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Amplified Single-Nucleotide Polymorphisms and a $(GA)_n$ Microsatellite Marker Reveal Genetic Differentiation between Populations of *Histoplasma capsulatum* from the Americas

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Carter, D. A., Taylor, J. W., Dechairo, B., Burt, A., Koenig, G. L., and White, T. J. 2001. Amplified single-nucleotide polymorphisms and a $(GA)_n$ microsatellite marker reveal genetic differentiation between populations of *Histoplasma capsulatum* from the Americas. *Fungal Genetics and Biology* 34, 37–48. *Histoplasma capsulatum* has a worldwide distribution but is particularly concentrated in the midwestern United States and throughout Central and South America. Genetic differences between isolates resident in separate parts of the world have been reported, but the relationship between the isolates and the level of migration between different endemic foci has not been clear. In this study we used multilocus genotypes based on amplified polymorphic loci and one microsatellite to quantify the level of genetic differentiation occurring between North and South American populations of *H. capsulatum*. Significant genetic differentiation occurred between isolates obtained from Indiana and Alabama, and a marked division was seen between the Indiana population and the Class 1 isolates from St. Louis. Strong genetic differentiation occurred between the two North American populations and the Colombian population. This study supports the separation of North and South American *H. capsulatum* into different species, which has been proposed under the phylogenetic species concept.

Index Descriptors: Histoplasma capsulatum; population differentiation; theta; histoplasmosis; molecular markers; SNP; fungal pathogen; microsatellite.

Histoplasma capsulatum is a dimorphic ascomycetous fungus, capable of saprophytic growth in soil and parasitic growth in humans and other mammals. Histoplasmosis occurs worldwide, but the major centers of endemism of the fungus are concentrated in the Americas, in particular the Mississippi and Ohio valley regions of the United States and the central and western regions of Central and South America (Kwon-Chung and Bennett, 1993). The fungus frequently occurs in soil enriched with bird guano and in caves occupied by colonial bat species (Hasenclever and Piggott, 1974). Birds do not appear to be infected with *H. capsulatum*, but viable cells have been isolated from various organs from different species of bats and from their gut contents (Klite and Diercks, 1965; M. L. Taylor *et al.*, 1999).

A number of different molecular markers have been developed for study of the epidemiology and genetic diversity of *H. capsulatum*. These have revealed that, far

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from being a single, well-resolved species, *H. capsulatum* consists of a number of genetically distinct groups or classes which frequently correlate with geographic origin (Vincent *et al.,* 1986; Spitzer *et al.,* 1989; Keath *et al.,* 1992). The predominant genotype found in North America has been defined as Class 2. A second distinct North American group consists of an unusual low-virulence isolate known as "Downs" (Gass and Kobayashi, 1969), and a small number of other closely related isolates, and is classified as Class 1 (Spitzer *et al.,* 1990). Isolates from throughout Central and South America are grouped together in Class 3; three additional very small classes are Class 4, comprising a single isolate from Florida soil, and classes 5 and 6, which are composed of small numbers of isolates from AIDS patients from New York and Panama, respectively.

A phylogenetic study by Kasuga and colleagues (1999) was done to attempt to resolve the relationships between the major classes and the two varieties of *H. capsulatum: H. capsulatum* var. *dubosii* and *H. capsulatum* var. *farciminosum*. Four unlinked genes were partially sequenced from 46 geographically diverse isolates that included the three varieties. The resulting analysis identified six separate clades, which included Class 1 and Class 2 from North America, two genetically distinct groups from South America, one from Panama, and *H. capsulatum* var. *dubosii*. Evidence of genetic recombination in one of the two South American groups was demonstrated. The authors argued that under the phylogenetic species concept each of these could represent a separate phylogenetic species.

Although the sequence data resolved 33 different multilocus genotypes within the 46 different isolates, almost all of the intraspecific diversity lay within the South American isolates. Very little diversity was seen among Class 2 isolates from North America despite a variety of geographic origins that ranged from Indiana in the northern midwest to Arkansas and Georgia in the southern United States. In contrast, RAPD[5] analysis (Kersulyte *et al.,* 1992) and single-nucleotide polymorphisms (SNPs) (Carter *et al.,* 1996) have found high levels of diversity within a collection of isolates from Indianapolis, Indiana. In addition, although the Kasuga *et al.* (1999) study implied a relatively recent population bottleneck in North America that might suggest recent clonal expansion in this region, the Indianapolis isolates have been found to have a population structure that suggests extensive genetic recombination (Carter *et al.,* 1996).

We began the current study to analyze in greater detail the genetic structure of *H. capsulatum* populations within North America. Using the SNP markers developed in Indianapolis isolates (Carter *et al.,* 1996) and a hypervariable $(GA)_n$ microsatellite (Carter *et al.,* 1997), we determined alleles in a collection of 69 isolates, including 18 clinical isolates from Birmingham, Alabama, the 5 isolates previously identified as belonging to Class 1, the 30 Indianapolis isolates, and 16 isolates from Colombia. In addition to examining the population genetic structure within North America and assessing whether genetic recombination has occurred in a second North American population, we test whether the resolution between the North and the South American populations found by Kasuga *et al.* (1999) can be supported using an independent dataset and analysis.

MATERIALS AND METHODS

Isolates

All isolates were obtained from clinical specimens. Each isolate was taken from a different individual, with the following exceptions: Birmingham isolates H123 and H132 were from one individual but were from different specimens and were taken 4 days apart, and H124, H133, and H134 were from a second single individual from different specimens collected over 6 days. Isolates were from patients with and without underlying conditions predisposing them to histoplasmosis. Further information on the individual isolates can be obtained from the authors.

Isolates from Indiana were provided by P. Connolloy and J. Wheat of the Indiana University Medical Center, Birmingham isolates were from W. Dismukes, S. Moser, and B. Hines of the University of Alabama. Colombian isolates H59–H64 and H73 were provided by Elisabeth Castañeda of the Instituto Nacional de Salud, Santa Fe de Bogota, and isolates H66–H76 were from the Hospital Pablon Tobon Uribe, Medillin provided by A. Restrepo and J. McEwen. Isolate H9 is the original "Downs" strain (Gass and Kobayashi, 1969) and was provided as DNA by E. Keath. H126–H128 were isolated from AIDS patients from St. Louis in 1987 and were determined to belong to Class 1 by Spitzer *et al.* (1990). H129 is also a Class 1 isolate but its origin and date of isolation are unknown, except that it was obtained subsequent to 1987. H126–

[5] Abbreviations used: RAPD, random amplified polymorphic DNA; SNPs, single-nucleotide polymorphisms; RFLP, restriction fragment length polymorphism.

H129 were provided by G. Kobayashi from Washington University, St. Louis.

Growth of Fungi and DNA Extraction

All manipulations of living fungal tissue were carried out in BL-3 containment facilities. DNA was extracted from heat-killed mycelia by a standard SDS–phenol–chloroform method (Burt *et al.,* 1995). Growth conditions and DNA extraction have been previously reported (Carter *et al.,* 1996).

Development and Application of Molecular Markers

Molecular markers were initially developed in a subset of isolates from Indianapolis and St. Louis and have been reported previously (Carter *et al.,* 1996), with the exception of L660.3/*Dde*I, which was developed in this study. Markers were found by analysis of arbitrarily amplified fragments of DNA by single-stranded conformation polymorphism, direct sequencing of fragments shown to be polymorphic, and determination of whether the polymorphism was associated with an RFLP or an indel of sufficient size to be assessed on an agarose gel. If so, specific primers were designed and were used to amplify the corresponding locus from additional isolates. Amplified DNA was digested with the appropriate restriction endonuclease and electrophoresed in 3% NuSieve agarose. RFLP alleles were scored 1 for presence of the enzyme site and 0 for absence of the site, with indels scored 1 for insertion and 0 for deletion. The amplification primers, thermocycler conditions, and method for restriction digestion have been presented in Carter *et al.* (1996, 1997). Primers for amplification of locus L660.3/*Dde*I are 660.3L 5′CCTGTAGTATTATTCTTGAAGC3′ and the M13-40 sequencing primer 5′GTTTTCCAGTCACGAC3′; amplification conditions were 94°C/1 min, 55°C/1 min, 72°C/1 min for 35 cycles.

The amplification of microsatellite polymorphism HSP-TC used primers HSP13-FAM and HSP373. Amplification conditions have been previously reported (Carter *et al.,* 1997). Amplified DNA was electrophoresed in an ABI 373 automated sequencer with ROX 1000 size standards. The size of migrating fragments was assessed with Genescan 2.1 software (Perkin–Elmer–ABI, Foster City, CA).

DNA to be sequenced was purified by polyethylene glycol precipitation (Rosenthal *et al.,* 1993) and was sequenced with the HSP373 primer. Sequencing was performed by the SUPAMAC facility (University of Sydney) with an ABI 377 automated sequencer and fluorescent dye terminator chemistry (Perkin–Elmer–ABI).

Data Analysis

Genetic recombination in the Birmingham population was assessed with the Index of Association (I_A) (Maynard Smith *et al.,* 1993; Burt *et al.,* 1996). Only loci that were polymorphic in the Birmingham population were included. This test was performed (1) on the entire population excluding H131 (see below) and extra isolates obtained from a single individual (H132, H133, H134) and (2) on the population excluding H131 and all isolates with replicated multilocus genotypes (clone corrected). Departure from recombination was assessed by comparison of the I_A for the Birmingham dataset with a range of I_A values calculated for artificially recombining datasets. The latter datasets were produced by randomization of alleles at each locus between members of the population while the original allele frequency was maintained, in effect, causing the population to undergo "virtual sex" in the computer. A total of 1000 independent recombining datasets were produced in this way. An I_A was calculated for each of these to give a distribution of recombining I_A values with which the observed I_A could be compared. Calculations were performed with the computer program MultiLocus PPc 1.0b, available at http://www.bio.ic.ac.uk/evolve/software/multilocus/.

The probability of a genotype occurring more than once in the dataset was calculated as

$$\sum_{x=n}^{G} \frac{G!}{x!(G-x)!} (P)^x (1-P)^{G-x},$$

where G is the number of genotyped isolates within the population, P is the probability of observation of the original genotype (which is the product of the frequency of each allele found at a locus), and n is the number of isolates with the same genotype as that in question. In our study, $n = 1$ and the formula reduces to $Pse = 1 - (1 - P)^G$ (Fisher *et al.,* 2000b).

Genetic differentiation between populations was analysed with Weir and Cockerham's theta (θ), an estimate of Wright's Fst, as

$$\theta = \frac{Q - q}{1 - q},$$

where Q is the probability that two different genes within a population are the same allele and q is the probability

TABLE 1
Multilocus Genotype for Each Isolate Included in Study

Locus	Indianapolis, IN																														
	H14	*H19*	*H20*	*H21*	*H22*	*H23*	*H24*	*H28*	*H30*	*H32*	*H33*	*H36*	*H39*	*H40*	*H41*	*H42*	*H43*	*H44*	*H46*	*H48*	*H49*	*H50*	*H51*	*H52*	*H53*	*H54*	*H55*	*H56*	*H57*	*H58*
L603/*Mnl*I	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	–	0	0	0	0	0
L604/*Dde*I	0	1	0	1	0	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0
L620.1/*Hae*III	1	0	0	1	0	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	1	0	0	1	0	0	1
L642/*Alu*I	1	1	0	0	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	1	0
L610.1/indel7	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	1	1	1	1	0
L649.3/*Dde*I	0	1	0	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0
L626/*Pvu*II	1	0	1	0	1	0	1	1	0	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	1	1	0	0	1	1
L652/*Spe*I	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
L655.3/*Cfo*I	1	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0	1	1
L667.1/*Hin*fI	1	0	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0	1	1	0	1	1	1	1	1	1	0	0	1
L660.3/*Dde*I*	0	1	0	0	0	0	0	0	1	0	0	0	1	0	–	0	0	1	1	1	1	0	0	0	0	0	0	1	1	0
Genotype based on biallelic markers	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	I	AB	AC
HSP-TC allele size	383	371	373	371	370	369	371	373	375	375	377	370	375	375	373	375	371	375	375	371	375	373	371	373	369	375	379	371	371	371
Overall multilocus genotype	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD

Amplified fragment contained 11-bp insert.
^† *Pvu*II site lost in these isolates, but genetic basis of loss differed in North American, Colombian, and Class I isolates
* Isolates shared distinctly different secondary amplification products.

that two genes in different populations are the same allele. If two different populations have the same allele frequencies, then $Q = q$, and $\theta = 0$. Conversely, if the populations are fixed for different alleles, then $Q = 1$, $q = 0$, and $\theta = 1$. The statistical significance of the theta values was calculated by recalculation of theta for 1000 datasets in which individuals were randomized across populations, with MultiLocus 1.2 (P. Agapow and A. Burt, 2001). Theta has the full range of values from 0 to 1 when polymorphic loci are discovered for all populations. It is often the case, as in this study, that polymorphic loci initially are characterized for one population and then applied to others. This approach biases theta so that genetic differentiation is underestimated and the maximum possible value for theta is less than 1 (Burt *et al.*, 1997; J. W. Taylor *et al.*, 1999).

The numerical index of discriminatory power (D) was calculated for the HSP-TC microsatellite as

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1),$$

where N is the size of the population, s is the number of different alleles, and x_j is the number of the population falling into allele class j (Hunter, 1991). The significance of the difference in allele size in different populations was calculated by two-tailed t test.

RESULTS

DNA Amplifications and Multilocus Genotypes

The 11 SNP loci and the $(GA)_n$ microsatellite were amplified from each isolate and scored (Table 1). Most loci were successfully amplified from all of the isolates. Loci L652/*Spe*I, L655.3/*Cfo*I, and L667.1/*Hin*fI were not amplified from H9 due to a very limited amount of DNA. Locus L652/*Spe*I had an 11-bp insertion in the Colombian isolates, but shared the same polymorphism inactivating the *Spe*I site as the other isolates, and was scored 0. Some amplifications produced secondary products that were seen in addition to the desired band; these were particularly prominent in the Class 1 isolates and in isolate H131 and were generally identical in these isolates.

Scoring of each isolate for the 11 SNP loci and the HSP-TC microsatellite produced an overall multilocus genotype for each isolate (Table 1). The majority of Class 2 isolates from North America had unique multilocus genotypes both with the SNP markers and with these plus the microsatellite marker. Only three overall genotypes were shared by two or more isolates: AE (isolates H97 and H101), AO (H123 and H132), and AP (H124, H133, and H134). For the latter two, all isolates were from a single

TABLE 1—*Continued*

Birmingham, AL																	Colombia																	Class I				
H97	*H98*	*H99*	*H100*	*H101*	*H102*	*H103*	*H104*	*H105*	*H106*	*H122*	*H123*	*H124*	*H125*	*H131*	*H132*	*H133*	*H134*	*H59*	*H60*	*H61*	*H62*	*H63*	*H65*	*H66*	*H67*	*H68*	*H69*	*H70*	*H71*	*H73*	*H74*	*H75*	*H76*	*H9*	*H126*	*H127*	*H128*	*H129*
0	1	1	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	1	0	0	0	0	1	0	0	0	0*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0*	0*	0*	0*	0*
1	1	1	1	1	0	1	1	1	1	0	1	1	1	1†	1	1	1	1	1	1	1	1	1^	1	1^	1	1	1	1	1	1	1	1	1†	1†	1†	1†	1†
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0*	0	0	0	0#	0#	0#	0#	0#	0#	0#	0#	0#	0#	0#	0#	0#	0#	0#	0#	0*	0*	0*	1*	0*
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1^	1	1	1	–	1	1	1	1	1
0	0	0	0	0	0	0	0	0	0	1	0	0	0	–	0	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
W	AD	AE	AF	W	AG	V	AH	AI	AF	AJ	K	AK	K	AL	K	AK	AK	AM	AM	AM	AM	AM	AN	AM	AN	AM	AM	AM	AM	AM	AM	AM	AM	AO	AL	AL	AP	AQ
375	369	370	371	375	373	375	369	369	373	371	369	371	367	361	369	371	371	361	349	350	349	354	346	365	349	361	349	361	350	350	349	361	356	361	362	361	361	361
AE	AF	AG	AH	AE	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR	AO	AP	AP	AS	AT	AU	AT	AV	AW	AX	AY	AZ	AT	AZ	AU	AU	AT	AZ	BA	BB	BC	AR	BD	BE

patient but were from different clinical specimens or were taken on different days. Nothing is known about the clinical history of isolates H97 and H101. As these isolates shared alleles that had a high frequency in the dataset, it is possible that they are not identical but share alleles by chance ($P > 0.1$). Although they were genetically diverse, the isolates from Birmingham were fixed at 3 of the 10 SNP loci: L652/*Spe*I, L655.3/*Cfo*I, and L557.1/*Hin*fI.

In contrast to the North American isolates, the genotypes of the Colombian isolates based on the SNP loci were very restricted. The only polymorphic locus was L626/*Pvu*II, which was not cut in isolates H65 and H67. However, when this locus was sequenced, the basis for the loss of the *Pvu*II restriction site was found to be different from that in the North American isolates (Fig. 1). Additional sequencing of the L626/*Pvu*II locus in the Class 1 isolates revealed a third polymorphism preventing restriction by *Pvu*II. These alleles were scored in the Colombian and Class 1 isolates as 1^ and 1†, respectively, and were treated as distinct alleles at locus L626/*Pvu*II in the calculation of θ. Isolates H65 and H67 were assigned multilocus genotype designations different from those of the remaining Colombian isolates to reflect this additional polymorphism (Table 1). The addition of the microsatellite allele allowed most of the Colombian isolates to be differentiated, indicating that these isolates are genetically diverse, but this diversity could not be detected with only the SNP loci.

Although most of the alleles were also fixed in the Class 1 isolates, these could all be differentiated by the combination of the SNP loci and the microsatellite. Isolate H128 shared multilocus genotype AR with Birmingham isolate H131. Because of its similarity to Class 1 isolates, this isolate was excluded from all subsequent analyses.

Ind:H49 CGTGTCTGAGGTG|CA**C**CTG|TTGAAACTGCTTGATCCCATGCCGG
Ind:H50 CGTGTCTGAGGTG|CAGCTG|TTGAAACTGCTTGATCCCATGCCGG
Col:H63 CGTGTCTGAGGTG|CAGCTG|TTGAAACTGCTTGATCCCATGCCGG
Col:H65 CGTGTCTGAGGTG|CAG**T**TG|TTGAAACTGCTTGATCCCATGCCGG
Col:H68 CGTGTCTGAGGTG|CAGCTG|TTGAAACTGCTTGATCCCATGCCGG
Col:H67 NGNGTCTGAGGAG|CAG**T**TG|TTGAAACTGCTTGATCCCATGCCGG
Cl1:H126 CGTGTCTGAGGTG|CAG**A**TG|TTGAAACTGCTTGATCCCATGCCGG
Cl1:H127 CGTGTCTGAGGTG|CAG**A**TG|TTGAAACTGCTTGATCCCATGCCGG
Cl1:H128 CGTGTCTGAGGTG|CAG**A**TG|TTGAAACTGCTTGATCCCATGCCGG
Cl1:H129 CGTGTCTGAG-TG|CAG**A**TG|TTGAAACTGCTTGATCCCATGCCGG

FIG. 1. Sequence polymorphism at the *Pvu*II restriction endonuclease site in locus L626/*Pvu*II in Indianapolis, Colombian, and Class 1 isolates of *H. capsulatum*. The *Pvu*II site is bracketed; underlined, cut by *Pvu*II; boldface, sequence polymorphisms preventing restriction.

HSP-TC Microsatellite Size Polymorphisms

The size of the amplified DNA fragment containing the HSP-TC microsatellite ranged from 346 to 383 bp, with a total of 19 different alleles (Fig. 2). The discriminatory power of the HSP-TC microsatellite varied from 0.4 in the Class 1 population to 0.839 in the Indianapolis population

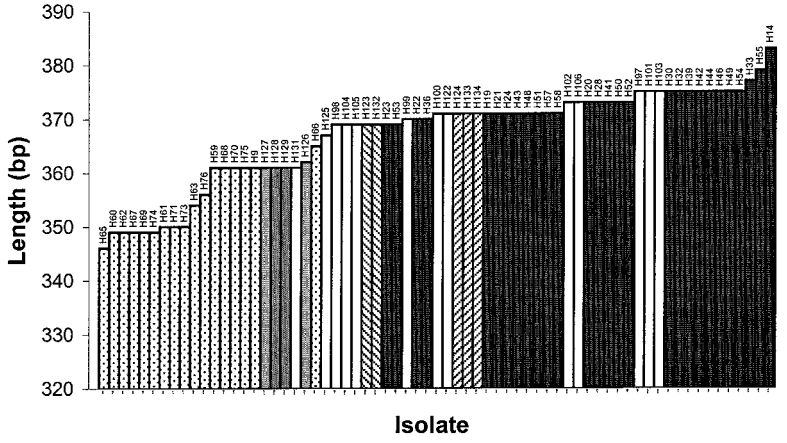

FIG. 2. Allele lengths (in bp) for the HSP-TC microsatellite in isolates of *H. capsulatum*. ■ Indianapolis, IN; □ Birmingham, AL; ▨ Colombia; ▩ Class 1; ▧ and ▨ indicate Birmingham isolates taken from single patients.

and across the entire population was 0.926 (Table 2). Although there was some overlap between the microsatellite allele sizes found in the different populations, each population was characterized by a distinct range of allele sizes. The significance of the difference in the allele sizes between populations ranged from $P = 0.04$ for Birmingham vs Indiana to $P < 0.001$ for all other pairwise comparisons. DNA sequencing revealed that size differences were not all due to variations in the number of tandem repeats of the $(GA)_n$ microsatellite (Fig. 3). In Class 1 isolates the microsatellite was reduced to $(GA)_4G_3(GA)_3$ and did not vary between the isolates; however, H126 had one extra T in a $(T)_n$ repeat which was downstream from the $(GA)_n$ microsatellite. The Colombian isolates H64, H66, and H70 also had short microsatellite motifs of 8, 7, and 7 repeat units, respectively, but large differences between their amplicon sizes. These were also due to highly variable downstream sequences, where there were numerous insertions and deletions. Often these had some repetitive structure such as mononucleotide and short dinucleotide repeats. Within the North American populations, however, length variation was largely due to the number of $(GA)_n$ repeats

TABLE 2
Properties of HSP-TC Microsatellite

Property	Population			
	Indiana	Birmingham	Colombia	Class 1
No. alleles	8	7	7	2
Size range (bp)	369–383	367–375	346–365	361–362
Mean	373.2	371.1	353.3	361.2
Variance	9.7	6.6	38.3	0.2
Index of discrimination (D)	0.839	0.813	0.808	0.4

Recombination in the Birmingham Population

Lack of polymorphism at most loci in the Colombian population and the small size of the Class 1 group meant that recombination could be assessed only in the Birmingham population. As loci L652/*Spe*I, L655.3/*Cfo*I, and L667.1/*Hin*fI were monomorphic in all Birmingham isolates, these could not be included in the analysis. Pairwise comparisons were made for all polymorphic loci to test for cosegregation of loci, as would be expected in clonally reproducing populations, and the combined data were used to compute an I_A across all loci. The I_A for both the entire population (excluding replicate isolates from single patients) and the clone-corrected population (excluding all isolates with identical genotypes) fell well within the range for I_A values generated for artificially recombining datasets (Fig. 4), indicating that recombination and genetic exchange has occurred in this population ($P > 0.05$).

Genetic Differentiation

Table 3 shows the results of the analyses of genetic differentiation between the following pairs of populations: Indianapolis vs Birmingham, Indianapolis vs Colombia,

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Ind:H53  : CGGGAGGGAGAGAGAGAGAGAGAGAGA--------AACAGCGCT 369
Ind:H51  : CGGGAGGGAGAGAGAGAGAGAGAGAGAGA------AACAGCGCT 371
Ind:H50  : CGGGAGGGAGAGAGAGAAAGAGAGAGAGAGA----AACNGCGCT 373
Ind:H42  : CGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA--CACAGCGNT 375
Bir:H125: CGGGAGGGAGAGAGAGAGAGAGAGAGA--------AACACCGCT 367
Bir:H123: CGGGAGAGAGAGAGAGAGAGAGAGAGAGA------AACAGCGCT 369
Col:H64  : CGGGACGGAGAGAGAGAGAGAGA------------AACAGTGCT 346
Col:H70  : CGGGAGGGAGAGAGAGAGAGA--------------AACAGCGCT 361
Col:H66  : CGGGAGGGAGAGAGAGAGAGA--------------AACAGCGCT 365
Cl1:H9   : CGGGAGGGAGAGAGAGGGAGAGA------------AACAGCGCT 361
Cl1:H126: CGGGAGGGAGAGAGAGGGAGAGA------------AACAGCGCT 362
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FIG. 3. Sequence of the HSP-TC microsatellite showing variation in the $(GA)_n$ repeat length and total allele size for 11 isolates of *H. capsulatum*.

Birmingham vs Colombia, Indianapolis vs Class I, and Birmingham vs Class 1. The Colombian and Class 1 populations were not compared as both populations were too genetically distinct from the Indianapolis population, in which the molecular markers were developed, to allow a meaningful value for differentiation to be assessed. Values for θ were calculated for each locus, and the total θ was computed to give an overall value for each population pair. θ values indicated a highly significant level of differentiation between the Colombian and both North American populations and between Indianapolis and Class 1 isolates and significant differentiation between the two North American populations and between Birmingham and Class 1 isolates. A much lower value for θ occurred between the Birmingham and the Indianapolis populations, but this θ still was significant at the $P < 0.01$ level.

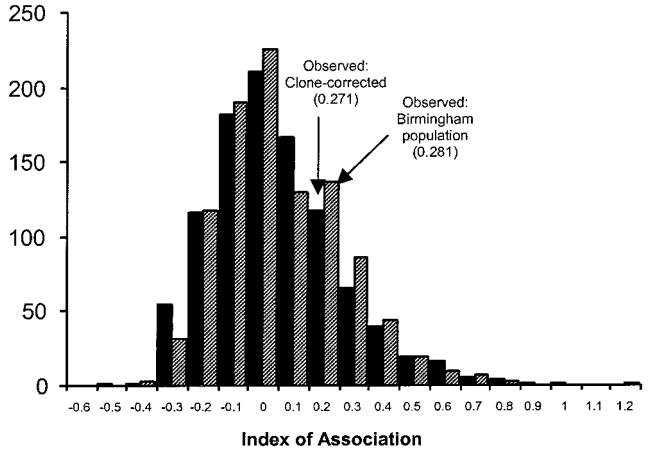

FIG. 4. Histogram showing the range of Index of Association values for 1000 recombined datasets based on the Birmingham population, ▨ and the same population after removal of identical genotypes ■. The values for the observed populations are indicated by arrows. Both fall well within the range expected for recombination ($P > 0.05$).

DISCUSSION

Genetic Differentiation Occurs between Geographically Separated Populations from North America

The most significant result of this study is that genetic differentiation can be detected between the populations of *H. capsulatum* from Indiana and Alabama ($\theta = 0.09$; $P < 0.01$). These two populations also possessed a range of microsatellite alleles that overlapped but were significantly different (Fig. 2 and Table 2; $P < 0.05$). Differentiation is likely to be due to the geographic separation (approx. 750 km) of the two regions. *H. capsulatum* can be spread by

TABLE 3
Theta Values Calculated between Each Different Population

Locus	Theta values				
	In vs Bir	In vs Col	Bir vs Col	In vs Cl. I	Bir vs Cl. I
L603/*Mnl* I	0.14	0.74	0.48	0.67	0.32
L604/*Dde* I	−0.04	0.21	0.17	0.11	0.03
L620.1/*Hae* III	0.01	0.37	0.25	0.27	0.10
L642/*Alu* I	—	0.27	0.17	0.17	0.03
L610.1/indel7	−0.03	0.31	0.25	−0.04	−0.14
L649.3/*Dde* I	−0.01	0.18	0.09	0.08	−0.04
L626/*Pvu* II	0.16	0.27	0.01	0.61	0.81
L652/*Spe* I	0.02	0.88	1.00	−0.07	0.22
L655.3/*Cfo* I	0.33	0.34	—	0.21	—
L667.1/*Hin* fI	0.20	0.21	—	0.08	—
L660.3/*Dde* I†	0.10	—	—	—	—
total θ	0.09**	0.46***	0.52***	0.31***	0.33**

† Locus 660.3 could not be amplified from Class 1 and Colombian isolates.
*** $P < 0.001$.
** $P < 0.01$.

infection of mammals, particularly bats, and is thought to be carried by the wind (Rippon, 1988); however, significant levels of migration of the fungus between these two areas is apparently not occurring. The contribution of geographic separation to genetic differentiation is supported by the difference in θ values between the two North American populations and the Colombian population (Col vs Bir, $\theta = 0.27$, $P < 0.001$; Col vs In, $\theta = 0.37$, $P < 0.001$; Table 3), where theta is highest between most geographically distant populations. These results are similar to those found by our analysis of genetic differentiation in *Coccidioides immitis* (Burt *et al.,* 1997) in which a gradient of differentiation occurred among isolates from California, Arizona, and Texas.

High values for θ were seen between the Class 1 isolates and the other two North American populations (Indianapolis, $\theta = 0.31$, $P < 0.001$; Birmingham, $\theta = 0.33$, $P < 0.01$). The lower P value for the Class 1 vs Birmingham comparison is probably due to the combined effect of a smaller number of Birmingham isolates and the fact that the Colombian and Birmingham populations were both fixed for the same allele at loci L655.3/*Cfo*I and L667.1/*Hin*fI. This discrepancy demonstrates the difficulties that can arise when markers that have been developed in one population are used to compare two other, genetically distinct populations. The HSP-TC microsatellite was distinctly different in both sequence and allele length in the Class 1 collection (Figs. 2 and 3). Differences between the majority of North American isolates and those characterized as Class 1 isolates also have been reported in DNA fingerprints, mtDNA, and rDNA RFLPs, karyotype, heat sensitivity, growth phenotype, and virulence characteristics (Spitzer *et al.,* 1990). Additionally, a number of the primers used in this study produced distinct secondary bands that were shared between the Class 1 isolates (and H131) but were not seen in any of the other populations. The extent of these genotypic and phenotypic differences makes it very unlikely that significant genetic exchange occurs between the Class 1 and the North American populations. As most of the Class 1 isolates originated from St. Louis, which is closer to Indianapolis and Birmingham than these centers are to one another, it is probable that genetic rather than geographic barriers exist which prevent genetic exchange with these isolates.

Isolates of H. capsulatum Are Genetically Diverse

Eleven polymorphic SNP loci and 1 multiallelic microsatellite locus have been used in this study to produce multilocus genotypes for 69 different isolates of *H. capsulatum*. A total of 56 different multilocus genotypes were found, of which 44 were present in the 48 Class 2 isolates obtained from North America (Indianapolis and Birmingham). This high level of diversity agrees with that in our previous study, in which isolates H14–H58 from Indianapolis, IN each had a different multilocus genotype (Carter *et al.,* 1996). The only isolates that were not known to have been obtained from a single individual and that could not be differentiated were H97 and H101. We cannot be certain that these are clonally related with the current marker set, and it is possible that these isolates would be differentiated if more loci were considered.

Isolates H124, H133, and H134 were obtained from the same patient and shared genotype AP, indicating that a single genotype of *H. capsulatum* was present in the blood and lung tissue of this patient. Likewise, isolates H123 and H132 were from a single patient and had identical genotypes. No data are available on the number of different isolates of *H. capsulatum* normally present during an episode of histoplasmosis, but studies on other mycoses that are acquired exogenously have found that in general only a single fungal strain is present and that the same strain persists throughout the infection (Spitzer *et al.,* 1993; Varma and Kwon-Chung, 1992; Burt *et al.,* 1996). These findings probably reflect the low success rate that most medically important fungi have of establishing a successful infection (Kwon-Chung and Bennett, 1993). The current set of molecular markers will allow the presence and maintenance of different strains of *H. capsulatum* to be assessed during the course of histoplasmosis.

The genetic diversity of the Class 1 isolates was substantially lower. Although each strain could be characterized by a unique genotype, only two of the SNP loci (L610.1/indel7 andL652/*Spe*I) varied between these isolates, and a single base change occurred in one strain in the microsatellite locus. This lack of differentiation is similar to that found in previous studies using rDNA RFLP fingerprinting, where Class 1 isolates H9 (“Downs”), H126, H127, and H128 were found to be very similar to one another but not all were identical (Spitzer *et al.,* 1990). All Class 1 isolates produced numerous secondary amplification bands with primers amplifying loci L649.3/*Dde*I, L626/*Pvu*II, and L652/*Spe*I, which is further evidence that they are genetically different from the Class 2 isolates in which the primers were developed. Birmingham isolate H131 shared these distinct secondary amplification products and had a genotype identical to that of H127; this isolate probably also belongs in Class 1. Until recently, all Class 1 isolates had been obtained from im-

munosupressed patients and were of lower virulence than the other classes; the latest addition to this class was an isolate from a striped skunk, which was obtained in the 1940s in Georgia (Kasuga *et al.*, 1999). Unfortunately, we do not have clinical information for H131. This isolate was removed from the Birmingham population before calculation of recombination and genetic differentiation (Table 3).

Kasuga *et al.* (1999) reported a substantially higher level of genetic diversity in South American than North American isolates of *H. capsulatum*. However, the current study, which included many of the same Colombian isolates used by Kasuga *et al.* (1999) found almost no differences between these isolates when only the SNP loci were considered. It is widely recognized that polymorphic markers that are not subject to selection pressure are likely to drift to fixation in segregated populations (Taylor *et al.*, 1999). Our result therefore indicates that genetic segregation has occurred between the Colombian population and the Indianapolis population, in which the SNP markers were originally developed. Variation in the Colombian population was confirmed by analysis of the HSP-TC microsatellite, which had seven different alleles and an index of discrimination of 0.808 in this population. This index was slightly lower than the discrimination indexes for the Indianapolis (0.839) and Birmingham (0.813) populations. However, microsatellite variation may have been underestimated in the Colombian population due to ascertainment bias, in which microsatellites are more likely to be polymorphic in the population in which they were developed through the preferential discovery and selection of long alleles (Goldstein and Pollock, 1997). The HSP-TC microsatellite was developed from the published Heat Shock Protein-60 gene, which was sequenced from strain G217B, a Class 2 type strain (Gomez *et al.*, 1991).

In addition to a high level of diversity within the Colombian isolates, Kasuga *et al.* (1999) found that these divided into two distinctly different groups: South American *H. capsulatum* group A (Sam *Hcc*A) and South American *H. capsulatum* group B (Sam *Hcc*B). The HSP-TC microsatellite supported this division; all the isolates characterized by Kasuga *et al.* (1999) as Sam *Hcc*A (H60, 61, 62, 64, 65, 67, 71, 73, and 74) had allele lengths of less than 360 bp, whereas the Sam *Hcc*B isolates (H59, 68, 70, and 75) were all 361 bp. This length difference was not due to the $(TC)_n$ microsatellite, but to a number of insertions and deletions in the region downstream from the microsatellite sequence. Sequences flanking microsatellites frequently also have some repetitive structure and can be very polymorphic (Tautz, 1989). H66 was an outlier in the Kasuga *et al.* (1999) study and is distinct here.

The Birmingham Population Has a History of Genetic Recombination

The eight SNP loci that were polymorphic in the Birmingham population were used to assess whether this population had a history of genetic exchange. Genetic exchange already has been demonstrated in the Indianapolis population by use of almost the same set of SNPs (Carter *et al.*, 1996) and in a subset of the Colombian population by use of sequence data (Kasuga *et al.*, 1999). Like these, the Birmingham population clearly had a recombining population structure, and from these results it appears that *H. capsulatum* regularly undergoes sexual reproduction in the environment. The exception to recombining populations may be the Class 1 strains. There are too few strains and too little variation to assess whether recombination occurs between them. Although low genetic diversity may be taken as evidence of clonality (Tibayrenc *et al.*, 1991), it does not rule out recombination.

Populations from North and South America Are Strongly Differentiated

The Indianapolis and Birmingham populations were both strongly genetically differentiated from the Colombian population. The latter was fixed at all SNP loci except L626/*Pvu*II, where it had a unique polymorphism that inactivated the *Pvu*II site and had an 11-bp insertion in locus L652/*Spe*I that was not seen in any of the North American isolates. The range of allele sizes for the HSP-TC microsatellite was also distinctly different in the North and South American populations (Fig. 2 and Table 2). The physical distance between Colombia and the North American populations makes it likely that geographic separation followed by genetic drift has caused this differentiation. Different environmental conditions in the two areas may have also contributed by promoting the selection and expansion of different genotypes. The North American midwest has the highest endemism of *H. capsulatum* in the world, and the very heavy infestation of this area is thought to be at least partly due to the presence of starlings and high levels of starling guano which are found under starling roosting sites (Rippon, 1988). In South America the starling is not yet common, and the habitat of *H. capsulatum* is restricted to chicken habitats and bat

caves, in which quite different environmental conditions would be encountered (Negroni, 1940). The level of differentiation between isolates from the two continents could mean that genetic barriers now exist to prevent gene flow and South American isolates belong to at least one separate phylogenetic species, as proposed by Kasuga *et al.* (1999). Mating tests have shown interactions among the different varieties of *H. capsulatum*, although not necessarily leading to viable and fertile offspring (Kwon-Chung *et al.*, 1974). Among fungi, there is evidence that phylogenetic methods can recognize genetic isolation in nature among individuals that retain the ancestral ability to mate (Vilgalys and Sun, 1994; Petersen and Hughes, 1999; Taylor *et al.*, 2000). It would be interesting to determine whether individuals assigned to different species by Kasuga *et al.* (1999) are capable of successful mating in cultivation or whether this ability has been lost as a consequence of their genetic isolation.

Different Molecular Markers Reveal Different Levels of Genetic Differentiation

An important first step in any study using molecular markers to characterize individuals and populations is to choose the marker that will reveal the right amount of variation to distinguish evolutionarily meaningful groups. Our previous work on *C. immitis* (Burt *et al.*, 1996, 1997) and the results of this study clearly show that markers characterized in one population may be substantially less variable in a second, geographically or genetically removed population. This disparity is the basis for the assessment of population differentiation; however, it also means that the level of diversity in the second population is underestimated. In an extreme case, as seen in the Colombian population of *H. capsulatum*, all alleles may be fixed and the differentiated population will appear to be completely clonal. The hypervariable and multiallelic nature of microsatellites means that they are much more likely to show variation in all populations; however, if the rate of mutation is too high, homoplasy may confound the effect of genetic differentiation. In a comprehensive study in *C. immitis*, in which microsatellites were compared with measures of genetic differentiation based on SNPs and DNA sequencing, Fisher *et al.* (2000a) found that microsatellites could reliably reconstruct phylogenetic and population structures, provided that several microsatellites were used and that the method of microsatellite analysis employed matched the age of the evolutionary events being studied. Microsatellites can also suffer from ascertainment bias and ideally should be developed from each genetically subdivided population if assessment of the level of diversity in each population is a goal.

Ascertainment bias does not occur in DNA sequencing analysis, provided that the sequences to be used are not subject to selection pressure in particular populations. Sequence data can therefore be used to characterize both variation and differentiation in populations (Koufpanou *et al.*, 1997; Kasuga *et al.*, 1999). However, as sequencing remains expensive and time-consuming to perform, sequencing studies on fungal populations have been limited to date to a few loci, and these few loci may underestimate diversity and fail to reveal differentiation between closely related populations. This problem is seen in the study by Kasuga *et al.* (1999), in which the North American populations appeared genetically homogenous despite the inclusion of more than 1500 bp of sequence data. The solution is to use either many more biallelic loci, as with the SNPs employed here, or multiallellic loci, as with the microsatellite used here. With increasing advances in genomics and a steady reduction in the cost of sequence analysis, future studies may be able to incorporate sufficient sequence data to answer questions of diversity and differentiation. Meanwhile it is clear that a set of complementary molecular markers should be used to accurately describe the genetic variation within and between populations.

The Biological and Clinical Relevance of Genetic Differentiation

Determination of whether different populations are genetically differentiated is useful for an understanding of the epidemiology of infectious diseases, as it allows clinical isolates to be assigned to geographic regions with a predictable level of certainty. Genetic differentiation may also have clinical relevance if there are associated differences in phenotypic characteristics that influence virulence or disease manifestation. It is clear that North American and Class 1 isolates are clinically and genotypically different, but no studies have reported clinical differences between North and South American histoplasmosis or between infections caused by other geographically separated populations. Studies of infections in bat species have found a high incidence of infection in bats from the Ohio–Mississippi Valley region, but much lower levels have been reported in Colombian bats, despite the presence of *H. capsulatum* in Colombian bat caves (Tesh *et al.*, 1968; M. L. Taylor *et al.*, 1999). Host differences may be responsible for this difference in infection as different bat species occur on the two continents, but there may be

differences in fungal virulence also. The importance of *H. capsulatum* as a pathogen of humans and other animals, and in particular its severity to immunocompromised hosts, should prompt further genotypic and phenotypic studies of this fungus.

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