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Multicentric Epidemiological Study of *Aspergillus fumigatus* Isolates by Multilocus Enzyme Electrophoresis

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The genotypes of 63 isolates of *Aspergillus fumigatus* obtained from three hospitals in different geographical areas and of eight culture collection strains were determined by multilocus enzyme electrophoresis. Twelve of the 17 enzymatic loci studied were polymorphic, giving rise to 48 different electrophoretic types. The existence of fixed multilocus genotypes, significant heterozygote deficits and excesses at the different loci, and linkage disequilibria within subpopulations strongly suggests a clonal reproduction mode for *A. fumigatus*. Numerical analysis of the comparison and disposition of the different electrophoretic types demonstrates a significant genetic differentiation between the three sampling sites. However, no correlation could be found between geographical distances and genetic differentiation. On account of the multiple discriminatory markers, multilocus enzyme electrophoresis typing seems to be a very powerful tool for epidemiological and reproductive mode studies of *A. fumigatus*.

Infections due to opportunistic fungal pathogens are an important cause of mortality among hospitalized, immunocompromised patients (8). Aspergillosis occurs less frequently than infections due to *Candida* species. However, *Aspergillus fumigatus* is one of the most frequent causes of nosocomial pneumonia, and the mortality from infection by this pathogen is 90% (37, 39). This fungus is commonly isolated from the environment (soil, organic debris, decaying vegetation, and water), from which it releases numerous conidia which can be inhaled by potential hosts (26, 29). This fungus induces a variety of illnesses, including allergic asthma, allergic bronchopulmonary aspergillosis, hypersensitivity pneumonitis, aspergilloma, and invasive aspergillosis.

The frequency and mortality rate of invasive aspergillosis have increased in recent years, in part as a result of the use of intensive chemotherapy and corticotherapy treatments for malignancies and transplantations (3, 30, 34). Simple species identification and colony morphology (morphotype) are not sufficient to confirm the epidemiological relationships between different isolates (19, 29). Isolates of *A. fumigatus* with different characteristics from the same patient could represent either a range of phenotypic variability in a single genotype or multiple strains or genotypes. The usual reason for strain analysis of *A. fumigatus* is to determine whether infections may have a common source or to ascertain whether certain strains are more frequently associated with human infection than others. There exist a number of molecular epidemiological typing methods based on genotypic variability. These include DNA fingerprinting methods, such as arbitrary fragment length polymorphism analysis (10, 11, 21, 22, 25), restriction fragment length polymorphism analysis (1, 4, 9, 21), and moderately repetitive sequence analysis (12). However, protein electrophoretic typing methods, such as immunoblotting (5, 40) and multilocus enzyme electrophoresis (MLEE) (21, 24, 27, 36), have proven to be useful in various studies. In this paper we describe the genetic variability observed at 12 loci of 71 strains of *A. fumigatus* by means of MLEE. The analysis of biodiversity has been used to investigate the population genetic structure and reproductive mode of *A. fumigatus.*

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MATERIALS AND METHODS

Isolates. The European Research Group on Biotype and Genotype of *Aspergillus* collected *A. fumigatus* clinical strains isolated at three European hospital centers. This collection was maintained by the Institute of Hygiene and Epidemiological Mycology of Brussels (IHEM; Brussels, Belgium). Our study was performed on 63 *A. fumigatus* isolates from patients suffering from aspergilloma, invasive aspergillosis, bronchial and bronchitis colonization, and semi-invasive aspergillosis, and it involved clinical, radiological, and mycological investigations of three different hospital centers (center A, Lyon, France; center B, Grenoble, France; center C, Milan, Italy). The identification of isolates from these hospital centers and IHEM was carried out by conventional techniques based on classical morphological identification (32). Eight monosporous reference strains of the IHEM culture collection were also used for the experimental typing investigation (Table 1). The isolates of *A. fumigatus* and the clinical aspects of the corresponding patients are summarized in Table 1. *Candida albicans* and *Saccharomyces cerevisiae* reference strains (Table 1) were used to assess the level of differentiation between *A. fumigatus* and these species. The isolates were cultivated at 42°C on minimal Sabouraud solid medium (glucose, 0.2% [wt/vol]; Bacto Peptone, 0.1% [wt/vol] [Gibco]; KH_2PO_4, 0.1% [wt/vol]; $MgSO_4$, 0.1% [wt/vol]; and select agar, 2% [wt/vol] [Gibco]).

Preparation of culture lysates. *A. fumigatus* isolates were grown on a gyratory shaker at 150 rpm and at 32°C in 500-ml flasks containing 150 ml of minimal Sabouraud liquid medium (Diagnostics Pasteur) (pH 8.0) sterilized by autoclaving. After 4 days of incubation, the mycelial growth was harvested and treated in phosphate-buffered saline (0.05 M sodium phosphate [pH 7.4] containing 1% [wt/vol] NaCl). Then, the mycelium was filtered through a 0.47-μm-pore-size Micronsep membrane filter (Bioblock) and the mycelial fragments were mechanically disrupted for 2 min with glass beads (0.25-mm diameter) in a ScienceTec

TABLE 1. Isolates of *A. fumigatus*

Patient or strain[a]	Isolate	ET	Clinical diagnosis	Sample source	Date of sampling (day/mo/yr)
A1	IHEM 9734	1	Bronchial colonization	Sputum[b]	26/08/92
A1	IHEM 9733	1	Bronchial colonization	Sputum[b]	24/08/92
A2	IHEM 9744	2	Bronchial colonization	Sputum[c]	26/02/93
A2	IHEM 9745	2	Bronchial colonization	Sputum[c]	30/03/93
A3	IHEM 9743	3	Bronchial colonization	Bronchoalveolar lavage[b]	28/09/93
A4	IHEM 9735	4	Bronchial colonization	Bronchial aspirate[b]	30/03/93
A5	IHEM 9778	5	Bronchial colonization	Bronchoalveolar lavage[c]	10/02/94
A6	IHEM 9740	10	Bronchial colonization	Sputum[b]	24/12/93
A7	IHEM 9748	6	Bronchial colonization	Bronchoalveolar lavage[b]	09/11/93
A8	IHEM 9736	7	Bronchial colonization	Pleural liquid[b]	24/11/93
A9a	IHEM 9750	8	Bronchial aspergillosis	Bronchoalveolar lavage[b]	02/06/93
A9b	IHEM 9751	8	Bronchial aspergillosis	Bronchoalveolar lavage[b]	09/06/93
A9c	IHEM 9779	8	Bronchial aspergillosis	Bronchoalveolar lavage[b]	21/06/93
A9d	IHEM 9752	8	Bronchial aspergillosis	Bronchoalveolar lavage[b]	30/06/93
A10a	IHEM 9730	9	Semi-invasive aspergillosis	Bronchoalveolar lavage[b]	11/06/93
A10a	IHEM 9731	9	Semi-invasive aspergillosis	Bronchoalveolar lavage[b]	23/06/93
A10b	IHEM 9732	10	Semi-invasive aspergillosis	Bronchoalvealar lavage[b]	14/12/93
A11	IHEM 9739	11	Invasive aspergillosis	Bronchial aspirate[b]	11/09/93
A11	IHEM 9737	11	Invasive aspergillosis	Bronchial aspirate[b]	17/09/93
A11	IHEM 9738	11	Invasive aspergillosis	Bronchial aspirate[b]	21/09/93
A12	IHEM 9741	11	Invasive aspergillosis	Pus[b]	07/09/93
B1	IHEM 7952	12	Aspergilloma	Surgical removal	22/01/93
B2	IHEM 7953	13	Aspergilloma	Bronchial aspirate	18/01/93
B3	IHEM 7954	14	Aspergilloma	Sputum	08/01/93
B4	IHEM 7955	15	Aspergilloma	Bronchoalveolar lavage	01/12/92
B5	IHEM 7956	16	Aspergilloma	Bronchoalveolar lavage	04/03/93
B6	IHEM 7957	17	Aspergilloma	Surgical removal	02/04/93
B7	IHEM 7958	17	Aspergilloma	Surgical removal	02/04/93
B8	IHEM 7959	18	Aspergilloma	Sputum	29/11/88
B9	IHEM 7961	19	Aspergilloma	Bronchial aspirate	21/06/88
B10	IHEM 7962	20	Aspergilloma	Bronchial aspirate	05/06/89
B11	IHEM 7963	21	Aspergilloma	Sinus drainage	15/03/93
B12	IHEM 7960	22	Bronchial colonization	Bronchial aspirate[b]	20/06/88
B13	IHEM 7964	23	Invasive aspergillosis	Bronchial aspirate[b]	31/12/92
B14	IHEM 7965	24	Invasive aspergillosis	Tracheal aspirate[b]	02/12/92
B15	IHEM 7966	25	Invasive aspergillosis	Vertebral biopsy[b]	11/01/90
B16	IHEM 7967	24	Invasive aspergillosis	Brain biopsy[b]	17/04/92
B17	IHEM 7968	26	Invasive aspergillosis	Tissue biopsy[b]	25/05/88
B18	IHEM 7969	27	Invasive aspergillosis	Sinus drainage[b]	19/05/87
B19	IHEM 7971	28	Invasive aspergillosis	Bronchial aspirate[b]	04/12/90
B20	IHEM 7972	29	Invasive aspergillosis	Sputum[b]	03/10/88
B21	IHEM 7973	30	Invasive aspergillosis	Bronchoalveolar lavage[b]	13/10/93
B22	IHEM 7974	30	Invasive aspergillosis	Bronchial aspirate[b]	26/10/93
B23	IHEM 7970	31	Semi-invasive aspergillosis	Sputum[b]	03/05/88
C1	IHEM 8065	32	Bronchial colonization	Bronchial secretions	?[d]
C2	IHEM 8086	33	Invasive aspergillosis	Skin surgical wound	?
C3a	IHEM 9010	34	Invasive aspergillosis	Abdominal drain	27/01/94
C3a	IHEM 9011	34	Invasive aspergillosis	Abdominal drain	27/01/94
C3a	IHEM 9012	34	Invasive aspergillosis	Abdominal drain	27/01/94
C3a	IHEM 9013	34	Invasive aspergillosis	Abdominal drain	27/01/94
C3a	IHEM 9014	34	Invasive aspergillosis	Abdominal drain	27/01/94
C3b	IHEM 9015	35	Invasive aspergillosis	Hepatic artery	28/01/94
C3b	IHEM 9017	35	Invasive aspergillosis	Hepatic hilum	02/02/94
C3b	IHEM 9018	35	Invasive aspergillosis	Hepatic hilum	02/02/94
C3b	IHEM 9019	35	Invasive aspergillosis	Liver	02/02/94
C3c	IHEM 9020	36	Invasive aspergillosis	Bile	02/02/94
C3d	IHEM 9021	37	Invasive aspergillosis	Lung biopsy	07/02/94
C3e	IHEM 9022	38	Invasive aspergillosis	Hepatic artery biopsy	07/02/94
C3f	IHEM 9023	39	Invasive aspergillosis	Portal vein biopsy	07/02/94
C3g	IHEM 9024	40	Invasive aspergillosis	Choledochojejunum biopsy	07/02/94
C4	IHEM 9487	41	Invasive aspergillosis	Bronchial secretions	20/12/94
C4	IHEM 9488	41	Invasive aspergillosis	Bronchial secretions	29/12/94
	CBS 144-89[e]	42	Invasive aspergillosis		
	CBS 1025-70[e]	43		Pleural liquid	

Continued on following page

TABLE 1—*Continued*

Patient or strain[a]	Isolate	ET	Clinical diagnosis	Sample source	Date of sampling (day/mo/yr)
	CBS 1079-74[e]	43	Meningitis		
C*	IHEM 8070	44		Intensive care unit environment (Italy)	
D	IHEM 8090	45		Aircraft fuel (United Kingdom)	
E	IHEM 8095	46		Dust mattress (Belgium)	
F	IHEM 9000	47		Desert oil (Iraq)	
E	IHEM 9005	48		Seeds of cereal (Belgium)	
S. cerevisiae	CBS 2978			Distillery	?
C. albicans	ATCC 7615			Candidosis	?

[a] A, patients from the Lyon center; B, patients from the Grenoble center; C, patients from the Milan center; C*, monosporous strain from Italy; D, monosporous strain from the United Kingdom; E, monosporous strains from Belgium; F, monosporous strain from Iraq. For patients having two or more ETs, designations have a lowercase letter corresponding to a specific genotype.

[b] Lung transplant.

[c] Heart-lung transplant.

[d] ?, unknown.

[e] CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

MSK homogenizer (B. Braun, San Francisco, Calif.). Cellular debris were removed by centrifugation at 12,000 × *g* for 30 min at 0°C. The supernatant of each strain was lyophylized, resuspended in a minimal volume of distilled water, aliquoted, and stored at −20°C before being used in MLEE.

Enzyme electrophoresis. Starch gel electrophoresis and specific enzyme staining were performed according to published protocols (16, 28, 35). In this study, 19 enzymatic systems were analyzed: peptidase A (PEP A; EC 3.4.11; substrate, Val-Leu), peptidase B (PEP B; EC 3.4.11; substrate, Leu-Gly-Gly), peptidase C (PEP C; EC 3.4.11; substrate, Lys-Leu), peptidase D (PEP D; EC 3.4.11; substrate, Phe-Pro), glucose phosphate isomerase (GPI; EC 5.3.1.9), malate dehydrogenase (MDH; EC 1.1.1.37), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), phosphoglucomutase (PGM; EC 2.7.5.1), lactate dehydrogenase (LDH; EC 1.1.1.27), hexokinase (HK; EC 2.7.1.1), isocitrate dehydrogenase (IDH; EC 1.1.1.42), aconitase (ACON; EC 4.2.1.3), Malic enzyme (ME; EC 1.1.1.40), aspartate aminotransferase (AAT; EC 2.6.1.1), glutamate dehydrogenase (GDH; EC 1.4.1.2), purine nucleoside phosphorylase (NP; EC 2.4.2.1), phosphogluconate dehydrogenase (PGD; EC 1.1.1.43), pyruvate kinase (PK; EC 2.7.1.40), and fructose kinase (FK; EC 2.7.1.4). Alleles were represented in increasing order of anodal mobility. Each isolate was characterized by its allelic combination at the different polymorphic enzymatic loci. Distinctive multilocus variants were designated electrophoretic types (ETs).

Data analysis. (i) Statistical analysis. F statistics are a set of tools devised by Wright to partition heterozygote deficits into within- and between-population components (42, 43). Wright's fixation indices are used by population geneticists to assess the levels of structuring of natural populations. F_{st} measures the genetic differentiation between samples. F_{is} measures the heterozygote deficit within a sample, i.e., it is a measure of deviation from random mating.

The F_{st} and F_{is} indices are given by the equations $F_{st} = \sigma^2(p)/\sigma^2_{max}(p)$ and $F_{is} = 1 - (H_{obs}/H_{exp})$, where $\sigma^2(p)$ is the variance of the allelic frequency among samples, $\sigma^2_{max}(p)$ is the maximum possible value for that variance, and H_{obs} and H_{exp} are the observed and expected heterozygote frequencies, respectively. F_{is} lies in the interval −1 to 1. Negative values indicate heterozygote excess, which generally reflects some presence of selective pressures, while positive values reflect some deviation from panmixia. However, the presence of both deficit and excess of heterozygote from one locus to another and from one sample to another is generally associated with clonality (15, 42, 43). F_{st} lies in the interval 0 to 1. When null, the index reflects genetic homogeneity between the different samples studied. The more F_{st} deviates from zero, the more differentiated the samples are. To estimate these indices, we have used the Weir and Cockerham unbiased estimators f (for F_{is}) and Θ (for F_{st}) (43). The deviation from zero of these indices was tested by using permutation procedures. Absence of heterozygote deficit or excess (i.e., $F_{is} = 0$) was determined by permuting alleles within sites. Population structuring (i.e., the null hypothesis [$F_{st} = 0$]) was tested by permuting individuals between sites. In each case the observed value was compared with that for the corresponding randomly generated distribution. Providing a sufficiently large number of permutations (5,000 in this case) gave an unbiased estimate of the exact probability of type I error (the probability of getting by chance values more extreme than the one observed). This test can be carried out over all loci and all samples. The unbiased estimators F_{is} and F_{st} were calculated with the F-STAT version 1.2 program (13).

Linkage disequilibria (42), expected to be found in a clonal mode of reproduction, were tested by the exact probability test for linkage disequilibrium performed with the GENEPOP-pc version 1.2 program (33). This test computes unbiased estimates, by the Markov chain method, of the exact probabilities of random association for all contingency tables corresponding to all possible pairs of loci in each population. Because multiple tests enhance type I error, the sequential Bonferroni procedure, which involves dividing the desired significance level (i.e., $\alpha = 0.05$) by the number of remaining comparisons (17), is used.

For all these tests, only one representative isolate per patient was used, except for patients whose samples displayed several clearly distinct ETs.

Analysis of relationship between ETs. Measures of Gregorius's genetic distances (14) between some ETs were calculated. This distance is linear, and its distribution is reasonably normal (18). The genetic distance per locus, d_k, is given by the relation

$$d_k = 1/2 \sum_{i=1}^{lk} |Pxi - Pyi|$$

where P_{xi} and P_{yi} represent the allelic frequencies of each allozyme and l_k is the allozyme number at each locus k. Then, Gregorius's genetic distance is given by the equation

$$D_g = 1/r \sum_{k=1}^{r} d_k$$

where r represents the number of loci and d_k represents the genetic distance per locus k. Gregorius (14) and Katz (18) considered this method the most appropriate for analysis of electrophoresis data because it is the only one which satisfies the axioms characteristic of a metric distance. Furthermore, because the distribution of its estimator fits the normal distribution, we were able to statistically test the significance of the distance between pairs of ETs. The computation of D_g was performed with the program DABS-pc version 2.2 (T. de Meeüs, Unité de Recherche Associée 698, Centre National de la Recherche Scientifique, Montpellier, France; J. Goudet, Institute of Zoology, Lausanne University, Dorigny, Switzerland). The significance of the departure from zero of these distances was tested by a t test.

To visualize the relationships between ETs, a dendrogram was constructed by the neighbor-joining method (38) with the TREECON-pc version 2.0 software package (Y. Van de Peer, University of Antwerp, Antwerp, Belgium) (see Fig. 3). The principle of this method is to find pairs of operational taxonomic units (i.e., neighbors) that minimize the total branch length at each stage of clustering of operational taxonomic units, starting with a starlike tree. Unlike other tree-making methods, this method produces a unique final tree by the principle of minimum evolution and gives the correct tree topology by means of computer simulations.

Genetic differentiation between isolates is also studied by discriminatory factorial correspondence analysis (DFCA) with the PRAXIS-pc version 2.0 software package (Praxeme R&D, Biométrie, Centre National de la Recherche Scientifique, Montpellier, France). DFCA is performed with a contingency table (samples versus alleles) in which each isolate is represented by its allelic makeup (6). This method of analysis characterizes each isolate simultaneously according to all the genetic variables (alleles) and shows the contribution of each allele to the overall variability of the samples. The calculation of the variance is based on the chi-square test, which is used as a measure of how a sample distribution deviates from a theoretical distribution and which is inherent to the correspondence analysis (2, 20). The first plane projection of DFCA in the two most informative axes allows us to see each allozyme's relative contribution to the

global explanation of correlations. Outside the differentiation circle we find allozymes which are the most explicative (discriminant allozymes). The projection giving the two most representative linear combinations of correlation between ETs and allozymes is realized. The barycentric center ET means lead us to a better visualization of the geographical discrimination (2, 20).

RESULTS

Isoenzyme analysis. MLEE reproducibility was first addressed by subculturing six isolates (two from each center) 16 times on Sabouraud's medium and performing electrophoresis on samples from every fourth isolate passage (data not shown). No differences in enzyme profiles were seen from the 1st to the 16th passages. Staining for PEP D (Phe-Pro substrate), ACON, and FK activities was unsuccessful. The remaining five loci (LDH, IDH, GDH, AAT, and PGD) were monomorphic. The G6PD locus exhibits two distinctive alleles (Fig. 1A). In the 63 isolates and the eight reference strains examined, 12 loci were polymorphic with two or three alleles yielding 48 different ETs (Table 2). The eight reference monosporous strains consistently exhibited heterogeneous isoenzyme patterns. Four loci (PEP A, PEP B, PEP C, and HK) displayed heterozygous patterns. Figure 1B presents an example of an enzyme system which displays a heterozygous pattern, HK. Overall percentages of heterozygotes were 9.8% for PEP A, 47.9% for PEP B and PEP C, and 36.6% for HK. For the multilocus patterns, 35 were represented by one strain each, 9 were represented by two strains, 2 were represented by four strains, and 2 were represented by five strains. It should be noted that patient 3 at center C, who presented with invasive aspergillosis, exhibited, within 1 week, infections caused by seven different isolates. Most isolates were recovered simultaneously from the privileged hepatic system.

Statistical analysis. (i) Population structuration analysis. The overall genetic differentiation of the three centers was significant (F_{st} = 0.24; probability that F_{st} is not >0, 0.0001). We observed significant values of differentiation between populations studied by pairs: between Lyon and Grenoble, F_{st} = 0.21; between Lyon and Milan, F_{st} = 0.17; between Grenoble and Milan, F_{st} = 0.17. This indicated that no relation between geographical distances and genetic differentiation existed.

Genetic differentiation between ETs was represented by DFCA in order to highlight the geographical structuration of the ETs (Fig. 2). The projection of the different ETs in two informative axes of DFCA was done for all centers and for all alleles compared. This analysis identifies discriminatory allozymes which are outside the unitary significant differentiation circle. By comparison, allozymes which are inside have never been used to highlight any geographical structuration of the ETs. Our results demonstrate by DFCA the existence of geographical discriminatory allozymes (Fig. 2a). For example, three allozymes (ME2, HK2, and PGM2) strongly differentiate center A from the others, PK3 discriminates center B from centers A and C, and NP2 and GPI2 allozymes differentiate center C from centers A and B (Fig. 2a). Patterns of NP, PK, and HK illustrating these discriminatory allozymes are presented in Fig. 1B. By using these discriminatory allozymes, the whole datum of the ETs was redefined. Then, DFCA representation separated ETs into three clear geographical clusters (Fig. 2b).

(ii) Expected and observed frequencies of heterozygotes. Departure from random mating was tested by using the fixation index f (F_{is} estimator) as a statistic. Table 3 displays the values and significance of F_{is}. One may notice the predominance of a total lack of heterozygotes at the multilocus level, leading to a large and highly significant global heterozygote deficit. However, PEP B, PEP C, and HK display significant heterozygote excess (Table 3). Figure 1B shows the phenotypic polymorphism of HK.

(iii) Genetic disequilibrium. Two locus pairs, PEP B-PEP C (Grenoble center) and PEP C-GPI (Milan center), display significant linkage disequilibria at the Bonferroni level: 0.0007 for Grenoble and 0.0078 for Milan.

(iv) Genetic relationships between ETs. With the allelic composition of the ETs for 12 enzyme loci, a neighbor-joining dendrogram was constructed on the basis of all enzyme bands detected (Fig. 3). The isolates were arranged in three clusters, each including two geographical populations (ETs from Lyon and Grenoble centers in cluster I, ETs from Grenoble and Milan centers in cluster II, and ETs from Lyon and Milan centers in cluster III). No subpopulations of *A. fumigatus* can be associated with invasive aspergillosis or with a particular type of aspergillosis as an origin. F-statistics analysis confirms this result (data not shown). However, possible variants from patients at center C can be associated with the same strain. Center C ET repartition in the dendrogram demonstrated that only isolates of ET37 and ET41, and to a lesser extent ET38 and ET39, are related and could be identified as variants of the same strain. Similar results were confirmed by the average Gregorius's genetic distance calculated. A genetic distance of zero between two ETs implies two identical genetic types, whereas a genetic index of one implies two absolutely different genetic types (dissimilarity, 100%). Genetic distance measurements give no significant percent dissimilarity for each pair of ETs compared when P is >0.05. There is only a 4.2% dissimilarity between ET37 and ET41 and 8.3% between ET38 and ET39 ($P > 0.05$). All the other genetic distances measured were significant (at least 17% genetic dissimilarity) for each pair of center C ETs compared ($P < 0.05$). It should be noted that isolates from patient 10 (ET9 and ET10) at center A are also genetically different. Genetic distance measurements confirmed this result, showing 21% genetic dissimilarity ($P < 0.05$), and a similar result is represented in the dendrogram. Nevertheless, the dendrogram representation demonstrated possible variants of the same strain (less than 9% dissimilarity; $P > 0.05$). For example, isolates included in ET42 and ET26 are quite similar, as are isolates included in ET14 and ET30 at center B and in ET8 and ET11 at center A.

DISCUSSION

Our study has focused on the genetic analysis of 63 clinical isolates from three geographical areas and eight reference strains of *A. fumigatus*. The results obtained reveal the extensive genotypic variability of this fungus, as observed in several other studies (21, 24, 36). Rinyu et al. found that 61 strains of *A. fumigatus* could be classified into 19 groups on the basis of the analysis of only seven enzyme systems (36). Matsuda et al. have also demonstrated a high degree of biodiversity in *A. fumigatus* and have clustered 34 strains into 14 ETs by using only three polymorphic enzyme systems (24). In the present study, we found 48 different allelic combinations among 71 isolates by using 12 polymorphic loci. Lin et al. have compared clinical and environmental *A. fumigatus* isolates by three typing methods (21). They concluded that the MLEE typing method had only limited discriminatory power. However, it may be necessary to obtain more polymorphic markers in order to increase the discriminatory power of all typing methods. These authors focused on only a limited number of isoenzymatic markers compared with those used in the present study and in the study of Rinyu et al. (36). In addition, they found only one allele for G6PD in the 35 isolates studied. Matsuda et al. had previously studied, in several *Aspergillus* species, some isoen-

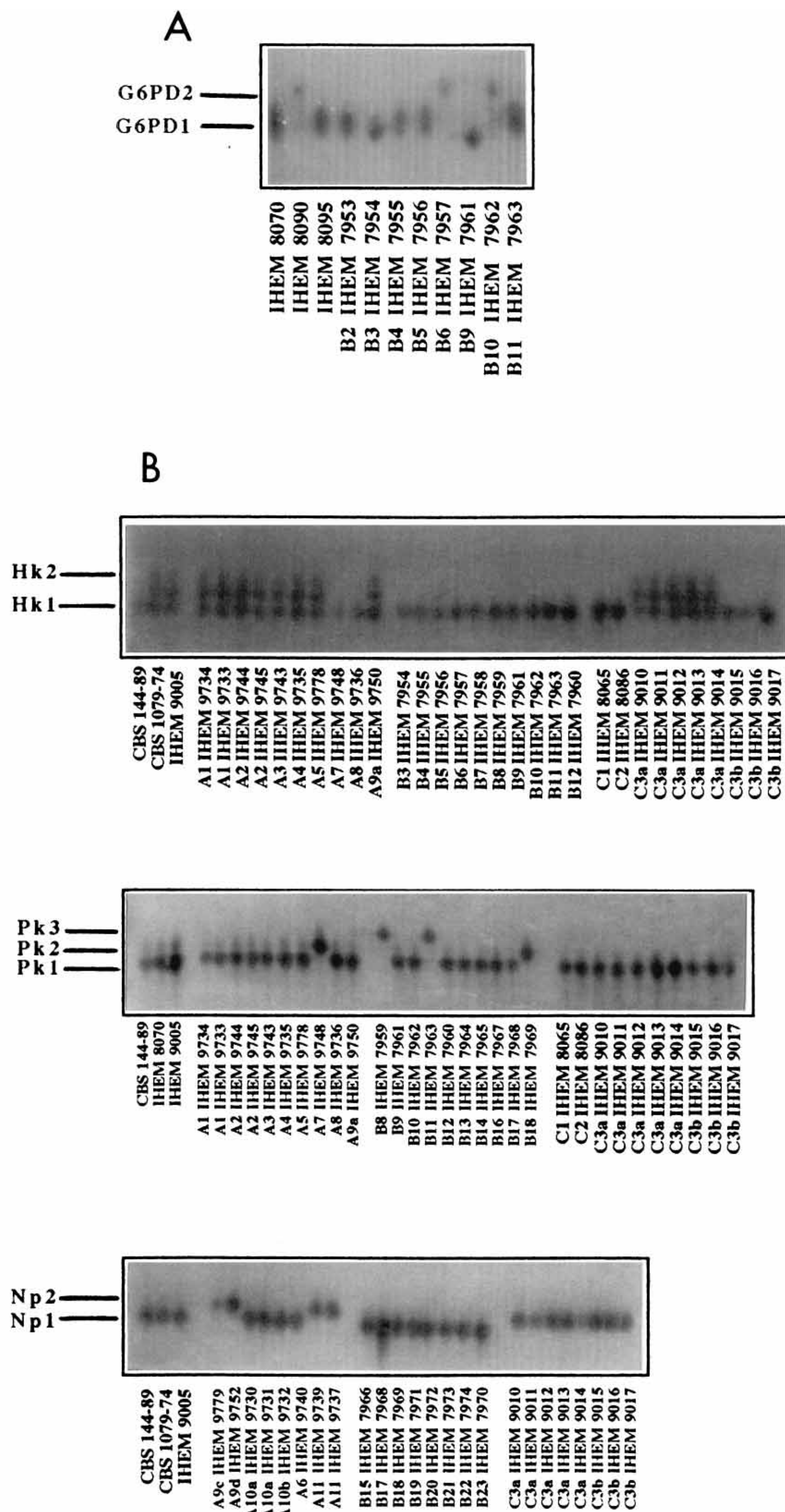

FIG. 1. (A) Polymorphism observed in *A. fumigatus* with G6PD as the enzymatic system. Alleles G6PD1 and G6PD2 are represented by their anodal migration. (B) Representation of three polymorphic enzyme staining systems exhibiting three discriminant alleles differentiating isolates from the three centers studied. Alleles are represented by their anodal migration. The discriminant alleles are Pk3, Np2, and Hk2. Np2 differentiates the Lyon and Milan centers from the Grenoble center. Hk2 differentiates the Lyon center from the Grenoble and Milan centers. Pk3 differentiates the Grenoble center from the Lyon and Milan centers. Designations of isolates and patients are as in Table 1.

TABLE 2. Allelic profiles of 48 ETs of 71 isolates of *A. fumigatus*[a]

Patient[b]	Isolate[c]	ET	Alleles at the indicated enzyme locus[d]											
			PEP A	PEP B	PEP C	GPI	MDH1	MDH2	G6PD	PGM	HK	ME	NP	PK
A1	IHEM 9734	1	2	1/2	1/2	1	1	1	1	2	1/2	2	1	1
A1	IHEM 9733	1	2	1/2	1/2	1	1	1	1	2	1/2	2	1	1
A2	IHEM 9744	2	1/2	1/2	1/2	1	1	1	1	2	1/2	1	1	1
A2	IHEM 9745	2	1/2	1/2	1/2	1	1	1	1	2	1/2	1	1	1
A3	IHEM 9743	3	2	1/2	2	1	1	1	1	2	1/2	1	1	1
A4	IHEM 9735	4	1	1/2	1/2	1	1	2	1	2	1/2	1	1	1
A5	IHEM 9778	5	1	1	2	1	1	1	1	2	1/2	1	1	1
A7	IHEM 9748	6	1	1	1	2	1	2	1	2	1	2	2	2
A8	IHEM 9736	7	2	1	1	1	1	1	1	1	1	2	2	1
A9a	IHEM 9750	8	1	1	1/2	2	1	2	1	2	1/2	1	2	1
A9b	IHEM 9751	8	1	1	1/2	2	1	2	1	2	1/2	1	2	1
A9c	IHEM 9779	8	1	1	1/2	2	1	2	1	2	1/2	1	2	1
A9d	IHEM 9752	8	1	1	1/2	2	1	2	1	2	1/2	1	2	1
A10a	IHEM 9730	9	2	1	1/2	1	1	1	1	2	1	1	1	1
A10a	IHEM 9731	9	2	1	1/2	1	1	1	1	2	1	1	1	1
A10b	IHEM 9732	10	2	1	2	1	1	2	1	2	1/2	1	1	1
A6	IHEM 9740	9	2	1	2	1	1	2	1	2	1/2	1	1	1
A11	IHEM 9739	11	1	1	1/2	2	2	2	1	2	1/2	1	2	1
A11	IHEM 9737	11	1	1	1/2	2	2	2	1	2	1/2	1	2	1
A11	IHEM 9738	11	1	1	1/2	2	2	2	1	2	1/2	1	2	1
A12	IHEM 9741	11	1	1	1/2	2	2	2	1	2	1/2	1	2	1
B1	IHEM 7952	12	2	1	1/2	1	1	1	2	1	1	1	1	1
B2	IHEM 7953	13	2	1/2	1/2	1	1	1	1	1	1	1	1	1
B3	IHEM 7954	14	1/2	1/2	1/2	1	1	2	1	1	1	1	1	1
B4	IHEM 7955	15	1/2	1	1	1	1	1	1	1	1	1	1	1
B5	IHEM 7956	16	1/2	2	2	1	1	1	1	1	1	1	1	1
B6	IHEM 7957	17	2	2	2	1	2	2	2	1	1	1	1	1
B7	IHEM 7958	17	2	2	2	1	2	2	2	1	1	1	1	1
B8	IHEM 7959	18	2	1/2	2	1	1	1	1	1	1	1	1	3
B9	IHEM 7961	19	1	1/2	2	1	1	2	1	1	1	1	1	1
B10	IHEM 7962	20	2	1/2	2	1	1	2	2	1	1	1	1	1
B11	IHEM 7963	21	1	2	2	1	1	1	1	1	1	1	1	3
B12	IHEM 7960	22	1	1/2	2	1	1	1	2	1	1	1	1	1
B13	IHEM 7964	23	1	1	1/2	1	1	1	2	2	1	1	1	1
B14	IHEM 7965	24	2	1/2	1/2	1	1	1	1	2	1	1	1	1
B16	IHEM 7967	24	2	1/2	1/2	1	1	1	1	2	1	1	1	1
B15	IHEM 7966	25	1	1/2	1/2	1	1	1	1	1	1	1	1	2
B17	IHEM 7968	26	1	1/2	1/2	1	1	1	1	1	1	1	1	1
B18	IHEM 7969	27	1	2	2	1	1	2	1	1	1	1	1	2
B19	IHEM 7971	28	1	1	1	1	2	2	1	1	1	1	1	1
B20	IHEM 7972	29	2	1	1	1	1	2	1	1	1	1	1	1
B21	IHEM 7973	30	2	1/2	1/2	1	1	2	1	1	1	1	1	1
B22	IHEM 7974	30	2	1/2	1/2	1	1	2	1	1	1	1	1	1
B23	IHEM 7970	31	2	1/2	1/2	1	1	1	1	2	1	1	1	3
C1	IHEM 8065	32	2	1/2	1	2	1	1	1	1	1	1	1	1
C2	IHEM 8086	33	1	2	2	2	1	1	1	1	1	1	1	1
C3a	IHEM 9010	34	1	1/2	1	2	1	2	1	1	1/2	1	2	1
C3a	IHEM 9011	34	1	1/2	1	2	1	2	1	1	1/2	1	2	1
C3a	IHEM 9012	34	1	1/2	1	2	1	2	1	1	1/2	1	2	1
C3a	IHEM 9013	34	1	1/2	1	2	1	2	1	1	1/2	1	2	1
C3a	IHEM 9014	34	1	1/2	1	2	1	2	1	1	1/2	1	2	1
C3b	IHEM 9015	35	2	1	2	2	2	2	1	1	1	1	2	1
C3b	IHEM 9016	35	2	1	2	2	2	2	1	1	1	1	2	1
C3b	IHEM 9017	35	2	1	2	2	2	2	1	1	1	1	2	1
C3b	IHEM 9018	35	2	1	2	2	2	2	1	1	1	1	2	1
C3b	IHEM 9019	35	2	1	2	2	2	2	1	1	1	1	2	1
C3c	IHEM 9020	36	1	1	2	2	1	1	1	1	1	1	2	1
C3d	IHEM 9021	37	2	1	1/2	1	2	2	1	1	1	1	1	1
C3e	IHEM 9022	38	1	1/2	1/2	1	1	2	1	1	1	1	2	1
C3f	IHEM 9023	39	2	1/2	1/2	1	1	2	1	1	1	1	2	1
C3g	IHEM 9024	40	1	1/2	1/2	1	2	2	1	1	1/2	1	2	1
C4	IHEM 9487	41	2	1/2	1/2	1	2	2	1	1	1	1	1	1
C4	IHEM 9488	41	2	1/2	1/2	1	2	2	1	1	1	1	1	1
	CBS 144-89	42	1	1/2	1/2	1	1	1	1	1	1	1	1	1

Continued on following page

TABLE 2—*Continued*

Patient[b]	Isolate[c]	ET	Alleles at the indicated enzyme locus[d]											
			PEP A	PEP B	PEP C	GPI	MDH1	MDH2	G6PD	PGM	HK	ME	NP	PK
	CBS 1079-74	43	2	1	1	1	1	1	1	2	1/2	1	1	1
	CBS 1025-70	43	2	1	1	1	1	1	1	2	1/2	1	1	1
C*	IHEM 8070	44	1	2	1	1	2	2	1	2	1	1	1	1
D	IHEM 8090	45	1/2	1/2	1	2	1	1	2	1	1	2	1	2
E	IHEM 8095	46	1/2	1	1/2	1	1	2	1	1	2	1	1	2
F	IHEM 9000	47	1	1/2	1	1	1	1	1	1	1	2	2	1
E	IHEM 9005	48	2	1/2	2	1	1	2	2	2	1/2	1	1	1
S. cerevisiae	CBS 2978		5	4	3	4	5	6	4	4	5	3	4	5
C. albicans	ATCC 7615		3	3	6	4	4	5	3	3	4	4	3	6

[a] Results of four independent experiments for each enzymatic system.
[b] Patient designations are as in Table 1.
[c] Strain designations are as in Table 1.
[d] 1/2, alleles 1 and 2 presented by heterozygote.

zyme markers of *A. fumigatus* strains (24). They demonstrated that G6PD and GDH were key enzyme markers for differentiating 31 clinical and nonclinical isolates of *A. fumigatus* from the other *Aspergillus* species. However, G6PD appeared to be polymorphic in our samples. Rinyu et al. demonstrated that polymorphisms for 61 *A. fumigatus* strains were observed in all isoenzymes studied except for LDH, superoxide dismutase (SOD), and GDH markers (36). Among the 17 enzymatic loci in this study, we found G6PD to be polymorphic and LDH, IDH, GDH, AAT, and PGD to be monomorphic for the 71 clinical and nonclinical *A. fumigatus* isolates.

Despite the lack of clinical environmental isolates of *A. fumigatus*, clinical isolates of different patients presenting the same ET seem to be epidemiologically related. This could be due to clinical conditions, which are often the result of a community-acquired infection. For example, *A. fumigatus* isolate variants could be assigned to the same strain at center A (patients A9, A11, and A12) and at center B (patients B3, B21, and B22). Isolates from patient 3 at center C provided the great majority of isolates from this center. This could constitute a limitation to our epidemiological study because the sampling size from this center, excluding patient 3, is very small. However, these isolates were included in the study because multiple infection episodes contributed to the variability analysis of *A. fumigatus*. Genetic distance calculations for each pair of ETs as well as dendrogram representations confirm the preexistence of these distinct strains. Because we have assumed that ET38 and ET39 were variants of the same strain, we conclude that a total of six different strains preexisted. This result can be related to a previous study of patients with invasive aspergillosis (9). Denning et al. showed that two isolates from each of the two patients studied were different strains on the basis of the restriction fragment length polymorphism pattern they displayed (9). Moreover, Burnie et al. (4) analyzed strains from multiple isolates from six aspergilloma patients. Analysis of restriction fragment length polymorphisms revealed three genotypes among six isolates recovered from one patient. The same genotype was found in four other patients. Thus, it is clear that more patients should be surveyed in order to confirm the uniqueness of the infective strains in patients with aspergillosis.

TABLE 3. Observed heterozygote deficits (F_{is}) and significance levels

Enzyme locus	F_{is} (significance [P])[a] at:		
	Center A	Center B	Center C
PEP A	0.81 (0.004)[s]	0.74 (0.002)[s]	1 (0.002)[s]
PEP B	−0.14 (0.001)[s]	−0.11 (0.821)[ns]	−0.18 (0.002)[s]
PEP C	−0.16 (0.017)[s]	−0.01 (0.666)[ns]	0.38 (0.040)[ns]
GPI	0.85 (0.002)[s]	NP[b]	1 (0.001)[s]
MDH1	1 (0.009)[s]	1 (0.005)[s]	1 (0.009)[s]
MDH2	1 (0.011)[s]	1 (0.014)[s]	1 (0.029)[s]
G6PD	1 (0.007)[s]	1 (0.010)[s]	NP
PGM	1 (0.007)[s]	1 (0.012)[s]	NP
HK	−0.34 (0.001)[s]	NP	−0.06 (0.002)[ns]
ME	0.83 (0.012)[s]	NP	NP
NP	1 (0.003)[s]	NP	1 (0.006)[s]
PK	1 (0.005)[s]	1 (0.010)[s]	NP
All loci (mean)	0.63 (0.005)[s]	0.69 (0.013)[s]	0.66 (0.003)[s]

[a] s, significant; ns, not significant.
[b] NP, not possible to test (no polymorphism observed at this locus).

MLEE is among the numerous typing methods developed to differentiate *A. fumigatus* isolates. This method not only distinguishes between *A. fumigatus* isolates with a relatively high discriminatory power but also allows assessment of the structure of the population studied, including its genetic diversity and its mode of reproduction. It was therefore used as the main technique for the present study.

Our results demonstrate significant genetic differentiation between the three sampling sites. Both significant discriminatory allozymes and significant F_{st} estimators of differentiation between populations studied by pairs demonstrated this geographical population differentiation. The differentiation, however, cannot be related to geographical distances because there is more genetic differentiation between the two cities nearest each other (Lyon and Grenoble) than between the two more distant pairs of cities (Lyon and Milan; Grenoble and Milan). The *A. fumigatus* isolates showed homogeneity (at least 65% similarity). This result is in accordance with earlier studies (24, 36) in which 75% similarity was detected. The difference between our results and those described in the past could be explained by the dendrogram method used as well as by the more numerous polymorphic markers we used in comparison with those used in previous studies. The neighbor-joining dendrogram showed a definite grouping into three clusters, each of which included isolates from two centers (I, Lyon and Grenoble; II, Milan and Grenoble; III, Lyon and Milan). This result could be related in part to F-statistics values. However, the genetic heterogeneity observed in each cluster and in each geographical population probably results from the aerial propagation mode of conidia of *A. fumigatus*. Nevertheless, DFCA,

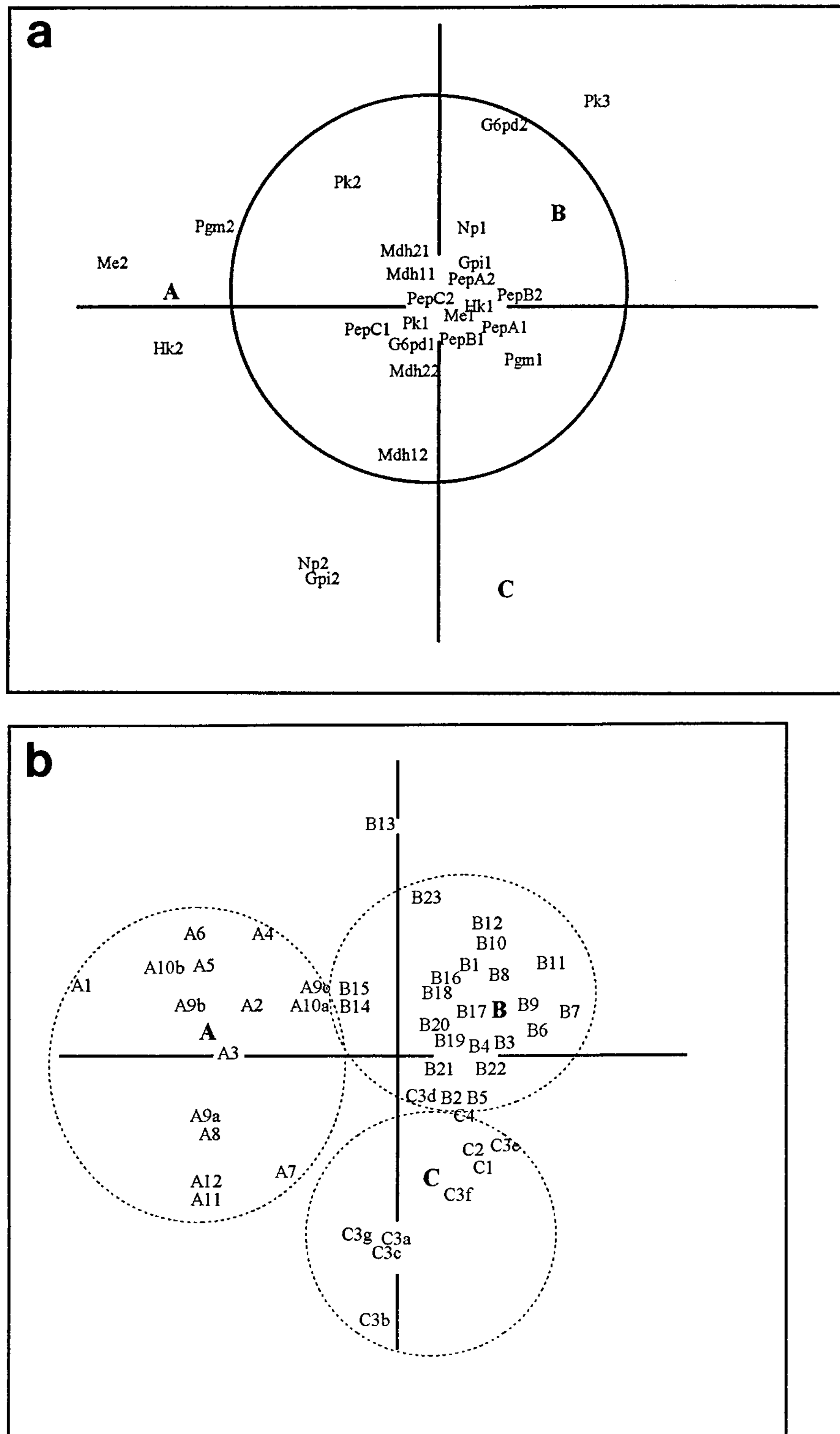

FIG. 2. (a) First plane projection of DFCA in two informative axes of all alleles observed. A, Lyon center; B, Grenoble center; C, Milan center. Differentiation circle divides allozymes that are not significant in the differentiation of the three populations studied (inside) from those that are significant discriminatory allozymes (outside). The alleles with a frequency of <1% for all individuals studied did not contribute to the formation of the factorial axes (2, 20). (b) Projection of all ETs by using significant discriminatory allozymes. The projection of centers of gravity of each ET population (2, 6, 20) is shown. ETs are numbered A1 to A12 for the Lyon center, B1 to B23 for the Grenoble center, and C1 to C4 for the Milan center. The significant discriminatory allozymes used are those shown in panel a. Each circle clusters a population studied.

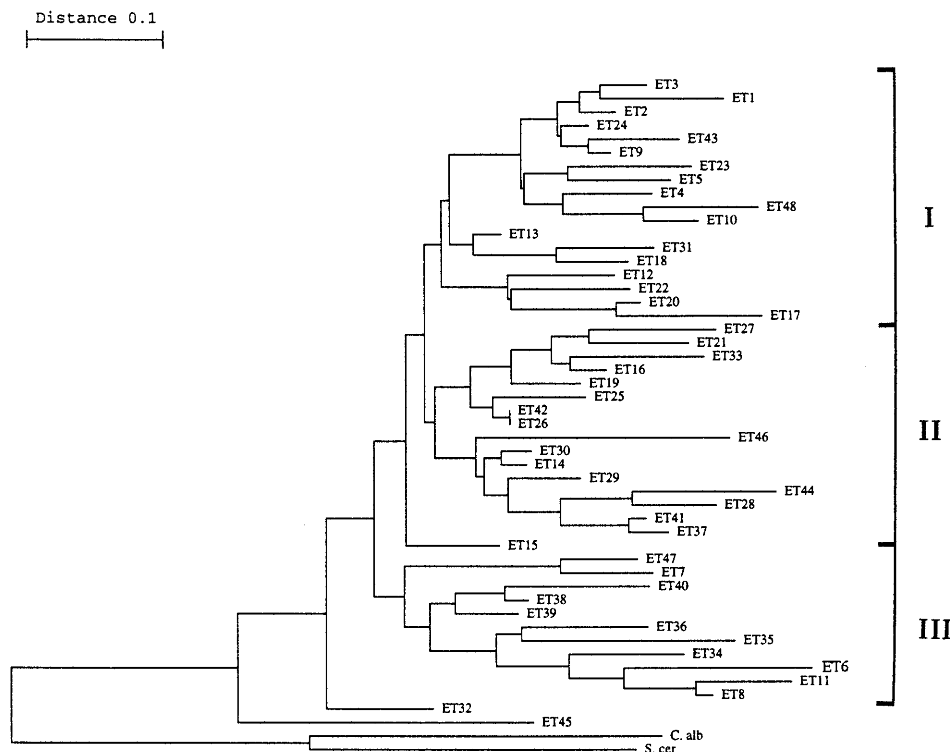

FIG. 3. Genetic relationships among 48 ETs of *A. fumigatus*. The dendrogram was realized by the neighbor-joining method (see Materials and Methods). ET designations are as in Table 1. I through III, subpopulation numbers; C. alb, *C. albicans*; S. cer, *S. cerevisiae*.

F statistics, in part dendrogram analysis, and the predominance of linkage disequilibria demonstrate a geographical genetic structuration and a genetic heterogeneity within each sampling site.

The reproductive mode of *A. fumigatus* is unclear (7, 41), and the clonal propagation of *A. fumigatus* has never been investigated in the past. Our results demonstrate the existence of significant heterozygote deficits and excesses between samples. The fixation indices (F_{is}) obtained in the present work are consistent with the lack of sexual reproduction observed in *A. fumigatus*. On the one hand, if sexual reproduction (and therefore recombination) occurs, then the different loci should recombine independently at each reproduction, unless they are located on a small segment of the same chromosome (which is unlikely). On the other hand, if there is no recombination, then the different loci should be in strong disequilibrium. Our results fully demonstrate the existence of significant linkage disequilibria between some pairs of loci. Finally, the existence of significant heterozygote deficit or excess between samples, coupled with the existence of significant linkage disequilibria between some pairs of loci, strongly suggests a predominantly clonal mode of reproduction for *A. fumigatus*, as has been previously suggested (9). However, it is possible that, during the growth of diploid individuals of *Aspergillus* species, homokaryotic diploid segregants may arise as a result of mitotic crossover. The ultimate effect of this is the creation of a possible variety of haploid strains displaying the characters of the mutant parents recombined in all possible ways (31, 32).

Our results demonstrate geographical structuration and genetic heterogeneity within each sampling site. This result could be related to a previous study (9). Denning et al. demonstrated widespread and localized distribution of *A. fumigatus* clones, with most isolates examined being heterogeneous (9). Such heterogeneity could be due to the predominance of several differentiated strains or taxa within each sampled area. Extensive genotypic biodiversity for *A. fumigatus* could be the result of such predominance operating over time. Aerial conidial propagation as well as genetic events could explain in part the origin of the high genotypic biodiversity observed for *A. fumigatus*. One difference between two strains could be the result of a genetic event, such as nucleic acid mutation, deletion, or insertion. This could also result in genotypic and phenotypic modifications (lack of expression of some alleles by genetic repression or by the allelic degradation process). As a consequence, all of these events could modify only one global protein electrophoretic charge and thus account for the differences observed. However, high clonal diversity in natural populations could be the result of these repeated genetic events, possibly with sporadic chromosomal alterations. This could then account in part for the extensive biodiversity observed.

Although *A. fumigatus* spreads by aerial conidial propagation, we demonstrated significant differentiation of each sampling site. However, the lack of correlation between genetic and geographic distances suggests the existence of selective pressures (23). These selective pressures could include host environmental adaptation and immunosuppressive treatment or other therapeutic influences. Much work remains then to describe the ecology of *A. fumigatus*, both in relation to the human environment and in nature. In this regard, our study focused on human clinical isolates. Therefore, conclusions from our results can apply only to *A. fumigatus* isolates capable of causing human colonization or infection. Thus, it seems very important to investigate other populations of *A. fumigatus* derived from various areas in order to ascertain whether the clonal hypothesis is generalizable to the whole *A. fumigatus*

taxon. This should also contribute to a better understanding of the relationship between genetic differentiation and geographical distances. Further studies using clinical and nonclinical isolates of *A. fumigatus* from other geographical areas are critically needed to confirm more accurately its biosystematics and population genetics.

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