

Phylogenetic Relationships of Varieties and Geographical Groups of the Human Pathogenic Fungus *Histoplasma capsulatum* Darling

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The phylogeny of 46 geographically diverse *Histoplasma capsulatum* isolates representing the three varieties *capsulatum*, *duboisii*, and *farciminosum* was evaluated using partial DNA sequences of four protein coding genes. Parsimony and distance analysis of the separate genes were generally congruent and analysis of the combined data identified six clades: (i) class 1 North American *H. capsulatum* var. *capsulatum*, (ii) class 2 North American *H. capsulatum* var. *capsulatum*, (iii) Central American *H. capsulatum* var. *capsulatum*, (iv) South American *H. capsulatum* var. *capsulatum* group A, (v) South American *H. capsulatum* var. *capsulatum* group B, and (vi) *H. capsulatum* var. *duboisii*. Although the clades were generally well supported, the relationships among them were not resolved and the nearest outgroups (*Blastomyces* and *Paracoccidioides*) were too distant to unequivocally root the *H. capsulatum* tree. *H. capsulatum* var. *farciminosum* was found within the South American *H. capsulatum* var. *capsulatum* group A clade. With the exception of the South American *H. capsulatum* var. *capsulatum* group A clade, genetic distances within clades were an order of magnitude lower than those between clades, and each clade was supported by a number of shared derived nucleotide substitutions, leading to the conclusion that each clade was genetically isolated from the others. Under a phylogenetic species concept based on possession of multiple shared derived characters, as well as concordance of four gene genealogies, *H. capsulatum* could be considered to harbor six species instead of three varieties.

The pathogenic ascomycete species *Histoplasma capsulatum* Darling [teleomorph, *Ajellomyces capsulatus* (Kwon-Chung) McGinnis et Katz] occurs throughout the world and causes histoplasmosis in various mammalian species, including humans (46, 61). The fungus grows as a saprobe in nature and is acquired by the inhalation of airborne microconidia or hyphal fragments. Once inhaled, the fungus transforms from a mycelium to a pathogenic yeast form. Histoplasmosis primarily affects the host's lungs, and its symptoms vary greatly. The vast majority of infected people are asymptomatic; however, the fungus can cause disseminated histoplasmosis in otherwise healthy people, and especially in immunocompromised individuals and AIDS patients (46).

Distinct genotypes and varieties of *H. capsulatum* which show different clinical manifestations and geographical distributions are known. Cases of histoplasmosis due to *H. capsulatum* var. *capsulatum* have been reported in at least 60 countries on all continents (1), but they are especially prevalent in the eastern half of the United States and most of Latin America (46). In North America, two prevalent groups (class 1 and class 2) of *H. capsulatum* isolates which showed differences in growth phenotype (53) and restriction fragment length polymorphisms in mitochondrial and genomic DNA have been identified (63, 68). The North American class 1 strains (NAM *Hcc1*; see Table 1 for this and other abbreviations) have been isolated mainly from patients with AIDS, whereas the North American class 2 strains (NAM *Hcc2*) have been isolated from patients both with and without AIDS (64). *H. capsulatum* var. *duboisii* Ciferri (1960) is the causal agent of African histoplasmosis and is endemic in the tropical areas of Africa (61). African histoplasmosis is characterized by the

presence of lesions, primarily in cutaneous, subcutaneous, and osseous tissues, and by the larger size of the yeast cells. *H. capsulatum* var. *farciminosum* (Rivolta) Weeks et al. causes subcutaneous and ulcerated lesions of the skin in horses and mules (46). The disease is widespread throughout Europe, North Africa, India, and South Asia. The morphology of the yeast cell of *H. capsulatum* var. *farciminosum* resembles that of *H. capsulatum* var. *capsulatum* (59).

Despite the clinical importance of the organism, the phylogenetic relationships among the varieties and geographical groups of *H. capsulatum* are presently unresolved. Leclerc et al. (49) and Guého et al. (37) have included representatives of the three *H. capsulatum* varieties in their phylogenetic studies of onygenalean fungi. However, the phylogeny of *H. capsulatum* varieties was not clearly resolved because there was not sufficient variation in the conserved rRNA gene sequences. In this research, we used 46 isolates comprising the three varieties and DNA sequences of four protein coding genes to analyze the evolutionary relationships of *H. capsulatum* varieties. In the process we also examined the mode of reproduction in isolates of one clade of *H. capsulatum* var. *capsulatum*.

MATERIALS AND METHODS

Fungal isolates, culture, and DNA isolation. Table 2 lists the isolates used in the study. H82 and H83 were isolated from a single patient who lived in Panama (9). H87 and H91 were also isolated from a single patient, who was infected at the Guinea-Liberia border (23). Each of the remaining clinical and veterinary isolates was derived from different individuals. Living isolates were manipulated and cultivated under biohazard level 3 containment. Mycelium was grown in liquid medium and heat killed for safety, and DNA was extracted as previously reported (14, 17).

Design of PCR primers. The DNA sequences of four nuclear genes of *H. capsulatum* available from GenBank were used to design PCR primers (Table 3). Internal transcribed spacer (ITS) primers were derived from White et al. (70). The primers (sequences) were as follows (5' to 3'): arf1 (agaatatggggcaaaaagga) and arf2 (cgcaattcatctctgtgag) (ADP-ribosylation factors); H-anti3 (cgcagtcacctccactactate) and H-anti4 (gcccgcagattaacc) (H antigen precursors); ole3 (ttt aaacgaagccccacgg) and ole4 (caccactccaacagcagca) (delta-9 fatty acid desatu-

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TABLE 1. Abbreviations of varieties and geographical groups of *H. capsulatum*

Abbreviation	Variety and population
NAm <i>Hcc1</i>	Class 1 North American <i>H. capsulatum</i> var. <i>capsulatum</i>
NAm <i>Hcc2</i>	Class 2 North American <i>H. capsulatum</i> var. <i>capsulatum</i>
Panama <i>Hcc</i>	Panamanian <i>H. capsulatum</i> var. <i>capsulatum</i>
SAM <i>Hcc</i>	South American <i>H. capsulatum</i> var. <i>capsulatum</i>

rases); tub1 (ggtggccaatcgcaaac) and tub2 (ggcagcttccgttctcagt) (alpha-tubulins); ITS4 (tctcgccttattgatgc) and ITS5 (ggaagtaaaagctgaacaagg) (internal transcribed spacers plus rRNA genes). PCRs were performed with 2 μ l of diluted genomic DNA template in 50- μ l reactions. Reactions consisted of 0.45 μ M of each primer, 1.0 U of AmpliTaq DNA polymerase (Perkin-Elmer), 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and 0.2 mM deoxynucleotide triphosphates with the following temperature profile: a 15-s DNA denaturation step at 94°C, a 30-s annealing step (see below), and a 1-min extension step at 72°C for 32 cycles, followed by a 5-min final extension step at 72°C. The annealing temperature in the first cycle was 65°C. This annealing temperature was subsequently reduced by 0.7°C/cycle for the next 12 cycles, and thereafter, the PCR was continued at an annealing temperature of 56°C for the remaining 20 cycles (Touchdown PCR [24]).

Sequencing. Automated sequencing was done with an ABI dye terminator cycle sequencing ready reaction kit and PCR primers in accordance with the recommendations of the manufacturer (Applied Biosystems Division, Perkin-Elmer, Foster City, Calif.). Sequences were generated from both strands and were edited and initially aligned with the SEQUENCE NAVIGATOR (v1.01; Applied Biosystems) software package, and the alignments were then optimized visually.

Data analysis. Phylogenetic analyses (both parsimony and neighbor joining) were performed by using PAUP* 4.0.0d62, a prerelease version generously provided by D. Swofford, Smithsonian Institute of Natural History. Most-parsimonious (MP) trees were generated by the heuristic search procedure using 1,000 replications of the random addition sequence option. Nucleotide sites were equally weighted, with character state transformations treated as unordered and of equal cost. Insertions and deletions were excluded from the data set. Indices of support (bootstrap values) for internal branches were generated by 500 replications of the bootstrap procedure (29). Neighbor-joining (NJ) trees were generated by using a maximum-likelihood correction for multiple hits with a transition/transversion ratio of 2 (40). Base deletions were treated as missing data. The Kimura two-parameter distance option gave the same topology as the maximum-likelihood option. The maximum sequence divergence value of each locus (Table 3) and the average and maximum sequence divergence values of combined loci (Table 4) correspond to the maximum and average values in the matrices of pairwise mean distances (nucleotide substitutions per nucleotide) of all isolates in Table 2.

RESULTS

Amplification and DNA sequence analysis of nuclear gene loci. Forty-six isolates of *H. capsulatum* representing the three varieties taken from clinical and environmental sources of diverse geographical origin were used in this study (Table 2). DNA sequences of published protein coding genes of *H. capsulatum* were used to design PCR primers. Primers were located in exons to amplify exons as well as flanking introns. The ITS region in the rRNA array was also examined. To select genes with sufficient variation to address questions of *Histoplasma* evolution with statistical confidence, a representative isolate of each variety and geographical group was chosen for testing. Each candidate gene was PCR amplified from the tester isolates. DNA sequences of successfully amplified products were then determined. DNA sequence divergence values are shown in Table 3. Genes for proteins arf (50), H-anti (22), ole (32), and tub1 (39) contained moderate levels (3.0 to 7.0% substitution per nucleotide at maximum) of DNA polymorphisms, which were useful for phylogenetic analysis of *H. capsulatum* varieties. The ITS region in the rRNA gene array is widely used for taxonomic and phylogenetic studies of fungi at subgenus or

subspecies level (8, 70). However, DNA polymorphism in the ITS region was not prevalent enough to resolve the varieties of *H. capsulatum*. The four protein genes were examined further for phylogenetic study by obtaining sequences for each gene from all 46 isolates (Table 2). Combining DNA sequence data for the four loci gave us 1,577 aligned sites, of which 208 were variable (Fig. 1). Introns were more variable than exons in all genes examined (Table 3). The 46 *H. capsulatum* isolates fell into 33 unique multilocus genotypes. Isolates with identical genotypes were found for NAm *Hcc1* (four of five isolates), Panama *Hcc* (all three isolates), and *H. capsulatum* var. *farcimosum* (all three isolates). Among 13 isolates of NAm *Hcc2*, 10 genotypes were found, which were very homogeneous with a maximum diversity of 0.38% nucleotide substitutions per nucleotide (Table 4). Isolate H79, taken from a striped skunk in the 1940s (27), grouped with the four NAm *Hcc1* isolates, from which it differed by a single T-C transition in 1,577 bp of sequence. H79 is therefore the first nonhuman NAm *Hcc1* isolate and predates by 20 years what had been thought to be the initial class 1 isolate, the Downs strain (33). Among the four *H. capsulatum* var. *duboisii* isolates, three genotypes with a maximum divergence of 1.02% were found. The SAM *Hcc* isolates were the most variable. Of 18 isolates examined, 16 unique genotypes were identified, and the maximum sequence divergence among the group is 2.81%.

Phylogenetic analyses of protein coding genes. From 208 variable sites in the aligned 1,577 bp DNA, all gaps, of which there were 36, and all uninformative sites, of which there were 58, were excluded. The total of 114 informative sites were distributed among the protein coding genes as follows: 23 positions in arf, 27 in H-anti, 33 in ole, and 31 in tub1. The data for each gene were used with a parsimony analysis to analyze the phylogenetic relationships of the 33 genotypes (Fig. 2a through d).

Parsimony analysis showed that isolates in the groups NAm *Hcc1*, NAm *Hcc2*, Panama *Hcc*, and *H. capsulatum* var. *duboisii* formed clades with all loci, with the exception of the location of genotypes H81 and H88 (Fig. 2a to d). On the other hand, SAM *Hcc* genotypes did not form such distinct clades. The majority of the Colombian *H. capsulatum* var. *capsulatum* genotypes (H60 to -64, -67, -71, -73, -74, and -76) formed a cluster (SAM *Hcc* group A), and Colombian *H. capsulatum* var. *capsulatum* genotypes H59, H68, and the Argentine genotype H85 formed the other cluster (SAM *Hcc* group B). Surprisingly, the horse pathogen *H. capsulatum* var. *farcimosum* was a member of SAM *Hcc* group A. South American *H. capsulatum* var. *capsulatum* isolates H66, H69, and H140 were distant from the two major SAM *Hcc* groups.

We attempted to root these parsimony trees with the fungal species thought to be the closest relatives of *H. capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* (11, 37). Of the four protein genes, arf and tub1 genes from *B. dermatitidis* and *P. brasiliensis* were successfully amplified with primers designed based on *H. capsulatum* sequences. However, DNA sequences of both *B. dermatitidis* and *P. brasiliensis* appeared to be very distant from *H. capsulatum* (Table 3). We were unable to align the DNA sequences of *B. dermatitidis* and *P. brasiliensis* with those of *H. capsulatum* without significant ambiguity. Therefore, we could not use these sequences to locate the root on the tree of *H. capsulatum* varieties and populations. Nonetheless, it is noteworthy that no matter where the tree is rooted, *H. capsulatum* var. *capsulatum* cannot form a monophyletic group; that is, no clade can be found that contains all *H. cap-*

TABLE 2. List of fungal isolates used in this study

Isolate	Variety/ population	Collection no. ^a		Location	Source	Year isolated	Provider of isolate ^b
		ATCC	Others				
H2	NAm <i>Hcc2</i>		D14	Indiana	Human with AIDS	1990	E. Keath from P. Connoly
H5	NAm <i>Hcc2</i>		D20	Indiana	Human with AIDS	1989	E. Keath from P. Connoly
H6	NAm <i>Hcc2</i>		E14	Indiana	Human	1980	E. Keath from P. Connoly
H8	NAm <i>Hcc2</i>	26032	M. D. Berliner G217B	Louisiana	Human	1973 or before	
H9	NAm <i>Hcc1</i>	38904	Downs	Missouri	Human	1968	E. Keath and G. Kobayashi
H10	NAm <i>Hcc1</i>			Missouri	Human with AIDS	1987	G. Kobayashi
H11	NAm <i>Hcc2</i>		848	Missouri	Human	1993 or before	G. Kobayashi
H18	NAm <i>Hcc2</i>		5-1MD	Missouri	Human	1993 or before	G. Kobayashi
H59	SAm <i>Hcc B</i>		H-0057-I-10	Bogota, Colombia	Human	1994 or before	A. Restrepo and J. McEwen
H60	SAm <i>Hcc A</i>		H-0057-I-11	Bogota, Colombia	Human with AIDS	1994 or before	A. Restrepo and J. McEwen
H61	SAm <i>Hcc A</i>		H-0057-I-14	Bogota, Colombia	Human	1994 or before	A. Restrepo and J. McEwen
H62	SAm <i>Hcc A</i>		H-0057-I-15	Bogota, Colombia	Human	1994 or before	A. Restrepo and J. McEwen
H63	SAm <i>Hcc A</i>		H-0057-I-18	Bogota, Colombia	Human	1994 or before	A. Restrepo and J. McEwen
H64	SAm <i>Hcc A</i>		H-0057-I-22	Bogota, Colombia	Human	1994 or before	A. Restrepo and J. McEwen
H66	SAm <i>Hcc</i>		13594, GH	Medellin, Colombia	Human	1986	A. Restrepo and J. McEwen
H67	SAm <i>Hcc A</i>		30177, JE	Medellin, Colombia	Human	1993	A. Restrepo and J. McEwen
H68	SAm <i>Hcc B</i>		30318, CH	Medellin, Colombia	Human	1993	A. Restrepo and J. McEwen
H69	SAm <i>Hcc</i>		21402, JVM	Medellin, Colombia	Human	1991	A. Restrepo and J. McEwen
H70	SAm <i>Hcc B</i>		30956, WS	Medellin, Colombia	Human with AIDS	1994	A. Restrepo and J. McEwen
H71	SAm <i>Hcc A</i>		21337, JJM	Medellin, Colombia	Human	1989	A. Restrepo and J. McEwen
H73	SAm <i>Hcc A</i>		H-0057-I-24	Bogota, Colombia	Human	1994 or before	A. Restrepo and J. McEwen
H74	SAm <i>Hcc A</i>		26760, GM	Medellin, Colombia	Human	1993	A. Restrepo and J. McEwen
H75	SAm <i>Hcc B</i>		14056, HC	Medellin, Colombia	Human	1986	A. Restrepo and J. McEwen
H76	SAm <i>Hcc A</i>		T29302, GC	Medellin, Colombia	Human	1993	A. Restrepo and J. McEwen
H77	NAm <i>Hcc2</i>	10886	C. W. Emmons 6613	Virginia	Brown rat	1940	
H79 ^c	NAm <i>Hcc1/2</i>	11408	C. W. Emmons 6617	Georgia	Skunk	1940	
H81	Panama <i>Hcc</i>	26028	M. D. Berliner G184B	Panama	Human	1967 or before	
H82	Panama <i>Hcc</i>	26029	M. D. Berliner G186A	Panama	Human	1967 or before	
H83	Panama <i>Hcc</i>	26030	M. D. Berliner G186B	Panama	Human	1967 or before	
H84	NAm <i>Hcc2</i>	26320	C. W. Emmons 6623	Georgia	Opossum	1940	
H85	SAm <i>Hcc B</i>	28308	CDC B923	Argentina	Soil	1966 or before	
H86	NAm <i>Hcc2</i>	32682	A. F. DiSalvo SC74	South Carolina	Soil	1974?	
H87	<i>Hc</i> var. <i>duboisii</i>	24294	D. Grigoriu 8107A	Guinea-Liberia border	Human	1970	
H88 ^d	<i>Hc</i> var. <i>duboisii</i>	32281	RV26821	Belgium	Human	1975 or before	
H90	<i>Hc</i> var. <i>farciminosum</i>	58332	CDC B-3786	Egypt	Horse	1983 or before	
H91	<i>Hc</i> var. <i>duboisii</i>	24295	D. Grigoriu 8123	Guinea-Liberia border	Human	1970	
H95	<i>Hc</i> var. <i>farciminosum</i>	58333	CDC B-3787	Egypt	Horse	1983 or before	
H96	<i>Hc</i> var. <i>farciminosum</i>	60358	A. F. DiSalvo 85-1610	India	Horse	1985?	
H97	NAm <i>Hcc2</i>		0001	Alabama	Human	1995 or before	W. Dismukes, S. Moser, and B. Hines
H126	NAm <i>Hcc1</i>			Missouri	Human with AIDS	1987	G. Kobayashi
H127	NAm <i>Hcc1</i>			Missouri	Human with AIDS	1987	G. Kobayashi
H130	NAm <i>Hcc2</i>		15	Alabama	Human	1995 or before	W. Dismukes, S. Moser, and B. Hines
H137	<i>Hc</i> var. <i>duboisii</i>	28536	Kwon-Chung Hd27	Zaire	Human	1962	
H138	NAm <i>Hcc2</i>	22635	Kwon-Chung T-3-1	Arkansas	Soil	1975	
H139	NAm <i>Hcc2</i>	22636	Kwon-Chung T-4-2	Arkansas	Soil	1975	
H140 ^e	SAm <i>Hcc</i>		MK9500885	Maryland; Peru	Owl monkey	1997	G. Miller
<i>Blastomyces dermatitidis</i>		60915	D. Stevens A				
<i>Paracoccidioides brasiliensis</i>			Pb18				Z. Pires de Camargo and E. Bagagli

^a ATCC, American Type Culture Collection, Manassas, Va.; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; CBS, Centraalbureau voor Schimmelfcultures, Baarn, The Netherlands.

^b E. Keath, St. Louis University, St. Louis, Mo.; P. Connoly, Indiana University Medical Center, Indianapolis, Ind.; G. Kobayashi, Washington University School of Medicine, St. Louis, Mo.; A. Restrepo and J. McEwen, Corporacion para Investigaciones Biologicas, Medellin, Colombia; W. Dismukes, S. Moser, and B. Hines, University of Alabama, Birmingham; G. Miller, National Institutes of Health, Bethesda, Md.; Z. Pires de Camargo, Universidade Federal de Sao Paulo, Sao Paulo, Brazil; E. Bagagli, Universidade Estadual Paulista, Botucatu, Brazil.

^c In this research, H79 was transferred from NAm *Hcc2* to NAm *Hcc1*.

^d Almost all cases of histoplasmosis *duboisii* in Europe are attributable to acquisition in areas in Africa in which this variety is endemic.

^e DNA was directly isolated from a yeast-infested monkey liver. The monkey was caught in the wild in Peru and then kept in Maryland.

sulatum var. *capsulatum* isolates, and only *H. capsulatum* var. *capsulatum* isolates.

We then examined the possibility of combining the data for the four protein coding genes into one phylogenetic analysis to improve the resolution. It is useful in phylogenetic analysis to test the congruence of separate data sets before combining them because incongruence of gene phy-

logenies may indicate problematic data sets (41, 58). However, when multiple individuals of a species are included in a phylogenetic analysis, incongruence among gene genealogies may be expected due to recombination (3). With this caveat in mind, we investigated congruence by two related methods, incongruence length difference (28, 54) and the partition homogeneity test (34, 41).

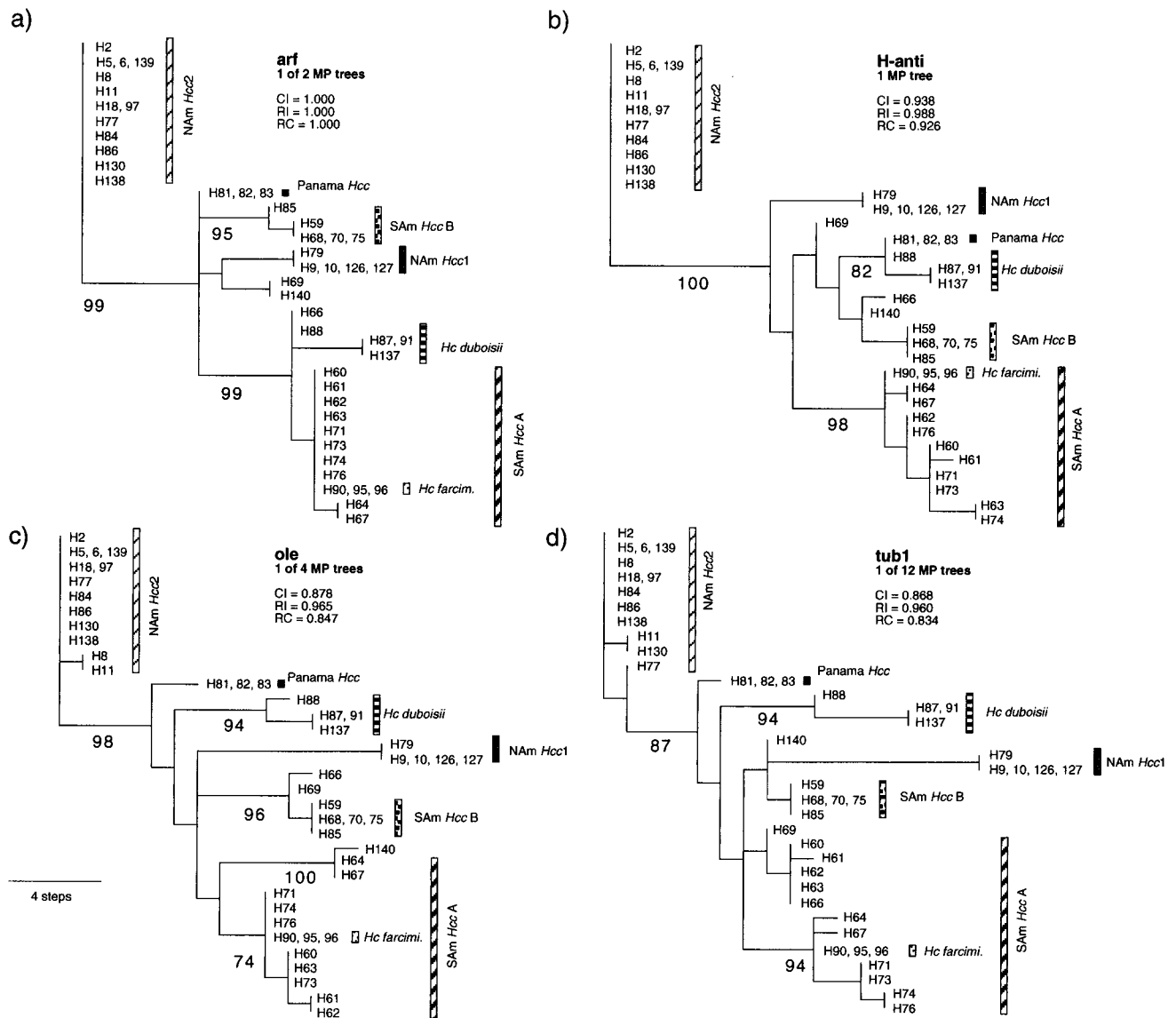


FIG. 2. A single MP tree resulting from analysis of DNA sequences of the 33 multilocus genotypes from each of the four gene regions sequenced. Isolates with an identical genotype are shown together with the representative isolate for each of the 33 genotypes. CI, consistency index; RI, retention index; RC, rescaled consistency index. Numbers below branches represent indices of support based on 500 bootstrap replications of the parsimony procedure. Only values $\geq 70\%$ are shown. Branch lengths are proportional to the numbers of changes in informative characters between nodes (scale at the lower left). SA*m Hcc* A, South American *H. capsulatum* group A; SA*m Hcc* B, South American *H. capsulatum* group B; *Hc farcim.*, *H. capsulatum farciminosum*. Abbreviations of other groups are listed in Table 1.

data set. In the SA*m Hcc* group A clade, some of the internal branches were collapsed, and only two of the branches are supported by bootstrap values above 70%. Therefore, we conclude that the deep and well-supported branches are congruent among the gene trees and lead to the species, whereas the shallow and poorly supported branches are incongruent and lie within the species. Under this interpretation, all isolates are accommodated in the six clades, except H66, H69, and H140, which we provisionally have attached to the South American *H. capsulatum* var. *capsulatum* group B (Fig. 4).

SA*m Hcc* group A has a recombining population structure. The observed poor resolution in the combined gene tree indicates genetic recombination in the SA*m Hcc* group A clade. Let us examine, for example, isolates H60, H71, and H73. In the *arf* and H-*anti* trees, these three isolates share an identical set of parsimony informative sites (Fig. 2). In the *ole* tree, H60

and H73 share identical sites and are one step distant from H71. In contrast, in the *tub1* tree, H71 and H73 are identical and seven steps away from H60. All genotypes in the SA*m Hcc* group A were subjected to parsimony analysis, and the values obtained for L_c , L_i , and I are listed in Table 6, which gives an I value of 6. The large I value is an indication of sexual recombination in the SA*m Hcc* group A population. The partition homogeneity test was also carried out by reconstructing 4,000 randomized data sets (Fig. 5). The observed sum of gene genealogy (tree) lengths of 24 was significantly smaller than those calculated from randomized data (range, 24 to 30; mean and mode, 28; $P < 0.0005$). This feature further corroborates the occurrence of genetic recombination in SA*m Hcc* group A. Note that this result does not address recombination in any of the other clades, where there are too few data (due to too few isolates or too little variation) to obtain a significant result. Of

TABLE 3. Lengths and maximum sequence divergence of the analyzed loci

Locus	Encoding	Length (bp) ^a		Maximum sequence divergence ^b		GenBank accession no.	Reference
		Exons	Introns	Exons	Introns		
arf	ADP-ribosylation factor	280	179	0.0107 (0.0558)	0.0608 (0.4280)	L25112	50
H-anti	H antigen precursor	237	171	0.0253	0.0760	U20346	22
ole	Delta-9 fatty acid desaturase	331	93	0.0423	0.0538	X85962	32
tub1	Alpha-tubulin	52	226	0.0578 (0.1047)	0.0734 (0.5349)	M28356	39
ITS	Internal transcribed spacers + rRNA genes	224	384	0.0045 (0.0134)	0.0584 (0.1620)	U18363	8

^a Lengths vary depending on the number of insertions or deletions. Lengths listed here are based on the isolate H8 (G217B).

^b For arf, H-anti, ole, and tub1, the maximum values of nucleotide substitutions per nucleotide obtained from pairwise comparisons of all isolates in Table 2 were used, whereas those of ITS were from comparison of representative isolates of each group, i.e., H2, H9, H62, H68, H71, H81, H88, and H90. Numbers in parentheses are the maximum distances between *H. capsulatum* and *Blastomyces dermatitidis*.

course, previous research on NAm *Hcc2* using arbitrary loci has supported recombination in this group (17).

DISCUSSION

Speciation in *H. capsulatum*. Phylogenetic analysis revealed that *H. capsulatum* var. *capsulatum* was not monophyletic. In each of the four protein gene trees, there were at least six distinct clades in the *H. capsulatum* isolates we examined: NAm *Hcc1*, NAm *Hcc2*, *H. capsulatum* var. *duboisii*, Panama *Hcc*, SAM *Hcc* group A, and SAM *Hcc* group B. No clear association between geographical groups of *H. capsulatum* var. *capsulatum* was observed. In the combined gene tree, each clade had a deep branch that was supported by a high bootstrap value (97 to 100%). The concordance between the four gene genealogies at the deep branches and their lengths suggests that these six groups have been isolated reproductively from each other for a long time. Hence, each of the six groups corresponds to a phylogenetic species. Phylogenetic species have been defined as the smallest clade grouping populations or lineages diagnosable by a unique combination of character states, in this case, shared, derived nucleotide substitutions (57). However, this definition could be too narrow if the gene upon which the species concept was based proved to be polymorphic in a randomly mating population, because individuals would be grouped by alleles. Our use of four different gene fragments guards against this possibility by using branches that are congruent in the four gene trees to group individuals into species and by recognizing that conflict among gene genealogies would be expected to occur within recombining species (4, 6).

The North American *H. capsulatum* populations. It was found that the genetic distance between the NAm *Hcc1* and NAm *Hcc2* is comparable to or larger than that of any other pair of groups, e.g., NAm *Hcc2* versus *H. capsulatum* var. *duboisii* or NAm *Hcc2* versus *H. capsulatum* var. *farcimosum* (Table 4). The two groups showed no close relationship in any

of the four loci examined. This implies that the NAm *Hcc1* and NAm *Hcc2* are not historically closely related and that there is no genetic exchange between the groups. By comparison with *Coccidioides immitis* (43, 56), it seems certain that NAm *Hcc1* and NAm *Hcc2* have been genetically isolated for at least several million years (20 million years, assuming a substitution rate of third-base positions of $\mu = 10^{-9}$ bp/yr), far older than the date when humans first encountered *H. capsulatum* in the New World (no earlier than 65,000 years ago, and usually estimated to be about 12,000 years before the present) (16, 35, 36). In the laboratory however, H9 (NA *Hcc1*) and H139 (NA *Hcc2*) have been mated, and gymnothecia were observed (45, 48). The gymnothecia contained ascospores, but the ascospores did not look healthy and their viability was not determined (45). Carter et al. (17) showed that clinical isolates of NAm *Hcc2* from Indianapolis formed a recombining population structure. Since NAm *Hcc1* and *Hcc2* have overlapping distributions and are sexually compatible, observation of hybrid genotypes in nature could be expected. However, there is no sign of hybridization in four isolates of NAm *Hcc1* and two isolates of NAm *Hcc2*, all from St. Louis, Mo. It may be that interspecific mating does not occur in nature, or it may be that hybrid progeny are less fit than those from either species alone (12). Examining many more isolates may shed light on this problem.

Genetic diversities within both populations of NAm *Hcc1* and *Hcc2* were much smaller than that of SAM *Hcc* or *H. capsulatum* var. *duboisii*. This implies that each North American group has recently emerged from a small evolutionary bottleneck, in which alleles became fixed or nearly fixed in all loci. Rippon (61) suggested an association between starlings and the spread of *H. capsulatum* in North America. The saprobic phase of *H. capsulatum* lives on guano of bat and bird species. However, not all guano serves equally well as a substrate, and the guano of starlings (*Sturnus vulgaris*) is one of the infested sources of *H. capsulatum*. In North America, the areas in which

TABLE 4. Within- and between-population sequence divergence values from combined data of arf, H-anti, ole, and tub1^a

Population	No. of genotypes/ isolates ^b	Avg (maximum) distance between isolates					
		NA <i>Hcc1</i>	NA <i>Hcc2</i>	Panama	SAM <i>Hcc</i>	<i>Hc farcimosum</i>	<i>Hc duboisii</i>
NA <i>Hcc1</i>	2/5	0.0006 (0.0006)					
NA <i>Hcc2</i>	10/13	0.0317 (0.0332)	0.0016 (0.0038)				
Panama <i>Hcc</i>	1/3	0.0218 (0.0230)	0.0267 (0.0238)	0.0000 (0.0000)			
SAM <i>Hcc</i>	16/18	0.0282 (0.0329)	0.0286 (0.0345)	0.0226 (0.0269)	0.0162 (0.0281)		
<i>Hc farcimosum</i>	1/3	0.0289 (0.0301)	0.0293 (0.0294)	0.0230 (0.0230)	0.0149 (0.0243)	0.0000 (0.0000)	
<i>Hc duboisii</i>	3/4	0.0288 (0.0313)	0.0337 (0.0351)	0.0201 (0.0211)	0.0257 (0.0307)	0.0256 (0.0268)	0.0055 (0.0102)

^a Average (maximum) distances (nucleotide substitutions per nucleotide) between isolates, obtained with PAUP* 4.0.0d62, are listed.

^b Number of unique genotypes and number of isolates in each population.

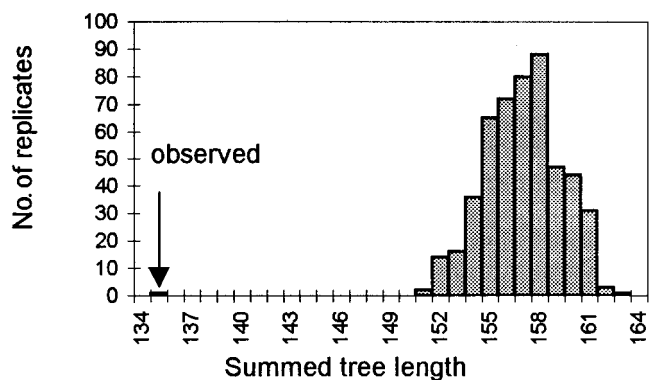


FIG. 3. Partition homogeneity test in the total data set comparing the observed summed tree lengths with the distribution of summed tree lengths calculated for 500 randomized data sets.

H. capsulatum is most highly endemic are also the areas with the greatest concentrations of starlings (61). Starlings were introduced into New York from Europe in 1890 and spread westward (7, 20). The low genetic diversity of *H. capsulatum* in North America might be explained by the recent spread via starlings of *H. capsulatum* already in North America. Of course, the long branches separating North American and South American *H. capsulatum* var. *capsulatum* make it certain that these two groups were genetically isolated long before the introduction of starlings into North America.

The low-virulence race, NAM *Hcc1*, became conspicuous after the AIDS pandemic. So far, NAM *Hcc1* has been isolated only from AIDS patients (64) and an 86-year-old woman (Downs isolate) (33). We have identified one additional strain, obtained from a striped skunk in the 1940s in Georgia, belonging to the NAM *Hcc1*, which suggests NAM *Hcc1* existed in nature long before the AIDS pandemic, as had been suggested by Spitzer et al. (64). The absence of NAM *Hcc1* isolates in collections of clinical isolates taken from patients with disseminated disease prior to the AIDS pandemic (68) suggests that these isolates seldom cause disease in otherwise healthy humans. As with NAM *Hcc2*, the genetic distance from NAM *Hcc1* to isolates in any other clade is large.

***H. capsulatum* var. *duboisii*.** *H. capsulatum* var. *duboisii* is distinct from *H. capsulatum* var. *capsulatum* not only in epidemiology and medical manifestations but also in the morphology of the yeast phase (1). *H. capsulatum* var. *duboisii* was described originally as the species *H. duboisii* (26) but was redefined as a variety because the morphological and immunological differences between the two fungi were insufficient (18, 25). As with *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii* is also associated with bat guano (38). Kwon-Chung (44) demonstrated that the sexual state of *H. capsulatum* var. *duboisii* was identical to that of *H. capsulatum* var. *capsulatum*. Furthermore, she successfully obtained sexual crosses between isolates of *H. capsulatum* var. *duboisii* and the reciprocal mating types of NAM *Hcc2* (H138 and H139). On agar media, the ascospores produced by the intervariety cross failed to germinate, but when the ascospore suspension was injected into mice, *Histoplasma* colonies were subsequently recovered from livers and spleens (47). Thus, *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii* were claimed to belong to a single biological species, in which the two varieties share a specific mate recognition system. However, Kwon-Chung (47) discussed the possibility that the progeny of the cross was an interspecies hybrid. In fact, the progeny isolates were sterile when backcrossed with the parents, suggesting

TABLE 5. Observed and minimum MP tree lengths for each of four loci among all groups used in this study

Locus	Observed MP tree length	Minimum possible MP tree length	Excess steps or homoplasy
arf	24	24	0
H-anti	32	30	2
ole	41	36	5
tub1	38	33	5
All four genes	170 (L_c)	135 (sum of L_i)	35 (I)

that the hybrids had lost their sexual ability. As in other cases, the biological species concept is difficult to apply to allopatric populations (71), which would be the case when crossing NAM *Hcc2* and *H. capsulatum* var. *duboisii*. Unlike sympatric species, allopatric species tend to lack mating suppression mechanisms and therefore are sexually compatible. Uniting taxa because they hybridize can lead to nonhistorical groups, which are problematic in speciation analysis (13, 71). Our data support Vanbreuseghem's view (see reference 47) that *H. capsulatum* var. *duboisii* and *H. capsulatum* should be recognized as two independent species.

The South American *H. capsulatum* populations. Isolates of SAM *Hcc* were extremely rich in polymorphisms, and most isolates fell into two well-supported clades. No significant differences in clinical manifestations of members of either group have been noted (52). The population genetic analysis revealed recombination in the SAM *Hcc* group A population. However, no recombination between the SAM *Hcc* group A and B has yet been identified. As the Panama *Hcc* was very distant from the SAM *Hcc* population, there may be more clades or phylogenetic species existing in Central and South America.

DNA of H140 was isolated directly from the liver of an owl monkey that lived in a research facility in Maryland. The monkey was caught in the wild and was of Peruvian origin. An autopsy revealed massive numbers of budding yeast in the liver, but the yeast was unculturable. Several efforts to identify the yeast have been made by others (55). The morphology of the yeast resembled *H. capsulatum* var. *duboisii* and *Loboa lobo*. An immunohistochemistry assay was conducted, but the results were inconclusive. Our phylogenetic analysis, however, conclusively identified the yeast as *H. capsulatum* and furthermore demonstrated a close relationship of H140 to Colombian *H. capsulatum*. This result was consistent with the Peruvian origin of the monkey and provided evidence that the infection was not acquired in Maryland. Our PCR-based approach does not require a pure culture of the pathogen, but only a small amount of infected tissue, and it demonstrates how the phylogenetic approach can help us to understand epidemiology.

TABLE 6. Observed and minimum MP tree lengths for each of four loci among South American *H. capsulatum* var. *capsulatum* group A

Locus	Observed MP tree length	Minimum possible MP tree length	Excess steps or homoplasy
arf	1	1	0
H-anti	5	5	0
ole	9	9	0
tub1	9	9	0
All four genes	30 (L_c)	24 (sum of L_i)	6 (I)

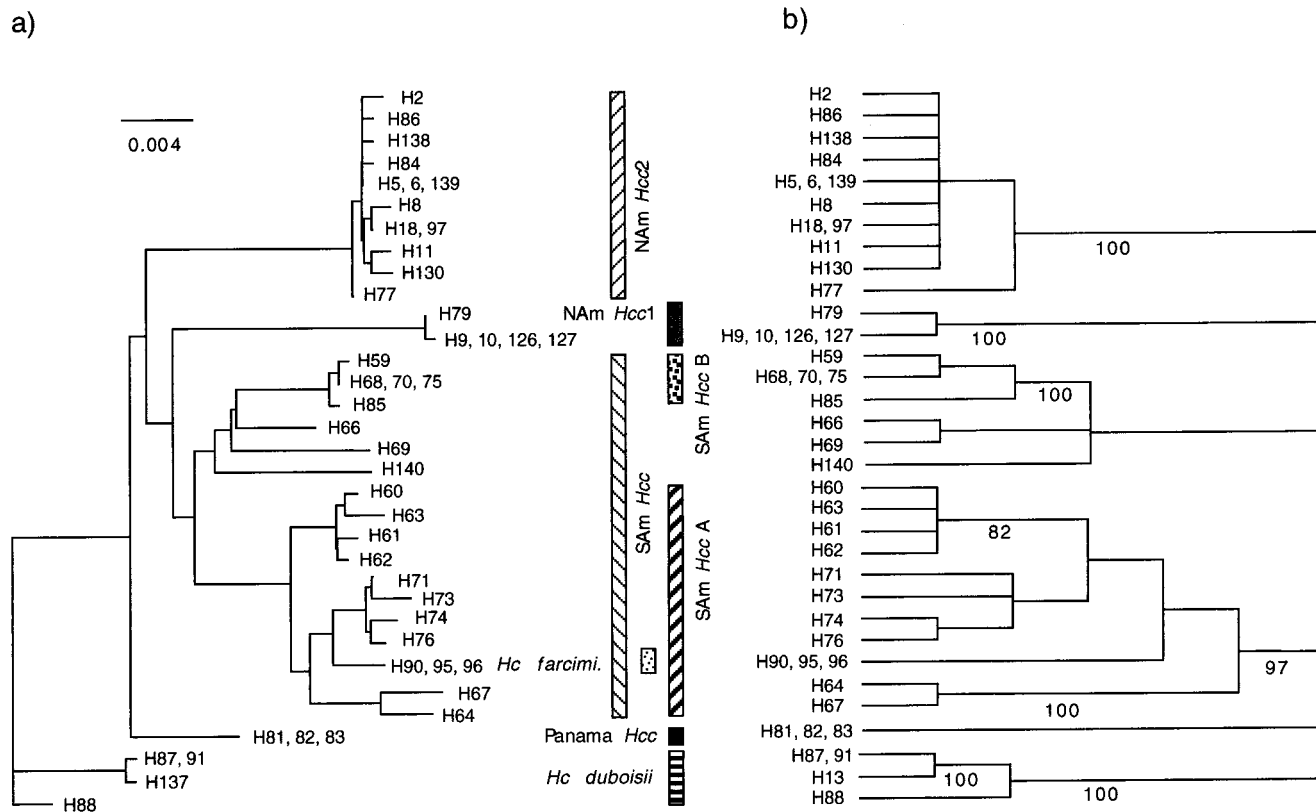


FIG. 4. (a) NJ tree from analysis of combined data of the four loci. Branch lengths are proportional to distance. (b) Strict consensus of 160 MP trees derived from analysis the same data of the four loci. Numbers below branches represent indices of support based on 500 bootstrap replications of the parsimony procedure; only values $\geq 70\%$ are shown. Trees were rooted using *H. capsulatum* var. *duboisii* as the outgroup taxon based on the morphology of pathogenic yeasts.

***H. capsulatum* var. *farciminosum*.** The most striking finding of this study is the phylogenetic position of *H. capsulatum* var. *farciminosum*. *H. capsulatum* var. *farciminosum* is deeply buried in the branch of SAM *Hcc* group A, both in the parsimony and NJ trees, looking as if it were an isolate of South American *H. capsulatum* var. *capsulatum*. *H. capsulatum* var. *farciminosum* is pathologically distinct; it infects horses and other Equidae and generally causes subcutaneous and ulcerated lesions of the skin. Primary infectious sites are thought to be skin injuries from harnesses, and infection through the pulmonary system is rare (46). *H. capsulatum* var. *farciminosum* was formerly described as an independent species but this assessment was changed to a variety of *H. capsulatum* due to the close morphological similarities of the mycelial and yeast forms (69). Antigenically, *H. capsulatum* var. *farciminosum* and *H. capsulatum* var. *capsulatum* are indistinguishable (65). We suggest that *H. capsulatum* var. *farciminosum* is not a separate taxon long adapted to Equidae but represents a recent infection of these animals caused by *H. capsulatum* var. *capsulatum*, presumably acquired in South America, transported to the Old World on an infected animal, and spread in the Old World from animal to animal via skin contact. Several observations pertain to this hypothesis. *H. capsulatum* var. *farciminosum* is prevalent in northern Egypt, where cases of histoplasmosis in humans are rare but do exist. Histoplasmin surveys showed that in Egypt and Israel, positive reaction of the skin test was infrequent or absent (2). However, Ajello et al. (2) successfully isolated *H. capsulatum* from soil samples from a bat-infested cave in Israel. These authors suggested the possibility that in these areas climatic conditions were not suitable for the proliferation and survival of *H. capsulatum* in the soil. The pro-

posed scenario would explain why *H. capsulatum* var. *farciminosum* isolates from Egypt and India are genetically identical at the four gene sequences and more closely related to some South American isolates than to others. It would also explain why animals kept in crowded conditions, such as those at race-horse maintenance facilities, are likely to become infected (31). It would be valuable to challenge this scenario by including clinical and soil isolates from Egypt or Israel in future investigations.

Phylogeny for epidemiological studies. The data presented here document the genetic differentiation of the different geographical and clinical groups of *H. capsulatum*. Each clade has

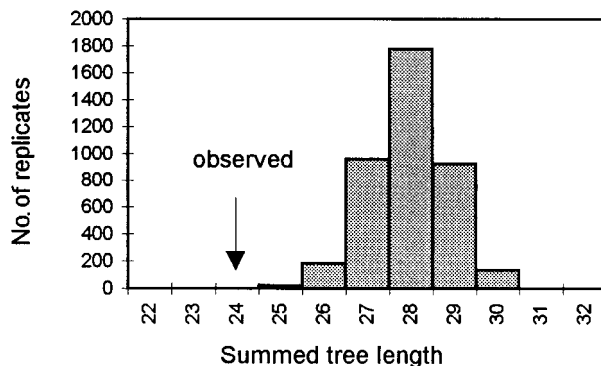


FIG. 5. Partition homogeneity test in the South American *H. capsulatum* var. *capsulatum* group A comparing the observed summed tree lengths with the distribution of summed tree lengths calculated for 4,000 randomized data sets.

many nucleotide positions that are unique shared characters in the language of phylogenetics, or fixed alleles in that of population genetics. This genetic differentiation indicates that the clades are phylogenetic species (5) and have the genetic differentiation expected of biological species (4). With the exception of the clade for *H. capsulatum* var. *duboisii*, members of the different clades do not show morphological differences. However, differences in pathogenicity in the case of members of the NAm *Hcc1* clade indicate that phenotypic differentiation is following the genetic differentiation and we might expect that further differences will be found. The emergence of hosts with different susceptibilities due to the human immunodeficiency virus pandemic and voluntary immunosuppression and to the progressive intensification of international travel and immigration has increased the number of cases of histoplasmosis acquired in areas in which *H. capsulatum* is endemic by hosts not expected to contract this disease (51). From the clinical point of view, it may become increasingly important to identify pathogens to their genetical background and geographic origin. PCR-based phylogenetic analyses based on the genetic variation demonstrated in studies like ours can provide this identification. However, such identification and typing methods are only as good as the sampling of individual fungi and genes used to discover the genetically isolated groups or species (66). Acquisition of additional isolates in poorly sampled geographic locations will improve our ability to identify and type, as well as our knowledge of the world distribution of *H. capsulatum*.

In addition to *H. capsulatum*, several other fungal pathogens, such as *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Coccidioides immitis* have become expanding threats due to immunosuppressive therapy and the AIDS pandemic. Genetic differentiation that correlates with geographic origin as well as allopatric species have been detected among populations of *Coccidioides immitis* (15, 43), *H. capsulatum* (42), and *Cryptococcus neoformans* var. *gattii* (10, 62). Conversely, genetic differentiation without geographic correlation has been seen in *A. fumigatus* (21, 60), *Candida albicans* (19, 67), and *Cryptococcus neoformans* var. *neoformans* (10, 30).

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