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Recombination and genetic differentiation in the mycorrhizal fungus *Cenococcum geophilum* Fr.

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Abstract: Population genetic analyses of the mycorrhizal fungus *Cenococcum geophilum* were conducted to test for a clonal or recombining population structure. Multilocus genotypes based on polymorphisms in 9 loci, identified in this study by PCR-SSCP techniques, were obtained for two populations. Genotypic variation occurred on a fine scale because unique genotypes were identified at most every transect point, and in some cases occurred even within one soil sample (equivalent to about a 500 mL volume). The largest genet observed occurred over a 30 meter transect space. The two population genetic methods employed to distinguish between clonality and recombination, (1) Index of Association; and (2) ''Parsimony Tree Length Permutation Test'' (PTLPT), could not reject the null hypothesis of recombination in either population. Wright's Fst, as estimated by theta, was used to examine gene flow between the two populations based on allele frequencies. Two of the nine loci had theta values that were not significantly different from what one would expect for the null hypothesis of panmixia. However, the other seven loci were consistent with reduced gene flow. The theta value for the Fisher combined probability (combining all 9 loci) was significant and indicated that there was genetic differentiation between these two populations.

Key Words: *Cenococcum geophilum*, clonality, gene flow, molecular markers, population genetics, recombination

INTRODUCTION

Asexuality is thought to be a common feature of fungal evolution because approximately 20% of fungi (mostly ascomycetes) lack the morphology of sexual reproduction and are thought to reproduce clonally (Reynolds 1993, Rossman 1993). Theoretically, clonal organisms are considered to represent an evolutionary dead end as explained by the inability of clonal species to produce progeny free of deleterious mutations, unlike recombining species (Maynard Smith 1978). Each time the clonal lineage with the fewest mutations is lost through drift, the genetic load increases for the species as a whole with no hope of decrease, like a ratchet held by a pawl (Müller 1964). The dead-end nature of clonal organisms has also been explained by the inability of clonal organisms to assemble advantageous genotypes as fast as their mating and recombining competitors or parasites, making the clonal organisms less fit in the constantly changing biological environment (Jaenike 1978). In spite of these theoretical arguments, there appear to be some old asexual organisms, the best example being the unisexual bdelloid rotifers (Maynard Smith 1992, Judson and Normark 1996, Welch and Meselson 2000). In support of clonality, conditions such as large population size are thought to allow asexual organisms to escape Müller's ratchet (Lynch and Gabriel 1990).

In fungi, phylogenetic studies that include sexual taxa and those that appear to reproduce only via asexual spores indicate that asexual taxa are not monophyletic but rather are evolving from sexual taxa (Guadet et al 1989, Berbee and Taylor 1992, LoBuglio and Taylor 1993, Rehner and Samuels 1994, Spatafora et al 1995, Geiser et al 1996, Ogawa et al 1997, O'Donnell et al 1998, Taylor et al 1999b). More recently, population genetic studies of several different fungi that lack sexual morphology have used the tools of population genetics to demonstrate that recombination is involved in the life cycle of these putatively asexual taxa (Burt et al 1996, Gräser et al 1996, Geiser et al 1998, Fisher et al 2000). Therefore, both the phylogenetic and population genetic approaches support the dogma that asexuality or clonality is rare in most cases and is an evolutionary dead end.

To test whether clonal fungi are an evolutionary dead end, we selected a putatively clonal fungus that lacks not only meiospores, but also mitospores, *Cenococcum geophilum* Fr. The only propagules made by *C. geophilum* are sclerotia, aggregations of mycelium

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(2 mm to 7 mm) that may be dispersed by water or animals (Trappe 1969). As a symbiotic mycorrhizal fungus, *C. geophilum* was also attractive for our study, because it has been proposed that mutualistic organisms can persist without sex (Law and Lewis 1983), an extreme example being the endosymbiotic bacterium that evolved into the mitochondrion (Birky 1995, 1996). Our first task with *C. geophilum* was to conduct a phylogenetic analysis of small subunit rDNA to search for close sexual relatives of *C. geophilum* among ascomycetes (LoBuglio et al 1996). This phylogenetic study showed that *C. geophilum* fell within the Loculoascomycetes, but that it was not closely related to any fungus whose rDNAs had been sequenced. In the present study, we aim to determine whether *C. geophilum* reproduces clonally or by recombination by conducting population genetic analyses as has been done for *Coccidioides immitis* (Burt et al 1996, 1997). We sampled *C. geophilum* from two North American sites, which allowed us also to test for genetic differentiation between these two populations of one of the most frequently encountered of all mycorrhizal fungi.

MATERIALS AND METHODS

Collection of C. geophilum *Isolates.*—Isolates of *C. geophilum* were collected from two sites, one in the New York Adirondack Mountains (State University of New York, College of Environmental Science and Forestry, Huntington Wildlife Forest, Newcomb, New York), and a second site in the Canadian Rocky Mountains (Jasper National Park, Geraldine Lake). At each site a 10-point transect was made with sampling points 10 meters apart. At each point 4 soil samples (roughly equivalent to a 500 mL volume) were collected 1 meter from the transect point to the north, south, east, and west. Additional samples were collected at three other sites in the Adirondacks: 1) a 5 point transect was made 1.6 km from the main transect, 2) random soil samples were collected on Arbutus island, about 0.5 km form the main transect, and 3) in 1990, 17 isolates were collected in a random fashion 0.4 km from the main transect (previously described in LoBuglio et al 1991).

Soil from each sample was sifted to remove debris, and water was added to create a soil slurry. *Cenococcum geophilum* sclerotia and mycorrhizae were retrieved from the slurry under a dissecting scope. The retrieved sclerotia and mycorrhizae were surface sterilized with 30% H_2O_2 and plated on 1% malt agar. Petri plates were incubated at 24 C and routinely screened for evidence of *C. geophilum* mycelial development from the sclerotia or mycorrhizae (which generally took at least several weeks to initiate). A total of 48 and 34 pure culture isolates of *C. geophilum* were obtained from the Adirondack and Canadian Rocky Mountain populations, respectively.

Development of molecular markers.—Genomic DNA from the pure cultures of *C. geophilum* was obtained as previously described (LoBuglio et al 1991). A set of six isolates (three from each population) was randomly selected for the development of the molecular markers. Ribosomal DNA primers (17–22 base pairs in length), known to anneal to the same DNA strand, were used in various pairwise combinations under low-stringency PCR amplification conditions to amplify arbitrary regions of *C. geophilum* DNA. In addition to the arbitrary regions primed by rDNA primers, we also developed one primer pair designed to amplify a portion of the 26S rRNA gene (locus 8), and another pair that amplified part of the calmodulin genic region (locus CMD) (Table I). The PCR reactions contained 1 μL of diluted genomic DNA template (approximately 1–5 ng) in 50 μL reactions. *Taq* DNA polymerase (0.5 unit, Cetus, 1 × *Taq* Buffer supplied by Cetus), 0.2 mM deoxynucleotide triphosphates, and 0.2 mM of each primer were used, with the following temperature profile: 2 min at 94 C, followed by 3 cycles of 94/35/72 C for 5/5/5 min, 30 cycles of 94/50/72 C for 1/1/1 min, and 7 min at 72 C. Primer combinations were sought that produced clearly separated bands of 100 to 600 bp that showed identical electrophoretic mobility in agarose among the six test isolates (see LoBuglio et al 1991 for electrophoresis conditions).

Single Strand Conformation Polymorphism analysis (SSCP) was used to detect polymorphisms among the amplicons. The SSCP analysis was carried out for each locus as follows: 1 μL of the PCR mixture was reamplified in 25 μL of the same cocktail as described above except that additional dNTPs were excluded, and 0.15 μL of [−[35S]thio]dATP (12.5 mCi/mL; 1 mCi = 37 MBQ) was added. The reamplification went for 8 cycles of 94/50/72 C for 1/1/1 min and then 7 min at 72 C. The reactions were electrophoresed on an MDE acrylamide gel (AT Biochem, Malvern, Pennsylvania) according to the manufacturer's instructions, except that 3 μL of labeled product was mixed with 3 μL of stop solution. Nine polymorphic DNA regions or loci were identified in this fashion.

Polymorphism among the loci were identified and confirmed by direct sequencing. Amplicons were gel-purified, reamplified, and sequenced with the Applied Biosystems 377 automated sequencer according to the manufacturer's instructions, using labeled terminators and the same primers as were used in the initial amplification. Knowledge of the amplicon's sequence allowed for the design of *C. geophilum* specific primers. *Cenococcum geophilum* specific primers (as described in Table I) were used to PCR-amplify DNA from the remaining *C. geophilum* isolates from both populations. A more stringent annealing temperature of 50 C was used rather than 35 C. Restriction enzyme sites that coincided with polymorphisms in the amplified DNA were identified using DNA Strider software. For all isolates, amplicons were made for each locus and were digested with the appropriate restriction enzyme to make a data set of biallelic multilocus genotypes (Table II).

Data analysis.—Genetic methods that are used to distinguish between clonality and recombination rely on the knowledge that all regions of the genome have the same history of inheritance under clonality, but that different regions have different histories under recombination (cf. Tay-

TABLE I. Loci from *Cenococcum geophilum* isolates used in this study: PCR primers, loci length, and polymorphisms present within each locus

Locus	Primer sequence	Length (bp)	Number of SNPs*	Restriction enzyme	Nucleotide polymorphism
59	5′ CACTGGAAGAGGCAGAAGC	398	15	*Scr*FI	T/C
	3′ GAGTTCAACCAGTCCAAC				
85	5′ GAATAGCTTGCACAAGTAAG	113	4	*Mse*I	A/G
	3′ CTGCTCACACTTATACCACG				
76	5′ CTGAGAAACCACTGCGTAG	178	1	*Rsa*I	T/C
	3′ CCATGAACAAATATGGC				
87	5′ GTGATGCGAGACGATGC	224	3	*Rsa*I	T/C
	3′ CATGAACATTCTACCACTG				
8**	5′ GCACCAGACTTGCCTGC	206	2	*Msp*I	T/C
	3′ CGCTTACAACCATTACG				
OR	5′ GTGCCGATTATCTACCG	148	3	*Mae*III	A/G
	3′ ATATTCAGACTGACCAT				
CMD***	5′ GACAAAGATGGCGATGGTCAG	310	5	*Bsm*AI	T/C
	3′ CCAGTGCTCCTTCCCAACAG				
20	5′ GCCAGCAGCCCCTTGTAGAC	324	2	*Mfe*I	A/G
	3′ GGTTGCTGATTCACTCG				
1	5′ CGGGAAGAACTAAGTAC	355	27	*Msp*I	A/G
	3′ GTCTCGTGGAATTATAG				

* SNP = Single Nucleotide Polymorphism.
** Locus 8 is part of the 28S rDNA
*** Locus CMD is part of the calmodulin gene.

TABLE II. Multilocus genotypes for *Cenococcum geophilum* individuals from a) Canadian Rocky Mountain and b) New York Adirondack Mountains

a) Multilocus Genotypes		a	b	c	d	e	f	g	h	i	j	k
Number of Isolates with Genotype:		6	2	1	2	2	7	1	7	1	4	1
Locus:	59	0	0	0	0	0	0	0	0	0	0	0
	85	1	1	0	1	1	1	0	1	1	0	0
	76	0	0	0	0	0	0	0	0	1	0	0
	87	1	0	0	0	0	1	0	1	1	0	1
	8	0	1	0	1	0	0	1	0	0	1	1
	OR	1	1	1	1	1	1	1	1	1	1	1
	CMD	0	0	0	0	0	0	0	1	0	0	0
	20	1	1	1	1	1	1	0	1	1	1	1
	1	1	0	0	1	0	0	0	0	0	0	0

b) Multilocus Genotypes		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Number of Isolates with Genotype:		6	1	2	2	2	1	5	1	2	1	4	2	1	2	7	2	3	1	3
Locus:	59	0	0	?	1	0	0	0	?	0	0	0	?	0	?	0	1	1	1	0
	85	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	76	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0
	87	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	8	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1	0
	OR	0	0	0	0	1	0	1	?	0	0	1	1	1	1	1	0	0	0	1
	CMD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	20	1	0	0	1	1	1	1	0	1	1	1	0	1	1	0	1	1	0	0
	1	1	1	0	1	1	0	0	?	1	1	1	0	1	0	0	0	0	0	1

lor et al 1999a). We used two methods. The Index of Association (I_A), the first method, is based on analysis of genetic distances among multilocus genotypes, and was developed by Brown et al (1980) for barley and applied to microbes by Maynard Smith (1993). Association between loci is evaluated by calculating the variance of the distances between all possible pairs of multilocus genotypes, which is low for recombining populations and high for clonal ones (for an example, see: Taylor et al 1999a, b). To establish the significance of the result, the Index of Association (I_A) for the observed data set was compared to the distribution of I_As calculated for 10 000 data sets that had been artificially recombined by resampling the alleles at each locus without replacement (Burt et al 1996). The null hypothesis of recombination would be rejected only if the I_A of the observed data set is significantly larger than the distribution of I_As for the randomized data sets.

The second method, Parsimony Tree Length Permutation Test (PTLPT) borrows on phylogenetic methods developed to test for signal in phylogenetic data sets (Archie 1989), and was adopted to test for reproductive mode (Burt et al 1996). Phylogenetic trees are built from the multilocus genotypes using parsimony (PAUP, Version 4, Swofford 1998) by treating the loci as characters and the alleles as character states. Significance is determined by comparing the length of the tree of the observed data to the distribution of tree lengths of 1000 data sets that have been artificially recombined as above. For clonal populations, the original tree should be significantly shorter than the distribution of tree lengths for randomized trees, but not so for populations with recombining alleles (Burt et al 1996, Taylor et al 1999a).

The extent of gene flow between the two populations was inferred from Wright's Fst statistic as estimated by theta using the method of Cockerham and Weir (1993). Values of theta range between 0 (no differentiation between the populations due to unrestricted gene flow) to 1 (complete isolation due to the absence of gene flow). The significance (p values) for population differentiation at individual loci was calculated by Fisher's exact test, while Fisher's combined probability was used to estimate the overall significance for population differentiation combined across loci (Sokal and Rohlf 1981: pages 738–743 and 779–782).

RESULTS

Identification of polymorphic loci.—PCR amplifications with rDNA primers in various arbitrary combinations resulted in 7 reactions that produced clearly separated bands (100 to 600 bp in length) of identical electrophoretic mobility in agarose for the six test isolates. Two additional reactions using primers for the 26S rDNA region and the camoldulin gene were also selected so that a total of 9 DNA regions were obtained for the population genetic analysis. Subsequent SSCP and sequence analysis of the DNA amplified from the test isolates confirmed the presence of polymorphic nucleotide sequence for each primer pair. When *C. geophilum* specific primers were designed for each region, the length of the amplified products decreased in length, and ranged from 113 to 398 (Table I). All of the polymorphisms observed among the DNA fragments were single nucleotide polymorphisms (SNPs) except for fragment 59 which also had a two bp insertion. The number of SNPs in the fragments ranged from 1–5, except for fragment 59, which had 15 SNPs, and fragment 1, which had 27 SNPs. All of the observed polymorphisms were transitions (either A/G or C/T; Table I). The sequences of the 7 arbitrary fragments were analyzed for homology to known sequences using the Blastx search; however, none was found. Although most of the fragments had two or more SNPs, only one could be used to define the locus because all of the SNPs on a single fragment must be physically linked. For each fragment, a SNP lying in the recognition sequence of a restriction enzyme was chosen as the locus. Where possible, SNPs with balanced allele ratios were chosen (Table II).

Table III. *Cenococcum geophilum* genotypes along transects in the NY Adirondack Mt. site (A–C, E–K) and the Canadian Rocky Mt. Site (a–k). Each point is separated by 10 meters. (Note: The Adirondack genotype "E" is identical to the Canadian Rocky Mt. genotype "a"; and the Adirondack genotype "G" is identical to the Canadian Rocky Mt. genotype "e")

Point	Adirondack Mts.	Canadian Rocky Mts.
1	A, A, (A, K)[a], J	k
2	C, K	a, g, h
3	B, G	i, b, c,
4	G, K	a, a, c
5	H, K	a, e, d, j
6		g, h, h, d
7	A, (F, G)[a], I, I	f, d, d, h
8	C, E	a, a, f
9		d, f, d, d
10	E	e, f, f, f

[a] Isolates collected from 1 soil sample.

Multilocus genotypes.—Nineteen unique genotypes were found among the 48 isolates collected in the Adirondack mountains and 11 unique genotypes were found among the 34 isolates collected in the Canadian Rocky Mountains (Table II). Two genotypes were present in both populations (Adirondack E = Rocky Mountain a, Adirondack G = Rocky Mountain e; Tables II and III). As shown in Table III, most of the genotypes were represented by just a few individuals and no large clones were identified. In the Adirondacks there were three most common genotypes (A, G, and O) represented by at least five

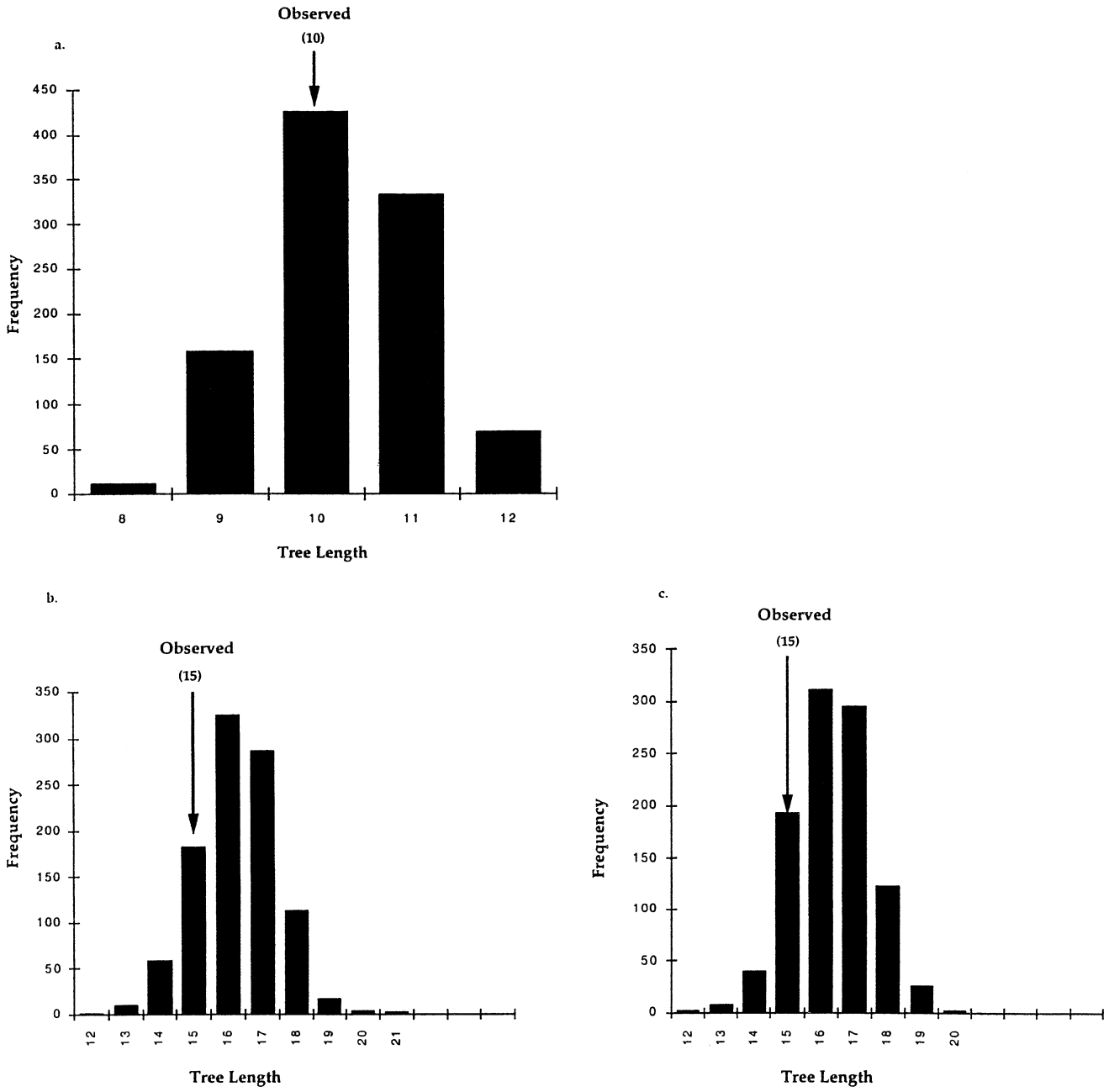

FIG. 1. Position of the observed tree length within the distribution of tree lengths for 1000 artificially recombined data sets using the Parsimony Tree Length Permutation Test; (a) Analysis of the complete Canadian Rocky Mountain data set, p = 0.60; (b) Analysis of the complete Adirondack Mountain data set, p = 0.172; and (c) Analysis of the Adirondack Mountain data set excluding genotype "H", p = 0.19.

or more individuals, and in the Rockies there were also three most common genotypes represented by six or more individuals (a, f, and h), one of which (a) was also present in the Adirondacks (E) (TABLE II). In general, genotypic variation occurred on a fine scale because unique genotypes were identified at most every transect point, and in some cases occurred even within one soil sample (equivalent to about a 500 mL volume). The largest genets were observed in the Canadian Rocky Mt. Site, where genotype f was observed at 4 continuous transect points (points 7–10, TABLE III), which covered 30 meters.

Reproductive mode.—Results from the analysis of the multilocus genotypes for both populations, using both the PTLPT and I_A methods, could not reject the null hypothesis of recombination (FIG. 1). For the PTLPT, the lengths of parsimony trees made from

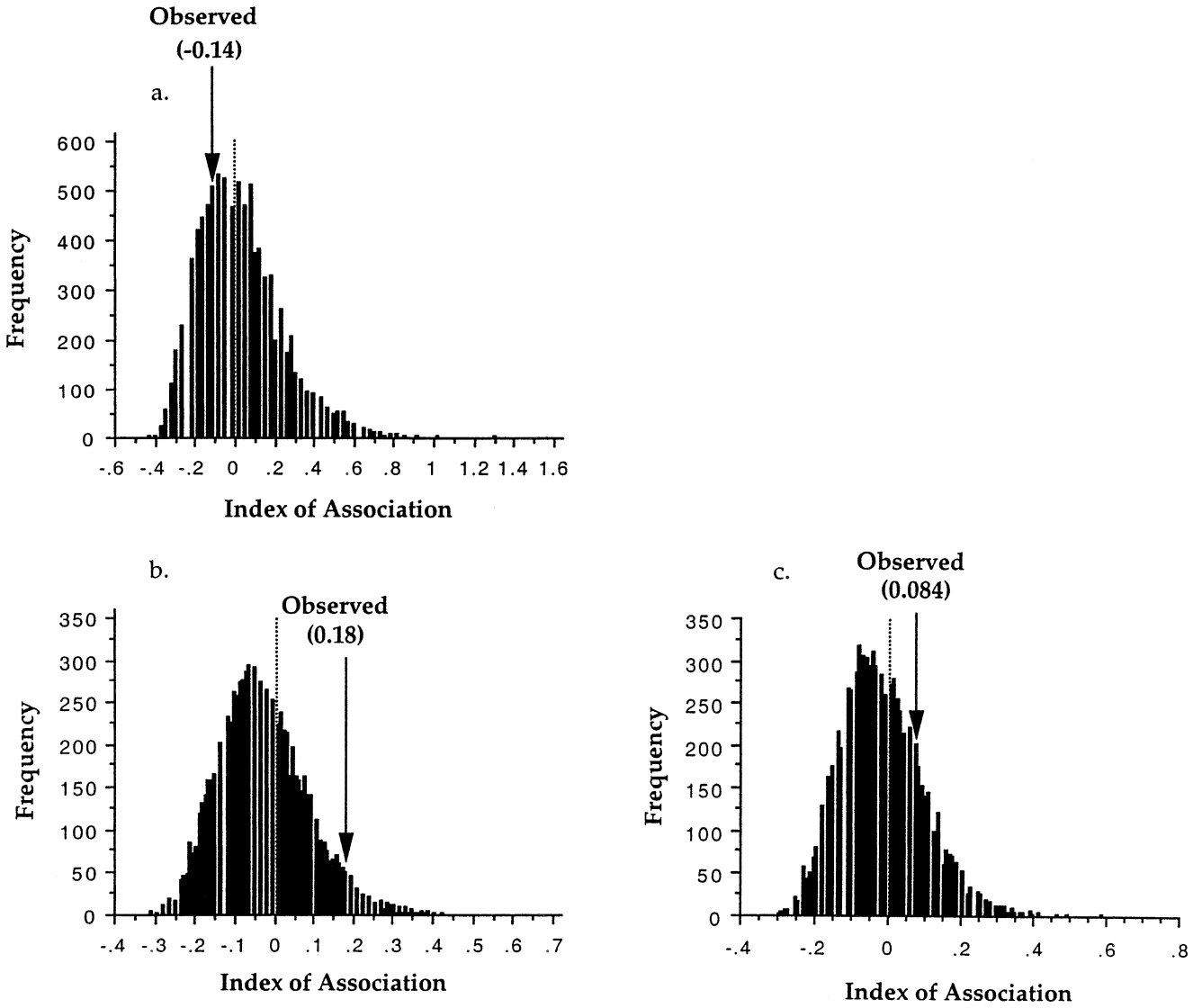

FIG. 2. Position of the observed Index of Association (I_A) relative to I_A values for 10 000 artificially recombined data sets whose mean is 0 and is represented by the dashed line; (a) Analysis of the complete Canadian Rocky Mountain data set, p = 0.78; (b) Analysis of the complete Adirondack Mountain data set, p = 0.05; and (c) Analysis of the Adirondack Mountain data set excluding genotype "H", p = 0.19.

the observed data set were not significantly different from the distribution of tree lengths calculated for 1000 randomizations of the observed data set (Canadian Rockies p = 0.232, FIG. 1a, Adirondacks p = 0.172, FIG. 1b). The observed I_A value for the Canadian Rocky Mt. population (−0.0803) was well within the distribution of I_A values for the 10 000 randomized data sets (p = 0.70, FIG. 2a). However, the I_A value for the Adirondack population was at the limit of significance (p = 0.05, FIG. 2b). This result could indicate that there may be some structure in this population or it could be due to genotype H, which had three unknown alleles. To test for the latter possibility, we removed genotype H from the I_A analysis, in which case the I_A was now near the mean of values for recombined data sets and would not permit rejection of the null hypothesis of recombination (p = 0.19, FIG. 2C).

Gene flow.—Wright's Fst, as estimated by theta (Cockeram and Weir 1993), was used to examine gene flow between the two populations based on allele frequencies. Two of the nine loci (76 and 8) had theta values that were not significantly different from what one would expect for the null hypothesis of panmixia (TABLE IV). However, the values for the other seven loci were consistent with reduced gene flow and the theta value for the Fisher combined probability

TABLE IV. Genetic differentiation of two *Cenococcum geophilum* populations inferred from Wright's Fst as estimated by Weir and Cockerham's Theta

Locus	Allele	Population NY	Population CA	Theta	Fishers P value
59	0	33	34	0.159	0.0067[ab]
	1	8	0		
85	0	0	7	0.2187	0.0014[a]
	1	48	27		
76	0	43	33	0.016	0.3927
	1	5	1		
87	0	46	12	0.598	0.0001[a]
	1	2	22		
8	0	32	24	−0.02	0.8113
	1	16	10		
OR	0	21	0	0.395	0.0001[a]
	1	26	34		
CMD	0	48	27	0.219	0.0014[a]
	1	0	7		
20	0	17	1	0.247	0.0003[a]
	1	31	33		
1	0	25	26	0.087	0.0382[a]
	1	22	8		
Sum:				0.2557	<0.001[ac]

[a] = significant at $P < 0.05$.
[b] = Fisher Exact test.
[c] = Fisher combined probability.

(combining all 9 loci) was significant, indicating that there is genetic differentiation between these two populations (TABLE IV).

DISCUSSION

The observation of repeated genotypes in each of the *C. geophilum* populations examined indicates that clonal reproduction has a significant role in *C. geophilum*'s mode of reproduction. However, population genetic analysis of the multilocus genotypes obtained for the two *C. geophilum* populations indicates that recombination is also occurring. This result is contrary to our original hypothesis that *C. geophilum* was a strictly clonal fungus. In this respect, the results with *C. geophilum* are similar to what has been observed in other putatively asexual fungi such as *Candida albicans*, *Coccidioides immitis*, and *Aspergillus flavus* (Burt et al 1996, 1997, Gräser et al 1996, Geiser et al 1998).

Recombination in *C. geophilum* populations presents the possibility that a cryptic sexual state exists for this mycorrhizal fungus. Of course mitotic or parasexual recombination could also account for the data, however, parasexuality has not been demonstrated in nature (Geiser et al 1996). Although the methods used in this study have identified recombination, they cannot indicate how frequently recombination occurs, or when the most recent recombination event occurred within these *C. geophilum* populations. Phylogenetic studies examining the evolution of putatively asexual fungi have generally identified close sexual relatives for the asexual taxa in question (LoBuglio et al 1993, O'Donnell et al 1998, Taylor et al 1999b). The inability to identify a close sexual relative for *C. geophilum* through molecular phylogenetic analysis (LoBuglio et al 1996) raises the possibility that the recombination identified in this study is the result of past sexual encounters.

Although *C. geophilum* appears not to be a strictly clonal fungus, there are other candidates for old, clonal fungi in the Glomales. Like *C. geophilum*, these fungi are mycorrhizal symbionts and they lack the morphology of sexual reproduction. In pot cultures their reproduction appears to be clonal (Rosendahl and Taylor 1996), but tests of reproduction on natural isolates have not been reported.

Analysis of the two *C. geophilum* populations indicates significant genetic differentiation between the *C. geophilum* populations in the Adirondack and Canadian Rocky mountains. This result is not surprising given the limited means of dispersal in *C. geophilum*. It may seem counterintuitive that some common genotypes could be present in both populations if there was little or no gene flow. However, one of each pair of the genotypes found in both locations was composed of the most common alleles, and these genotypes may represent those of the population ancestral to the present Adirondack and Rocky Mountain populations. The presence of unique genotypes in both populations would not be expected if gene flow was high, and in the Adirondacks, 42% of the multilocus genotypes were unique and 32% were so in the Canadian Rockies. The simplest interpretation of our data is that we have found two locations between which genes of *C. geophilum* are not exchanged at a rate high enough to counter drift. Genetic differentiation may have progressed to isolation, however, no locus was fixed for alternative alleles in the two populations, arguing that they still are part of one phylogenetic species (Taylor et al 2000). A more complicated explanation could be that the two populations examined are not at equilibrium. However, the individuals in each population had similar amounts of allelic variation, unlike a population that recently has expanded from another (Fisher et al 2001). Extensive sampling between the Adirondack and Canadian Rocky Mountain populations would likely clarify the population dynamics of *C. geophilum*, as has been the case for *Schizophyllum commune* (James et al 1999) and *Coccidioides immitis* (Fisher et al 2001).

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