5% malt extract agar plates. Five replicates of each dilution were made, and the plates were sealed with parafilm and incubated at 25 C. The plates were monitored daily for germination for two weeks.

Several collections were chosen to study the ability of C. subnuda sporocarps to revive and produce spores after drying. Sporocarps were exposed to ambient laboratory conditions until dry (various periods) and rehydrated with double-distilled H_2O. Revived caps were placed with the lamellae oriented downward on glass slides and covered to maintain high humidity. Pilei were alternately air-dried and rehydrated until no spore discharge was noted. Periodically, the slides were examined for the presence of discharged spores, and sections of the pileus were removed for microscopic examination and nuclear staining with DAPI.

RESULTS

Spore-catching: C. subnuda regional distribution

Microscopic examination showed that many of the spore-catching plates lacked clamp-bearing mycelium after incubation, indicating that no compatible spore landed on the plate. Table 8 shows that in most cases, when no sporocarps of C. subnuda were collected, no spores were trapped. In three cases, sporocarps were collected without spores being trapped, but in these cases collecting conditions were poor and only a few sporocarps in poor condition were observed.

Microscopic examination also showed that some of the spore-catching plates had clamp-bearing mycelium after incubation, indicating the presence of mating compatible spores in the atmosphere during the exposure period. Spores were trapped when no sporocarps of C. subnuda were observed on two occasions. On one of these occasions (Sherwood Lake, WV; Table 8) collecting was of brief duration, and it is likely that sporocarps would have been found if collecting had not been interrupted by heavy rain. On the other occasion (Coweeta, NC), however, collecting was intensive and yielded no collections of C. subnuda, yet ISG 1 spores were caught. Interestingly, ISG 1 was collected in this study only twice in the Appalachian Mountains south of Virginia: once in Coweeta (RHP 1818) and once in an adjacent

watershed (JFM 1285). Therefore, the presence of ISG 1 in the southern Appalachian Mountains is known only from three data points: two sporocarp collections and one spore-catching plate.

Some spore-catching plates developed sectors after incubation (Fig. 20). Microscopic examination showed the presence of clamp connections throughout the mycelium within the sectors, indicating that the monokaryon had been dikaryotized and that the method was useful for determining the presence or absence of spores of the ISGs. Control plates never contained clamped hyphae. Control plates occasionally produced pigments in the agar following prolonged incubation, but distinct somatic incompatibility zones never formed. Only a few of the spore-catching plates were discarded due to contamination. Even grazing by unidentified invertebrates did not result in visible contamination or inhibit dikaryotization. Contaminating fungi eventually overwhelmed the C. subnuda mycelium, but this occurred only after several months' incubation at room temperature and long after the data were collected.

Spore-catching: spatial and temporal variation in spore rain

1. Preliminary experiment

The preliminary experiment to investigate the variability in the spore-catching method resulted in no

sectors in the five samples of both ISGs on Brush Mt. This was not surprising, since conditions during this period were increasingly dry, and a sample taken the previous year indicated that spore load decreases rapidly as humidity drops (see below). In spore-traps exposed near Pandapas Pond, however, sectors formed in samples of both ISGs. The mean number of sectors formed in spore-traps of ISG 1 was 2.6 (s.d. 1.2), and the mean number in ISG 2 spore-traps was 36.8 (s.d. 9.7). Based on these observations, the sample size for the transect experiment was adjusted to 10 plates per ISG.

2. Transect experiment

Sporocarps of C. subnuda were observed in the vicinity of the transect sampling sites, but by the end of the experiment, fruiting of C. subnuda on Brush Mt. was not as great as expected.

The highest sector count in this experiment was less than 80, which is a countable number. As sector counts in preliminary experiments approached 100, the incompatibility zone between adjacent sectors became indistinct.

Contamination rates were low. Of the 160 plates exposed in this experiment, only a few plates developed contamination and even in these, sectors could be scored. Usually the contaminant only developed after prolonged

incubation.

Figure 21 shows that over a distance of a few hundred meters, spore rain can vary significantly. During the second sampling period shown in Fig. 21, the average spore rain of ISG 2 was 22 spores/plate/sampling period at site 1, then dropped to 2 spores/plate/sampling period at site 2, and was zero at site 3.

Spores of ISG 2 are present in the spore rain on Brush Mt., although no collection of ISG 2 has been made in 5 years of sampling Brush Mt.

Figure 22 shows a nocturnal surge in the spore rain of both ISGs. Spore rain approached its maximum value near midnight. Spore rain changes rapidly over time. In a preliminary experiment, spore rain on Brush Mt. went from 61 to 15 sectors/plate in ISG 1 and from 17 to zero in ISG 2 (no replicates, 24 h exposure, consecutive days). During this period, a high pressure system passed through and humidity levels dropped. During the 24 hour period shown in Fig. 22, spore rain rates of ISG 2 varied from 0.8 - 5.4 spores/cm^2/h, with a mean of 2.9 spores/cm^2/h.

Spore viability and sporocarp rehydration studies

Germination time varied among spores, but most germinated by seven days. It was noted during hemacytometry of the day five sample that many spores had already

germinated on the spore print (i.e., nearly immediately after deposition), and apparently had undergone plasmolysis, possibly due to desiccation. The optimal time for taking final germling counts was 8 to 10 days after inoculation, when the germination rate slowed and the faster-growing germlings began to overgrow the slower ones. Figure 23 shows the percent germination of the spores at different sampling times. Only 15.6% (s.d. 0.95%) of the spores inoculated on day 0 germinated. Germination dropped to 2.2% (s.d. 0.22%) by day 5.

Pilei from collections JFM 1474 (ISG 2) and JFM 1475 were air-dried for 2 d. Approximately 10 h following rehydration, both pilei produced a light spore discharge at their margins. The pilei were air dried for an additional three days. Upon rehydration, no spores were discharged from these pilei nor from a pileus which had been air dried for 5 days without prior rehydration.

Pilei from collections JFM 1553-1558 (probably a mixture of both ISGs, based on habitat and location) were rehydrated approximately 4 h after collection. Microscopic examination 2 h after rehydration revealed that no mature basidia were present in any of these collections. At 8 h, mature basidia were observed in 4 of the 6 collections. The basidia were 2 and 4-sterigmate, and DAPI staining showed the spores were 1- and 2-nucleate. Approximately 1-10% of

the spores were binucleate.

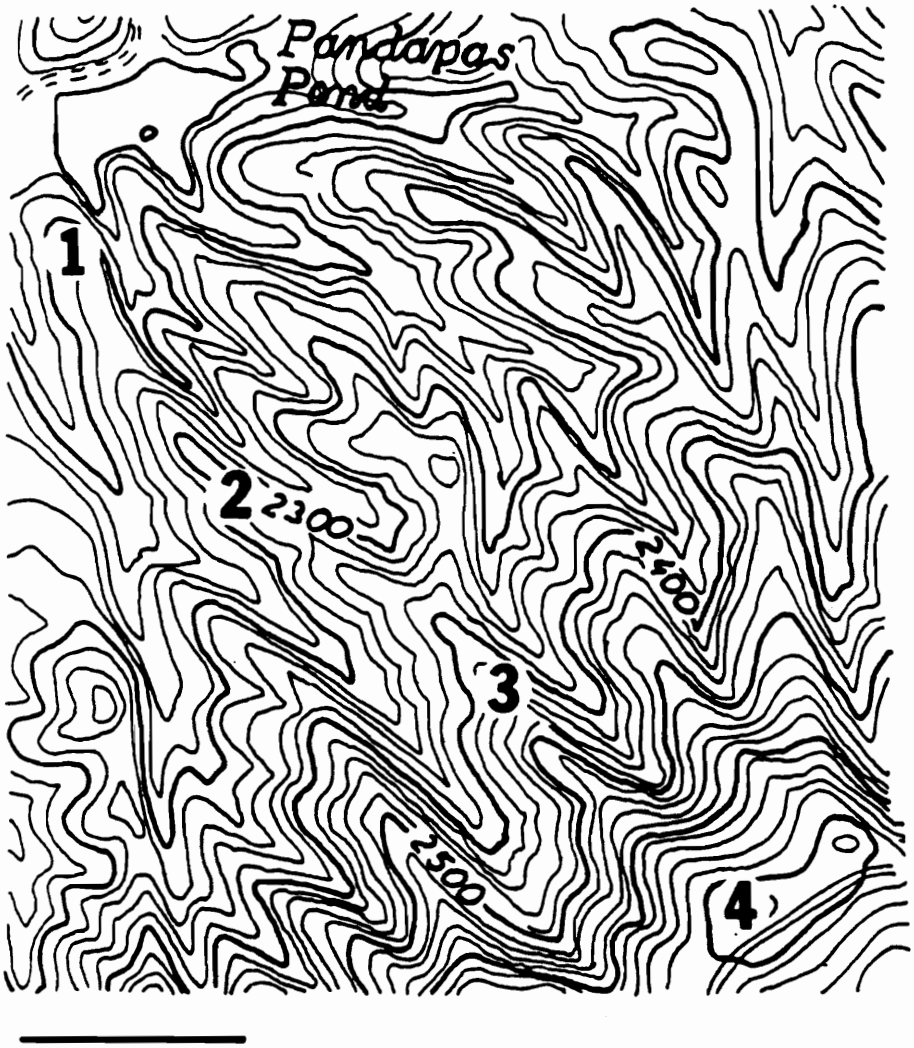

Figure 19. Spore-catching sampling sites 1 - 4. Bar = 1000 m. This map is a copy of a portion of the Newport, VA 7.5' quadrangle, United States Geological Survey reference # N3715-W8022.5/7.5.

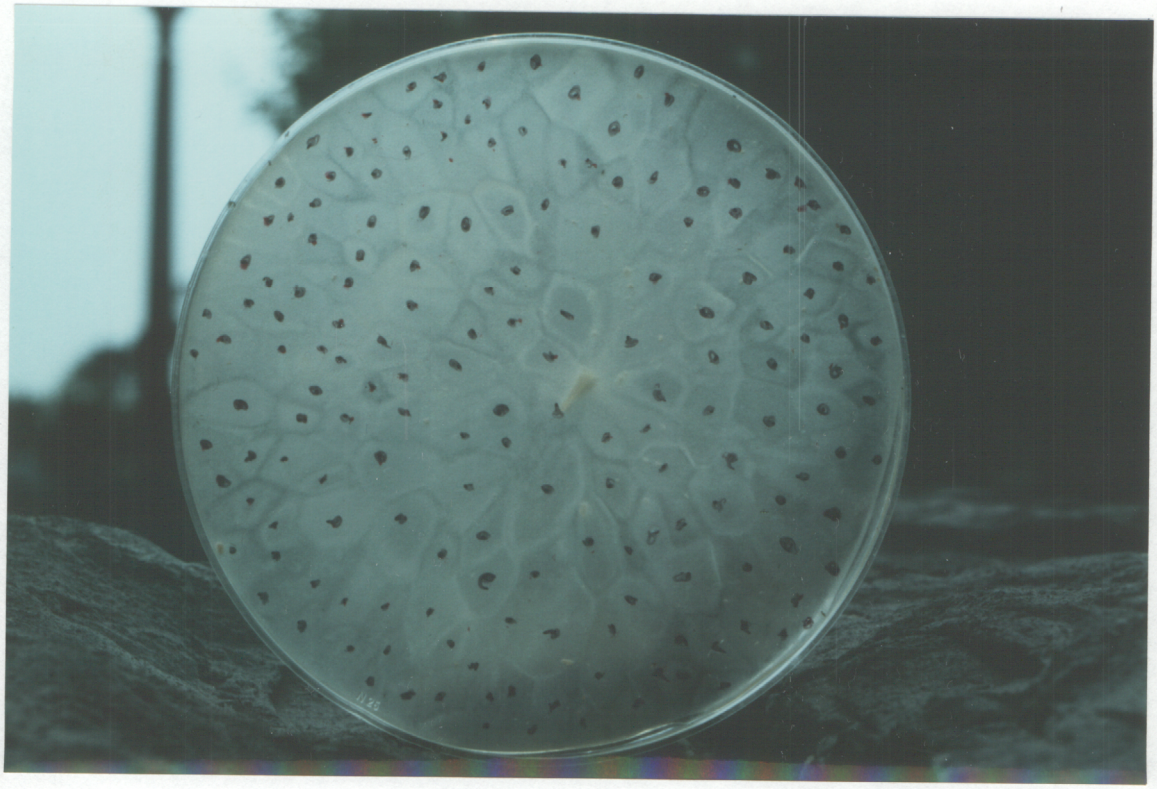

Figure 20. Sectors in Collybia subnuda spore-catching plate. Each sector is marked with an ink spot.

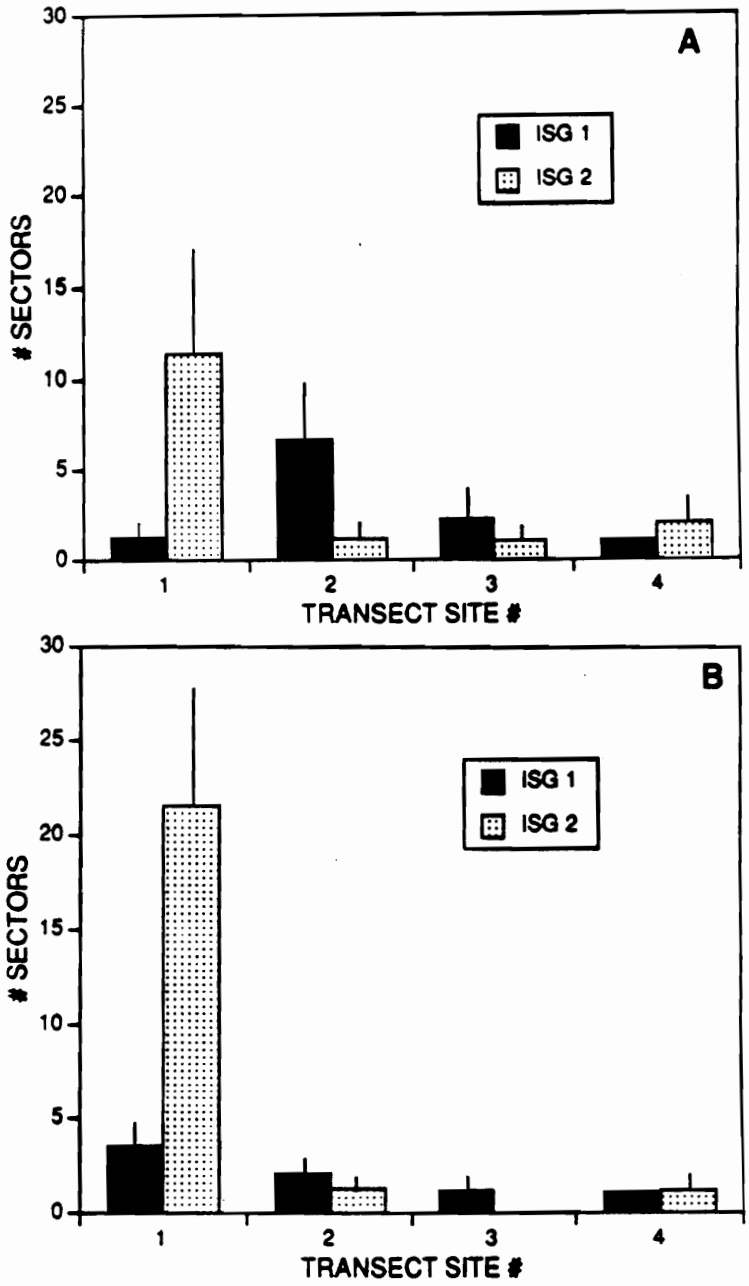

Figure 21. Spatial and temporal variation in the spore rain of Collybia subnuda. A) First 4 h sampling period (Day 1, 2PM-6PM). B) Second 4 h sampling period (Day 2, 10AM-2PM).

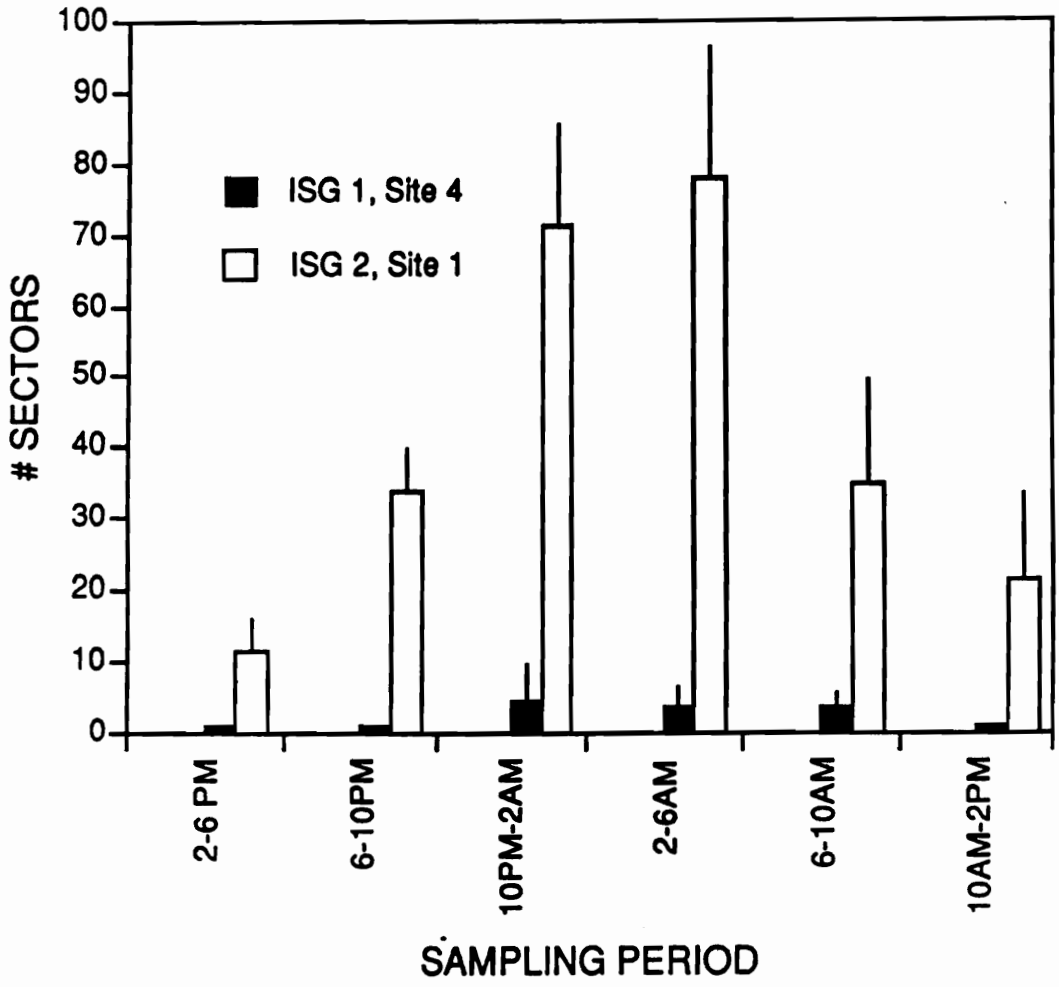

Figure 22. Collybia subnuda spore rain over a 24 hour period.

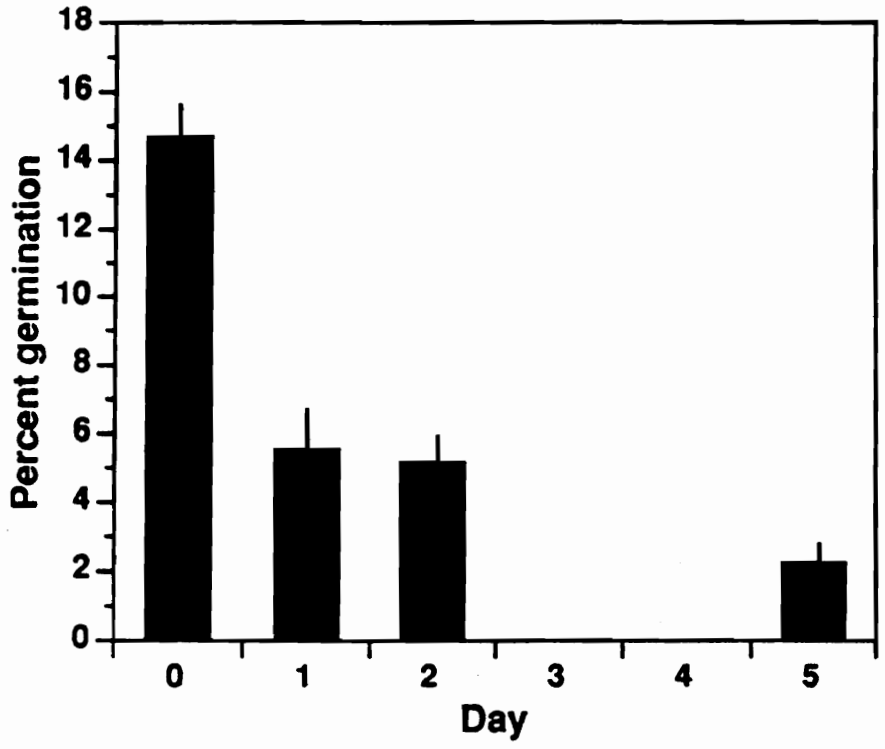

Figure 23. Spore viability of Collybia subnuda.

Table 8. Sampling for C. subnuda via spore-catching, versus conventional sporocarp collection, single-spore isolation, and crossing with tester isolates.

Location	Sporetrap results[1]		Sporocarp collections		Collecting conditions
	ISG 1	ISG 2	ISG 1	ISG 2	
Highlands, NC	0	0	0	0	poor
Oconee, SC	0	0	0	X[2]	poor
Standing Indian, NC	0	0	X	0	poor
Overflow Cr., NC	0	7	0	X	poor to fair
Highlands, NC	0	0	0	0	poor. 12 hr. exposure
Kilmer Forest, NC	0	0	0	0	excellent
Coweeta, NC	2	0	0	0	fair
Pocahontas, WV	+/-170[3]	0	X	0	excellent
Brush Mt, VA	0	0	0	0	poor
Delaware Water Gap	0	0	0	0	poor
Dutchess Co., NY	0	0	0	X	poor to fair
Blue Bend, WV	0	0	0	0	excellent
Sherwood Lake, WV	70	14	0	0	excellent

[1] Reported in number of sectors per sporetrap. A zero indicates no sectors and no clamp connections observed on the plate.

[2] Indicates that sporocarp collections were made and were subsequently identified to ISG after single spore isolation and crossing with tester strains.

[3] This number is an estimate from a plate with too many sectors to count accurately.

DISCUSSION

Spore-catching has been shown to be a useful technique in the study of the distribution of spores of Coriolus versicolor by Williams, et al. (1984) and for Pleurotus spp. by Vilgalys and Sun (1994). Because of the rapid in vitro growth rate of C. subnuda, its resistance to contamination, and the easy differentiation of monokaryon from dikaryon by the formation of abundant clamp connections, C. subnuda is also a good candidate for spore-catching experiments. Furthermore, the development of somatic incompatibility zones in spore traps allows the method to be used to quantify spore rain. It is concluded from these observations that spore-catching is a valuable addition to the methods used in studying the population biology of fungi.

The simplest use of spore-catching is to investigate the presence or absence of a fungus in the vicinity. It is not necessarily superior to traditional collecting methods, but it is simple enough that non-mycologists can use it to determine if a species is present. It must be emphasized, however, that an undikaryotized spore trap is not sufficient evidence to conclude that the fungus is absent from the locale sampled, since the fungus simply might not be fruiting during the sampling period. In a few instances in this study, sporocarps of C. subnuda were collected although

spore-traps did not detect the presence of spores. In these instances, however, the weather was on a drying cycle and the sporocarps were beginning to dry out. The reliability of the method can be increased by frequent sampling and careful timing, since spore rain is dependent upon the presence of sporocarps and weather conditions.

In the preliminary experiment, the low number of sectors in the ISG 1 spore-traps was not surprising, since ISG 1 was rarely collected in Poverty Hollow. For the same reason, the high number of sectors in the ISG 2 spore-traps in the preliminary experiment was expected. Ten replicates per sample appears to be a satisfactory number based on the observed standard deviations given in figures 21 and 22. The four-hour sampling period was satisfactory. However, under different environmental and fruiting conditions, other intervals might be optimal.

In the transect experiment, the spore rain of ISG 2 in Poverty Hollow was high, as expected. The spore rain of ISG 1 on Brush Mt. was much lower than expected. Since observations of sporocarp production during the sampling period indicated that ISG 1 did not fruit as heavily as expected nor as heavily as has been observed many times, its low spore rain is not surprising. Replicate experiments at different times during the year could test whether there is a difference in the phenology of the two ISGs.

The sectoring of ISG 2 spore-traps exposed on Brush Mt. indicates that the apparent absence of ISG 2 on Brush Mt. is not due to the absence of colonizing potential, but rather to unidentified ecological factors or to incomplete sampling. Furthermore, the presence of spore rain of both ISGs along the transect indicate that in this region, at least, the two ISGs are truly sympatric.

The occasionally high rate of spore deposition suggests that competition among monokaryons for substrate and for mates can be high. The temporal variability in spore rain of C. subnuda contrasts with the data presented by Williams, et al. (1984) for C. versicolor, in which spore-catching experiments detected the presence of viable spores in the spore rain year round. In C. subnuda, spore dispersal is affected by the ephemeral nature of spore production, low spore viability, and, possibly, short range spore dispersal, which may be a consequence of the liberation of spores close to the ground.

The sectoring observed following exposure and incubation of spore-traps was particularly significant. Similar sectors were observed by Williams, et al. (1984), who interpreted them as evidence of somatic incompatibility reactions between the heterokaryotic dikaryons resulting from the fertilization of the monokaryon by different spores. Each sector represented a unique fertilization

event. Therefore, in addition to simply determining the presence or absence of spores of each ISG, the method also has the potential to quantify spore rain. Quantitative approaches to spore-catching can provide important information about spore dispersal and the potential for gene flow between presumably isolated populations of fungi. Timing of exposure periods is critical, and reliability of the data can be increased by performing replicate experiments.

The use of the sectoring phenomenon to quantify spore rain in C. subnuda involves several assumptions. The first is that the tester strains distinguish ISGs perfectly. Mating tests between ISGs show partial compatibility in some crosses, but these require prolonged incubation. It is unlikely that a partially compatible spore of a different ISG would be able to compete successfully with nuclei from spores of the same ISG. Support for this assumption comes from the very different results for the two ISGs obtained at the same sampling station and time. The second assumption is that the monokaryotic tester strains do not discriminate among spores within ISG. Evidence that selective mating might occur between monokaryons in natural populations has been presented for Pleurotus (Liou, et al., 1994; Petersen, 1995) and in Marasmius (Gordon and Petersen, 1992). If this is so, then instead of using replicate plates of the same

single spore isolate, a variety of single spore isolates of the same biological species should be used. Nevertheless, the results from using even a single tester strain should indicate trends in spore rain.

Gregory (1973) showed in wind tunnel experiments that turbulence at the rim of Petri plates caused non-random deposition of spores on the plates. Nevertheless, he reported that many researchers have used Petri plates in outdoor studies. He also described an improved design which puts the plates in a bottom of a cylinder 13 cm deep. In regards to spore-catching, it is not clear how relevant wind tunnel experiments are to natural conditions. Nevertheless, future studies should incorporate field tests of both approaches.

Williams, et al. (1984) did not use the development of sectors to assay spore rain because of the possibility that interactions among spores landing on a monokaryon could result in deviations from a Poisson distribution. For instance, if nuclear division and migration of the invading nucleus is rapid (or if the spore rain is heavy or the exposure time is too long), spores landing on an already dikaryotized mycelium would not become established or counted.

Nuclear migration rates in different fungi are given in Table 9 and discussed by Snider (1965). In the

Table 9. Literature reports of nuclear migration rates in fungi.

Williams et al. (1981)	Coriolus versicolor	1.7 - 2.3 mm/h (estimated, not determined empirically).
Buller (1931)	Coprinus cinereus	0.5 - 1.0 mm/hr
Snider and Raper (1958)	Schizophyllum commune, (radiate mycelium)	1.5 - 5.4 mm/hr
Snider and Raper (1958)	Schizophyllum commune, (reticulate mycelium)	0.42 mm/hr
Oikawa (1939)	Galera tenera	0.5 - 0.63 mm/hr
Murphy (1992)	Marasmiellus praeacutus	0.08 mm/hr

basidiomycetes which have been studied, nuclear migration rates vary from 0.5 mm/h in "reticulate" cultures of Schizophyllum commune to 3.2 mm/h in Coprinus macrorhizus. The nuclear migration rate in C. versicolor is 1.7 - 2.3 mm/h (calculated from the data presented by Williams, et al., 1984). Although the rate of nuclear migration in C. subnuda has not been measured, it is considerably less than the rate of C. versicolor (pers. obs. of di-mon crosses). Within the 4 h sampling period in this study, a nuclear migration rate of 3 mm/h could theoretically produce a dikaryon of 12 mm diam., which is approximately the size of the sectors produced in the most heavily inoculated spore-catching plates in this experiment (similar to the plate illustrated in Fig. 20). However, the delay imposed by spore germination, plasmogamy, and the slower nuclear migration rate of C. subnuda makes significant interactions between deposited spores unlikely. Even if interactions do occur among the "trapped" nuclei, the data should show trends in spore rain and still provide valuable information concerning changes in spore rain over time and distance.

A nocturnal peak in spore rain was not observed by Williams, et al. (1984) in C. versicolor, but their methods may not be directly comparable to the ones used here. Ingold (1971) reported on the influence of light and temperature on fungal spore production. Some fungi, such as

Sordaria spp., are stimulated by light to release spores. In other fungi, particularly xylariaceous fungi such as Hypoxylon and Xylaria, spore production is directly inhibited by light. Gregory (1973) reported that the greatest atmospheric concentration of spores of hyaline-spored basidiomycetes is between sunset and sunrise. Miller, et al. (1980) report that spore production in sporocarps of the Basidiomycete Rhodotus palmatus is inhibited by blue light (410-440 nm), but the effect of ambient light under natural conditions is not known in this species. The observed nocturnal peak in the spore rain of C. subnuda may also be caused by a temperature effect. While temperature and humidity were not measured in this experiment, it is likely that the dew point reached a minimum during the observed spore rain maximum.

The low viability of C. subnuda spores, and the rapid loss in their viability over time, probably limit the dispersal ability of the fungus. In a natural system, high temperatures, low humidity, and ultraviolet radiation probably act to decrease the viability of spores even more than in this experiment. Low spore viability over time decreases the likelihood of long-range dispersal, and increase the possibility of isolated populations and allopatric speciation.

If the spores produced by a fungus have a brief life,

then the timing of spore release is critical. Miller (1962) showed that spores of the Basidiomycete Echinodontium tinctorium are sensitive to desiccation and that the timing of spore release appears to coincide with optimal germination conditions. The ability C. subnuda sporocarps to revive and produce spores following desiccation increases the ability of the fungus to take advantage of favorable environmental conditions. A rapid response to the quickly changing humidity levels during a typical summer day in the southeastern U. S. is clearly advantageous.

These studies in spore behavior allow reproduction in C. subnuda to be characterized as follows. Individual genets of C. subnuda are rather small, occupying a few square meters of the litter layer or a small portion of the freshly cast limb of an oak tree. In summer, when humidity levels in the substrate and in the atmosphere are sufficiently high, C. subnuda produces small sporocarps which rapidly develop a fertile hymenium. If humidity levels drop, hymenial development is suspended but can be resumed if conditions improve. Spores are shed and disperse. The timing of spore dispersal may be related to temperature and light as well as humidity, since spore-catching experiments showed a peak near midnight. Spore rain may be locally high. Since spore viability is low, it is likely that germination and dikaryotization occur rapidly

after spore release, and the new dikaryon becomes established quickly after spore production. Spore dispersal is probably sufficient for outcrossing to occur, but dispersal is limited by low spore viability, and this may limit the ability of C. subnuda to colonize isolated habitats.

GENERAL CONCLUSIONS

The population biology of two litter-decomposing Basidiomycetes was studied because of the paucity of information concerning fungal population biology in general and fungi occupying this ecological role in particular. Both were shown to have interesting characteristics which add to our understanding of the diversity of fungal ecology and population biology. Marasmiellus praeacutus was remarkable by having a lower than expected mating allele diversity at both local and regional levels of sampling. It is possible that its small stature and delicate spore morphology are factors which limit its spore dispersal, but more research is required before the causes and implications of its low mating allele diversity can be elucidated.

Collybia subnuda has a typically high mating allele diversity, a random distribution of mating alleles, and it appears to be a regularly outcrossing species. Collybia subnuda is composed of two intersterility groups. The two ISGs are sometimes intercompatible in vitro, but this is unusual and the barrier to gene flow between the two ISG's appears strong. The two ISGs were not distinguishable by morphology, base pair sequence of the ITS region of the ribosomal RNA gene, or biogeography. Local observations indicate that there may be differences in fine-scale distribution and in the timing of sporocarp production, but

more data are required before conclusions can be made about this. The two ISGs have different substrate preferences; ISG 1 is usually associated with oak leaves alone and ISG 2 is usually associated with the cortical layers of fallen oak trees, but there is overlap between the two ISGs. The data indicate a relatively recent divergence of the two ISGs, and the two ISGs provide an excellent model for the observation of speciation in progress.

The spore-catching method was tested and found to be a useful method for investigating distributions and spore dispersal in C. subnuda. The sectors which developed on spore traps following multiple inoculations by spores allowed a quantitative approach to the analysis of spore rain. Measurements were made of spore deposition rate, and, in a limited experiment, spatial and temporal variation along a transect was observed in the spore rain of the two C. subnuda ISGs. The spore-catching method promises to be a useful tool in the study of population biology of higher fungi, providing the species of interest meets the criteria for its use.

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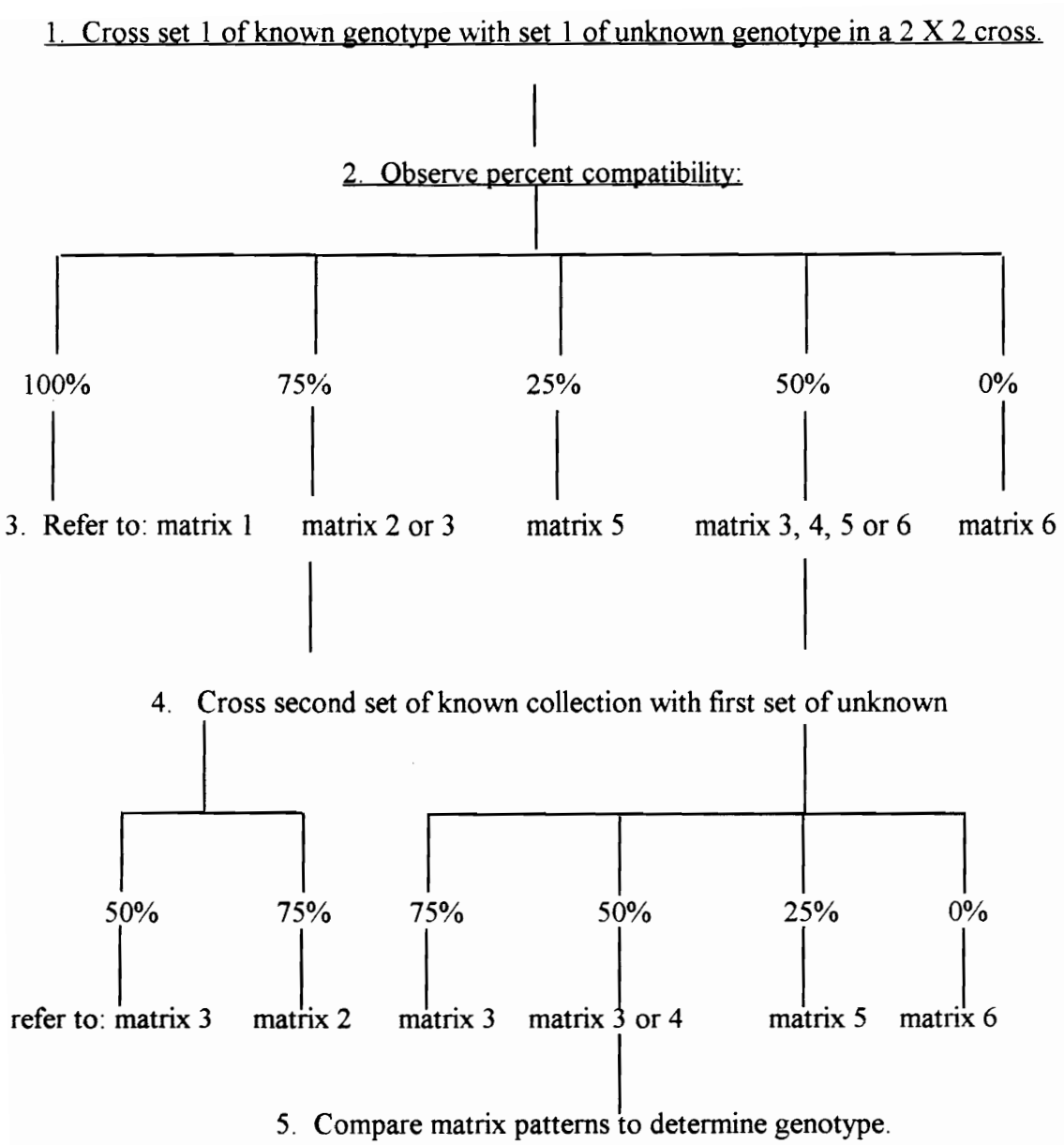
Appendix 1: Flow chart and rationale for determining mating allele genotypes of tetrapolar fungi.

If a set from a collection of known genotype is crossed with a set from an unknown genotype, the following possible results can be obtained:

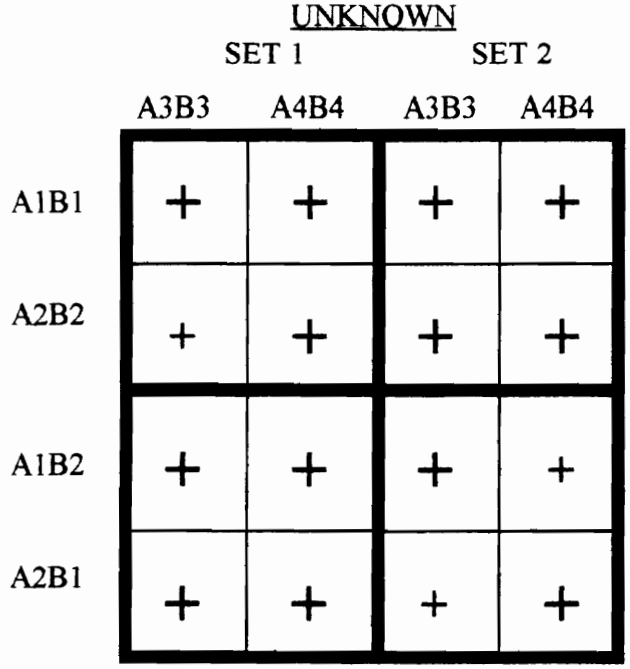
genetic relationship number[1]	Possible observed % compatibility	refer to matrix
Zero shared alleles	100	1
One allele shared at one locus	75	2
1 allele shared at each locus	50 or 75	3
2 alleles shared at 1 locus	50	4
2 alleles shared at 1 locus and 1 shared at the other.	25 or 50	5
All alleles shared	0 or 50	6

1 Matrices are apppended to the flowcharts.

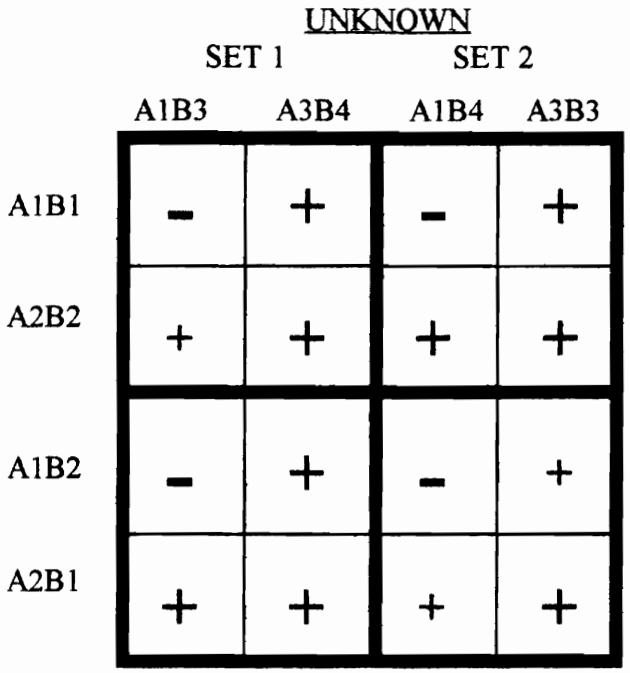
The mating allele genotype of an unknown collection can be determined by using the flowchart presented on the following page.


1. Cross set 1 of known genotype with set 1 of unknown genotype in a 2 X 2 cross.
2. Observe percent compatibility:
100%
75%
25%
50%
0%
3. Refer to: matrix 1
matrix 2 or 3
matrix 5
matrix 3, 4, 5 or 6
matrix 6
4. Cross second set of known collection with first set of unknown
50%
75%
75%
50%
25%
0%
refer to: matrix 3
matrix 2
matrix 3
matrix 3 or 4
matrix 5
matrix 6
5. Compare matrix patterns to determine genotype.

Matrix 1. Zero shared alleles.



Matrix 2. One allele shared at one locus



Matrix 3. One allele shared at both loci

UNKNOWN

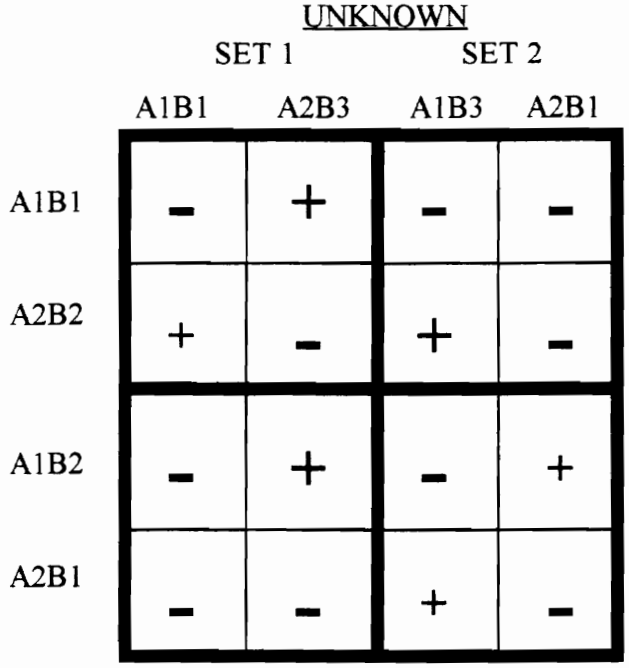
	SET 1		SET 2	
	A1B1	A3B3	A1B3	A3B1
A1B1	-	+	-	-
A2B2	+	+	+	+
A1B2	-	+	-	+
A2B1	-	+	+	-

Matrix 4. Two alleles shared at one locus

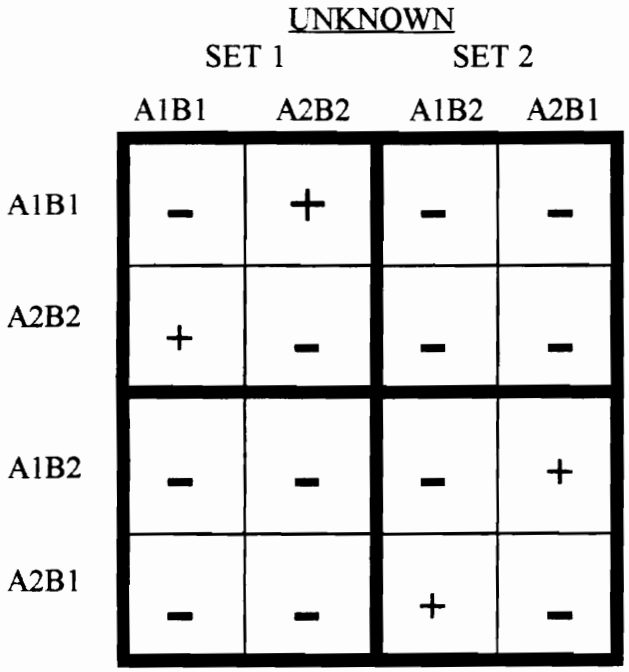
UNKNOWN

	SET 1		SET 2	
	A1B3	A2B4	A1B4	A2B3
A1B1	-	+	-	+
A2B2	+	-	+	-
A1B2	-	+	-	+
A2B1	+	-	+	-

Matrix 5. Two alleles shared at one locus; one allele shared at the other locus



Matrix 6. All alleles shared



VITAE

John (Jack) Murphy was born near Woodstock, New York, on October 17, 1960. His mother, Anna Sochor Chapman, encouraged outdoor activities, travel, and biological investigations and instilled in him a love of nature and strong environmental ethic. After graduating from high school in 1978, Jack attended Deep Springs College, a small (24 student) combination ranch/college located on the edge of the Great Basin in eastern California. The combination of intense, interdisciplinary academics, small classes, hard labor, and wilderness experience at Deep Springs set an educational standard to which Jack will always aspire. After Deep Springs, Jack attended Humboldt State University in Arcata, California, where he earned a Bachelor of Arts in Biology. It was at Humboldt that Jack learned about mushrooms from Dr. David Largent and from the northwest coastal Sitka Spruce, Douglas Fir, Coast Redwood forest association. For two years after graduating, Jack worked as a Teacher/Naturalist at an environmental education center, an experience which affirmed his commitment to education, and which ultimately led to his enrollment at Virginia Tech. Jack received a M.S. in Biology from Virginia Tech. in 1992.