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Genotyping of *Madurella mycetomatis* by Selective Amplification of Restriction Fragments (Amplified Fragment Length Polymorphism) and Subtype Correlation with Geographical Origin and Lesion Size

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One of the causative organisms of mycetoma is the fungus *Madurella mycetomatis*. Previously, extensive molecular typing studies identified Sudanese isolates of this fungus as clonal, but polymorphic genetic markers have not yet been identified. Here, we report on the selective amplification of restriction fragment (AFLP) analysis of 37 Sudanese clinical isolates of *M. mycetomatis*. Of 93 AFLP fragments generated, 25 were polymorphic, and 12 of these 25 polymorphic fragments were found in a large fraction of the strains. Comparative analysis resulted into a tree, composed of two main (clusters I and II) and one minor cluster (cluster III). Seventy-five percent of the strains found in cluster I originated from central Sudan, while the origin of the strains in cluster II was more heterogeneous. Furthermore, the strains found in cluster I were generally obtained from lesions larger than those from which the strains found in cluster II were obtained (chi-square test for trend, $P = 0.03$). Among the 12 more commonly found polymorphisms, 4 showed sequence homology with known genes. Marker A7 was homologous to an endo-1,4-beta-glucanase from *Aspergillus oryzae*, 97% identical markers A12 and B3 matched a hypothetical protein from *Gibberella zeae*, and marker B4 was homologous to casein kinase I from *Danio rerio*. The last marker seemed to be associated with strains originating from central Sudan ($P = 0.001$). This is the first report on a genotypic study where genetic markers which may be used to study pathogenicity in *M. mycetomatis* were obtained.

In the 1840s, physicians of the Royal Army stationed in the Madura region in India reported an invasive disease which severely affected the foot. The foot degenerated into "one mass of disease of a fibrocartilaginous nature, with entire destruction of the joints, cartilages and ligaments." This disease is now known as mycetoma and can be differentiated into advanced cases and moderate to minor cases, with 80% of cases affecting the dorsal part of the foot (9, 17). However, it is also possible for other areas to become infected, including the hand, knee, arm, legs, head, and neck (9) Since the first reports on mycetoma, it appeared that mycetoma is not restricted to India but has a worldwide distribution (9). It is endemic around the Tropic of Cancer, between latitudes 15°S and 30°N (4). In these regions the climate is relatively arid and hot, with alternating short rainy seasons and longer dry seasons (15). The countries where mycetoma has been reported include Argentina, Colombia, Venezuela, Yemen, Tunisia, Senegal, Somalia, and Sudan (7–9). Sudan appears to have the highest number of cases per capita per year, which amounts to about 300 to 400 actual infections (17).

Mycetoma can be caused by a great variety of microorganisms, but these are not evenly distributed throughout the "mycetoma belt" (17). In Sudan the most frequently encountered causative organism is *Madurella mycetomatis. M. mycetomatis* is a slowly growing fungus which forms a dark, sterile mycelium (4). Only two reports on genetic variability or the lack thereof in *M. mycetomatis* isolates have appeared. In a report published by Lopes et al., it was shown that random amplification of polymorphic DNA (RAPD) and restriction endonuclease assays differentiated *M. mycetomatis* strains from different countries (14). In contrast, a large set of clinical *M. mycetomatis* isolates obtained from Sudan showed little genetic variation based upon classical high-throughput RAPD tests and PCR-restriction fragment length polymorphism analysis tests, and the species was identified as a clonal organism (2). Neither of these studies presented genetic markers which could be used to generate epidemiologically relevant information or taxonomic frameworks. Another microbial DNA-based typing method is the amplification of restriction fragments (AFLP) technique, a selective restriction fragment amplification method which establishes the absence or presence of DNA restriction sites by means of selective PCR (19, 21). Genomic DNA is completely

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TABLE 1. *Madurella mycetomatis* polymorphisms detected by AFLP and the PCR primers under condititions designed for detection of each individual polymorphism

Fragment	GenBank accession no.	Sequence length (bp)	% Isolates in which fragment is present	Homology	Primer	Sequence (5′ → 3′)	Annealing temp (°C)
A3	—[a]	—	46.1	—	— —	— —	—
A4	AY918173	65	61.5	No homology	A4 fw A4 rv	CCT TCA TCG TGC CAC AGG CG CGC TGA GTA AAC GAG TTT TC	52
A5	AY918174	223	53.9	No homology	A5 fw A5 rv	TCA CAC TTA TCG AGG CAG AT GTA TGT CCA CGC AGG GCA TG	55
A6	—	—	51.3	—	— —	— —	—
A7	AY918175	161	15.4	Endo-1,4-beta-glucanase	A7 fw A7 rv	ATG AGC TGG CTT GAT GGC GG TCC TGA GTA AAC GTC GAT CC	55
A8	—	—	35.9	—	— —	— —	—
A9	—	—	51.3	—	— —	— —	—
A10	AY918172	180	69.2	No homology	A10 fw A10 rv	GAG GAT GCG ACT TCG CCG CT CGC GAC GAG GGA CGT GAA TC	60
A11	AY918176	168	80.6	No homology	A11 fw A11 rv	GTA TAG TAC TAC GAC CAC CA CTT AGG ACT AGT ATA ACT AG	50
A12	AY918177	235	71.8	Hypothetical protein[b]	A12 fw A12 rv	CAG CGA AGC ACT AAT GAG GT CCA TGT TAG TAT CCT ACA AG	52
B3	AY918178	233	28.2	Hypothetical protein[b]	B3 fw B3 rv	AAG CAT CTA TGA GGT GAC CA CCA TGT TAG TAT CCT ACA AG	52
B4	AY918179	387	33.3	Casein kinase 1 delta	B4 fw B4 rv	TTC GCC ACA TAC ATT GAC TA TTC TAA ATC GGC TGT AAG TC	50

[a] —, no sequences determined.
[b] The sequences obtained for these two fragments are 97% homologous.

digested with two restriction enzymes, after which double-stranded adaptors are ligated to the resulting DNA fragments. The resulting fragments are then amplified by using primers complementary to the adaptor and restriction site sequences. To limit the number of amplified fragments, selective nucleotides can be added at the 3′ ends of the primers (19, 21). AFLP is a useful technique for the differentiation of strains within a species, even when they are clonal (19). The aim of the current study was to test whether the AFLP technique could differentiate clinical *M. mycetomatis* isolates obtained from mycetoma patients in various regions of Sudan. Another important question addressed was whether genetic differences among *M. mycetomatis* isolates could be used to link isolates with demographic and clinical characteristics.

MATERIALS AND METHODS

Strains and patients. A total of 39 fungal isolates from black grain eumycetoma patients were included. Thirty-eight of these strains were obtained from patients seen in the Mycetoma Research Centre, University of Khartoum, Khartoum, Sudan. One additional strain was obtained from a patient from Mali. The strains were isolated from biopsy specimens and were maintained on Sabouraud dextrose agar (Difco Laboratories, Paris, France). The strains were previously identified to the species level on the basis of morphology, PCR-based restriction fragment length polymorphisms, and sequencing (1, 3). Thirty-seven of the isolates were identified as *M. mycetomatis*, one was identified as a fungal species belonging to the *M. mycetomatis* cluster without appropriate species definition (strain mm27), and one was identified as *Leptosphaeria senegalensis* (strain mm3) (6). The following characteristics were recorded for the patients: geographical origin, lesion size, sex, age, and duration of the disease. Assessment of the lesion size was done by visual interpretation. Small lesions were those without sinuses and whose volumes were less than average. Large lesions clearly exceeded the average size and had multiple sinuses. No objective size parameters were developed.

Antifungal susceptibility. MICs were determined after 7 days by using the colorimetric Sensititre YeastOne method (Trek Diagnostic Systems, Ltd., East Grinstead, England), as reported elsewhere (20).

DNA isolation. DNA was isolated as described before (2).

AFLP analysis. AFLP analysis was performed as described before (21). In short, DNA was restricted with the endonucleases EcoRI and MseI. After restriction, adaptors were ligated to the resulting fragments. The resulting fragments were preamplified with primers E (5′-GACTGCGTACCAATTC-3′) and M (5′-GACGATGAGTCCTGAGTAA-3′), after which a selective PCR was performed. The selective primers were identical to primer E or M but were extended with selective dinucleotides at the 3′ terminus. Two primer combinations were used: primers E12 and M12 and primers E20 and M12. Primers E12 and M12 were extended with AC, and primer E20 was extended with GC. Primers E12 and E20 were radioactively labeled, and the amplified material was

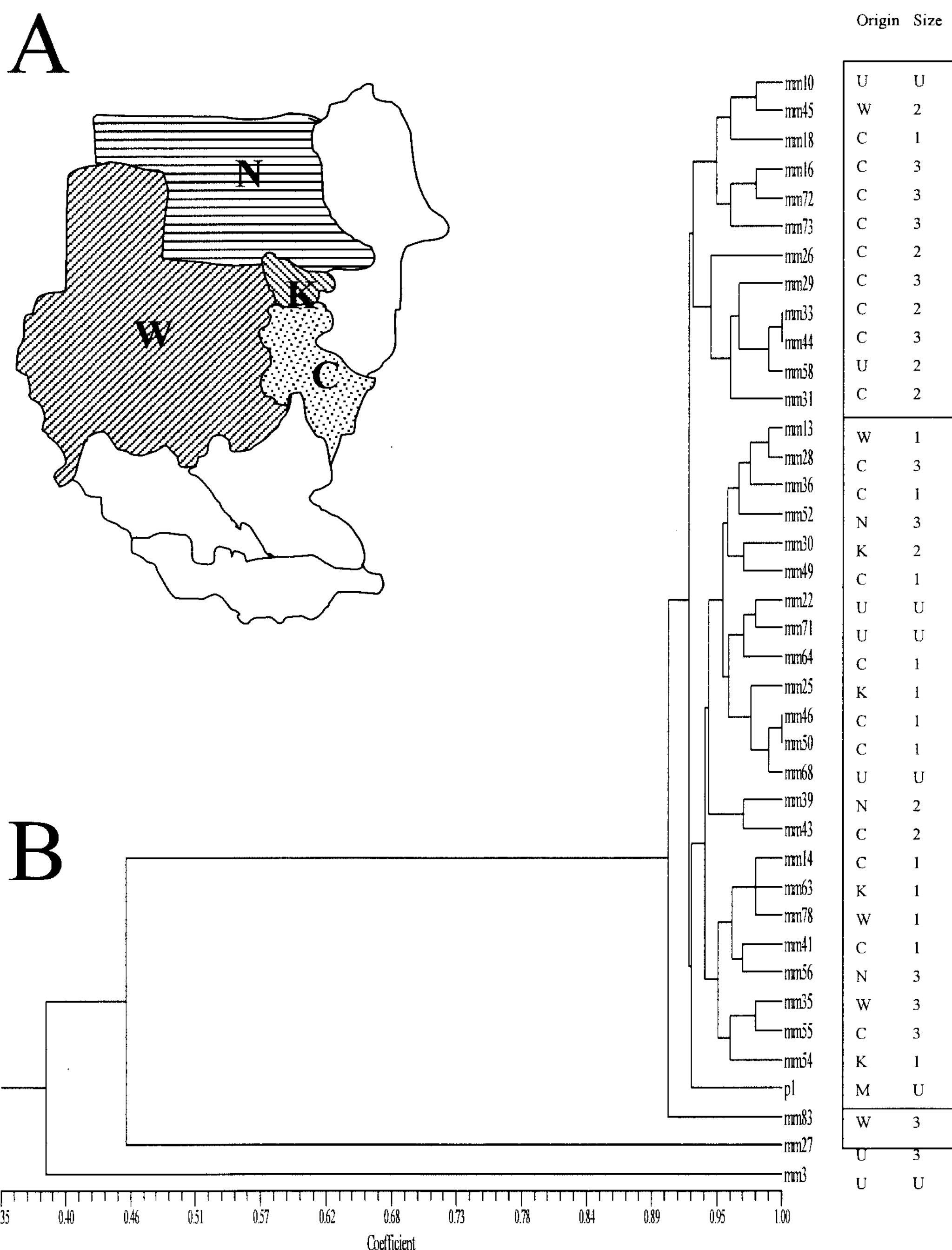


FIG. 1. (A) Areas of Sudan sampled for clinical *Madurella mycetomatis* isolates. Sudan is divided into central Sudan (C), western Sudan (W), northern Sudan (N), and Khartoum (K). (B) Phylogenetic tree based on the AFLP data obtained for 39 clinical black grain mycetoma fungal isolates. Thirty-seven of these isolates are *Madurella mycetomatis*. Strain mm3 represents the fungus *Leptosphaeria senegalensis*, and strain mm27 represents a fungal species belonging to the *M. mycetomatis* cluster without precise species definition. The *M. mycetomatis* strains are divided into clusters I, II, and III. For each isolate the geographic origin and the size of the lesion are presented. Strains originated from central Sudan (C), western Sudan (W), northern Sudan (N), Khartoum (K), and Mali (M). The lesion size is designated as small (size 1), moderate (size 2), and large (size 3). U, unknown.

analyzed on 4.5% polyacrylamide slab gels. The presence or absence of markers is scored in a table, which could be transformed into a dendrogram with the program NTsys (Exeter Software, Sekautet, N.Y.).

Sequencing and primer design. Selected markers were excised from the gel and reamplified with the following primers: 5′- AGCGGATAACAATTTCAC ACAGGACACACTGGTATAGACTGCGTACCAAT-3′ and 5′-GACGATGA GTCCTGAGTAA-3′. These PCR fragments were sequenced, aligned, and compared to each other and to other sequences in the National Center for Biotechnology Information data bank by use of the BLASTN 2.2.8 and BLASTX 2.2.8 programs (5). Internal primers were designed for screening purposes (Table 1).

PCR analysis of the selected polymorphisms. The PCRs were performed in 50-μl reaction volumes containing 50 ng DNA, 1× Supertaq PCR buffer 1 (HT Biothechnology Ltd., United Kingdom), 0.2 mM PCR nucleotide mix (Amersham Life Sciences, Roosendaal, The Netherlands), 25 pmol forward primer, 25 pmol reverse primer, and 1.2 U Supertaq (HT Biothernology Ltd.). The PCR

TABLE 2. Comparison of ITS sequences of mm27 and mm3 with those of true *Madurella mycetomatis* isolates[a]

Name	ITS difference (%)	Difference (bp)	Identity at the species level
Reference	0	0	*Madurella mycetomatis*
mm55	0.6	3	*Madurella mycetomatis*
mm39	0.6	3	*Madurella mycetomatis*
mm27	7.3	39	*Madurella species*
mm3	30.8	164	*Leptosphaeria senegalensis*

[a] ITS differences are stated as the percentage of base pair differences with the previously published sequence with GenBank accession no. AF162133. Variability within the genus *M. mycetomatis* was noted at 8 positions (6).

consisted of a predenaturation step of 4 min at 94°C and 40 cycles, each of 1 min of denaturation at 94°C, 1 min of annealing at variable temperatures, and 1 min of elongation at 72°C. This was followed by a postelongation step of 7 min at 72°C. The annealing temperatures differed for each fragment and are stated in Table 1. The PCR products were visualized by electrophoresis on 3% agarose gels (Hispanagar; Sphaero Q, Leiden, The Netherlands).

Statistical analysis. Associations between fungal DNA polymorphisms, demographics, and disease characteristics were tested for significance by Fisher's exact test (two sided). The association of genetic features with the geographic origin of the strain was studied by comparing each region with all others. The association with the size of the lesion (small, medium, or large) was tested by the chi-square test for trend. Correlations with age, disease duration, and MICs to antifungals were tested by the Mann-Whitney test (two tailed). All statistical calculations except for the adjustment of the lesion size for disease duration were performed with GraphPad InStat, version 3.00 (GraphPad InStat Software, Inc., San Diego, Calif.). Adjustment of the lesion size for disease duration was done by linear regression analysis with SPSS, release 10.1.0 (SPSS, Inc., Chicago, Ill.).

Nucleotide sequence accession numbers. The sequences reported here are deposited in the GenBank database under accession numbers AY918172 (fragment A10), AY918173 (fragment A4), AY918174 (fragment A5), AY918175 (fragment A7), AY918176 (fragment A11), AY918177 (fragment A12), AY918178 (fragment B3), and AY918179 (fragment B4).

RESULTS

AFLP fragment analysis. Ninety-three AFLP markers were generated with the two primer combinations used for the 39 clinical black grain mycetoma isolates. Comparative analysis of the markers resulted in a score table and the phylogenetic tree shown in Fig. 1. From the tree it can be concluded that strains mm3 and mm27 differ considerably from the other strains. Strain mm3 appeared to be the fungus *Leptosphaeria senegalensis*, and strain mm27 is an as yet ill defined species but still a close relative of *M. mycetomatis* (see Materials and Methods and reference 6). Consequently, mm27 is more closely related to the *M. mycetomatis* isolates than mm3. This was verified by comparisons of internal transcribed spacer (ITS) sequences. In Table 2 it can be seen that the ITS sequences obtained for strains mm39 and mm55 differ by only 0.6% from the already published ITS sequence for *M. mycetomatis* (GenBank accession number AF162133). The ITS sequences for mm27 (7.3% difference) and *L. senegalensis* (30.8% difference) differ considerably more.

In the *M. mycetomatis* cluster, 25 markers were polymorphic. Thirteen of these markers were seen only incidentally in one to three strains, while the rest of the markers were seen in at least 15% of the strains. This means that of the *M. mycetomatis* markers, 26.9% were polymorphic, which is a relatively large fraction compared to that from our previous RAPD data (2).

Two of the strains used in this study, strains mm72 and mm73, were isolated from a female patient with two independent large lesions, one on the sole of the foot and one on the knee. The lesion on the sole of the foot had been there for over 13 years, while the lesion on the knee joint was just 4 years old. Figure 1 shows that both isolates are found in cluster I and were closely related but not identical. Strains mm33 and mm44 appeared to be 100% identical by AFLP analysis. Those strains originated from two different central Sudanese patients (a 24-year-old male with a moderate lesion and a 28-year-old female with a large lesion) belonging to the same tribe. They had been infected for 2 years, which could imply that they were infected with the same strain originating from somewhere in that area. In cluster II, strains mm46 and mm50 had exactly the same AFLP banding pattern. Those two strains derived from central Sudanese patients (a 28-year-old male and a 35-year-old male). Those two patients had both been infected for 1 year and had only small lesions.

The *M. mycetomatis* strains were divided into two main clusters: clusters I and II (Fig. 1). Minor cluster III consists of only one strain (mm83) which originated from western Sudan and which caused a large lesion. Cluster I and cluster II have several distinctive features, but the most striking one is that in cluster I, fragment A12 is largely absent and fragment B3 is largely present, while in cluster II this is the other way around. Seventy-five percent of the strains found in cluster I originated from central Sudan, while only 45.8% of the strains in cluster II originated from central Sudan. The other 54.2% of the strains encountered in this cluster were divided as follows: 16.7% of the strains originated from Khartoum, 12.5% originated from northern Sudan, 8.3% originated from western Sudan, and 4.2% ($n = 1$) originated from Mali. Data on lesion size are displayed next to the phylogenetic tree in Fig. 1; and it appeared that among the strains in cluster I, only one strain caused a small lesion. All other strains in cluster I caused moderate (41.7%) or massive (41.7%) lesions. Among the strains in cluster II, half of the strains caused small lesions, while 12.5% and 33.3% of the strains caused moderate and large lesions, respectively. This may point to differences in virulence between the strains from the two main clusters.

DNA sequencing of polymorphic markers. Of 93 amplimers, a total of 25 markers were polymorphic in the *M. mycetomatis* strains. Thirteen of these markers were only seen in one to three strains, while the rest of the markers were seen in at least 15% of the strains. Eleven of these more common polymorphic markers were reamplified and sequenced. No useful sequence could be obtained from four of these fragments, probably because mixtures of DNA fragments were excised from the gel. After BLASTN and BLASTX analysis, it appeared that the sequences obtained for fragments A12 and B3 were 97% identical. A tribase substitution and two deletions were the only differences obtained, suggestive of gene duplication or heterozygostiy. Fragment A7 showed the highest homology (66% identity) with an endo-1,4-beta-glucanse gene from *Aspergillus oryzae*. Fragment B4 showed similarity with a casein kinase 1 isoform delta gene. The highest homologies for this gene, namely, 55%, were obtained with the species *Danio rerio*. The closely related fragments A12 and B3 matched a gene for a noncharacterized hypothetical protein from *Gibberella zeae*. The other four fragments showed no significant homology with any other known sequence.

TABLE 3. Presence of AFLP amplimers versus PCR amplicons

Fragment and method	Presence of amplicon in strain[a]:																																				
	10	13	14	16	18	22	25	26	28	29	30	31	33	35	36	39	41	43	44	45	46	49	50	52	54	55	56	58	63	64	68	71	72	73	78	83	p1
A10																																					
AFLP	0	1	1	1	1	1	1	2	1	1	0	0	1	0	1	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0
PCR	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′
A11																																					
AFLP	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1
PCR	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1
B4																																					
AFLP	0	0	1	1	0	1	0	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0
PCR	0	0	1	1	0	1	0	1	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0

[a] 0, no amplicon present; 1, amplicon present; 1′, amplicon present but slightly smaller; 2, very faint amplicon present.

Development of PCR tests. Because no genome data are available for *M. mycetomatis*, internal PCR primer pairs were designed for all of the sequences obtained. PCR with the novel primers for fragments A4, A5, A7, A12, and B3 resulted in equally sized amplicons for all strains except mm3 and mm27. Size-variable amplicons were obtained for fragments A10, A11, and B4. As stated in Table 3, PCR for fragments A11 and B4 resulted in positive PCR signals only for those strains in which the original AFLP fragment was present. For fragment A10, all strains gave a positive PCR signal, but in the strains in which the original AFLP fragment was not present, the PCR products were smaller. This size difference still allowed the discrimination of different types.

Analysis of correlations. Strains from central Sudan belonged to cluster I significantly more often ($P = 0.049$) (Table 4). With respect to the AFLP markers tested, we found the B4 fragment (a casein kinase delta homologue) in half of the strains originating from central Sudan but in none of the strains from the other regions ($P = 0.001$). The lesions caused by cluster I strains tended to be larger than those caused by the other strains ($P = 0.03$). The size of the lesion appeared to be strongly associated with the duration of disease ($P = 0.001$, Pearsons's correlation coefficient). However, the association between lesion size and cluster did not decrease after adjustment for disease duration. No linkage between strain genetic features and the sex or the age of the patients was found with either cluster or with the duration of disease.

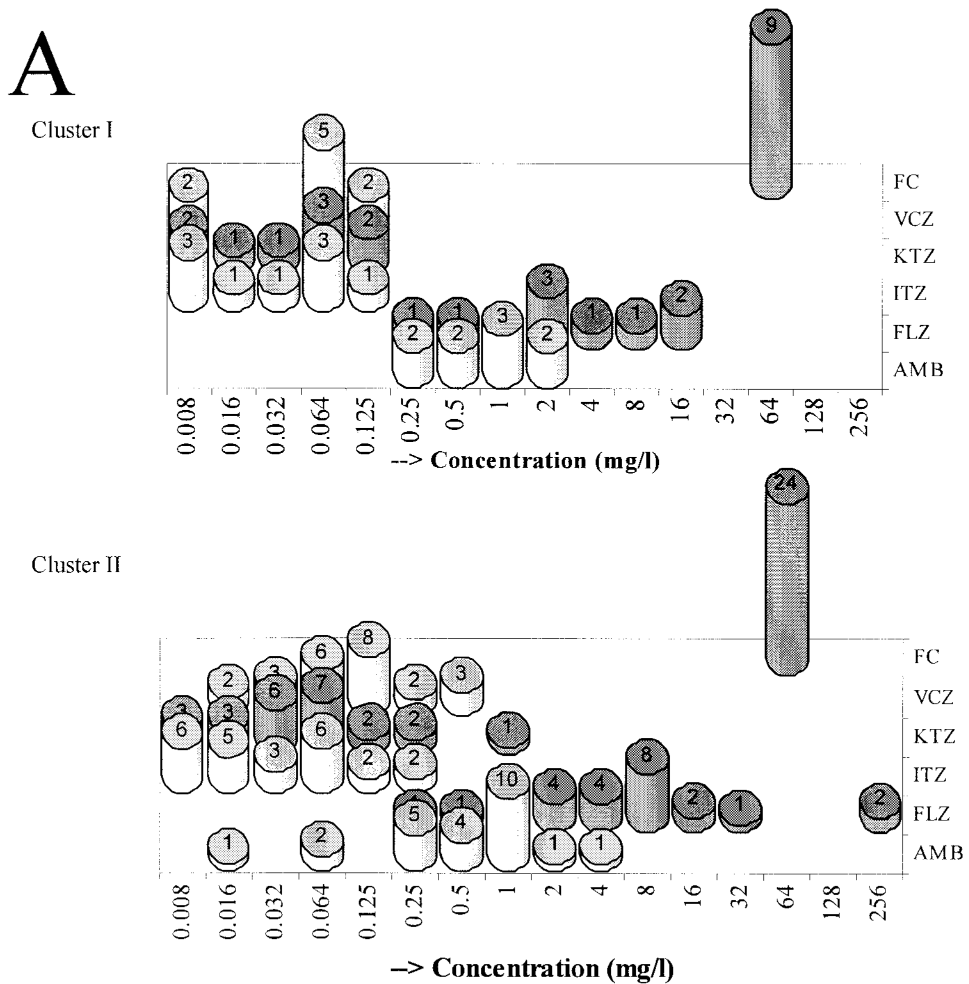
Additionally, associations between the genetics of the strains and antifungal susceptibility were studied. The distribution of the antifungal susceptibilities of cluster I and II strains are shown in Fig. 2A. MICs were obtained for the azoles itraconazole, ketoconazole, fluconazole, and voriconazole as well for the polyenic compound amphotericin B, but not for flucytosine. As was already seen in the MIC distributions in Fig. 2A, there was no obvious correlation between antifungal susceptibility and strain clustering. This was confirmed with the Mann-Whitney test. The same analyses were performed for the individual markers. After statistical analysis of two of the

TABLE 4. Correlations of *Madurella mycetomatis* strain genetic clusters and polymorphic markers and various demographics, disease characteristics, and antifungal susceptibility

Trait	Cluster		P value for cluster[a]	Marker			
	I	II		Marker name	P value for marker	Absent	Present
Geographic origin							
Central Sudan versus the rest of Sudan	9/1[b]	10/10	0.049	B4	0.001	8/12	11/0
Western Sudan versus the rest of Sudan	1/9	3/17	NS		NS		
Northern Sudan versus the rest of Sudan	0/10	3/17	NS		NS		
Kharthoum versus the rest of Sudan	0/10	4/16	NS		NS		
Lesion size (no. of small/moderate/large lesions)	1/5/5	12/3/5	0.03		NS		
Sex (no. of males/no. of females)	6/6	17/6	NS		NS		
Age of the patients (yr [median])	19	21.5	NS		NS		
Duration of the disease (yr [median])	3	3	NS		NS		
Antifungal susceptibility (median MIC [mg/liter])							
Amphotericin B	1	0.5	NS	A4	0.02	1	0.5
	0.5	1		B4	0.02	0.5	1
Fluconazole	2	8	NS		NS		
Itraconazole	0.032	0.032	NS		NS		
Ketoconazole	0.032	0.064	NS		NS		
Voriconazole	0.125	0.125	NS		NS		

[a] *P* values were obtained by Fisher's exact test, the chi-square test for trend (size of the lesion), and the Mann-Whitney test (age, duration, and comparison of MICs). NS, not significant.

[b] Data represent number of strains from first location listed/number of strains from second location listed.



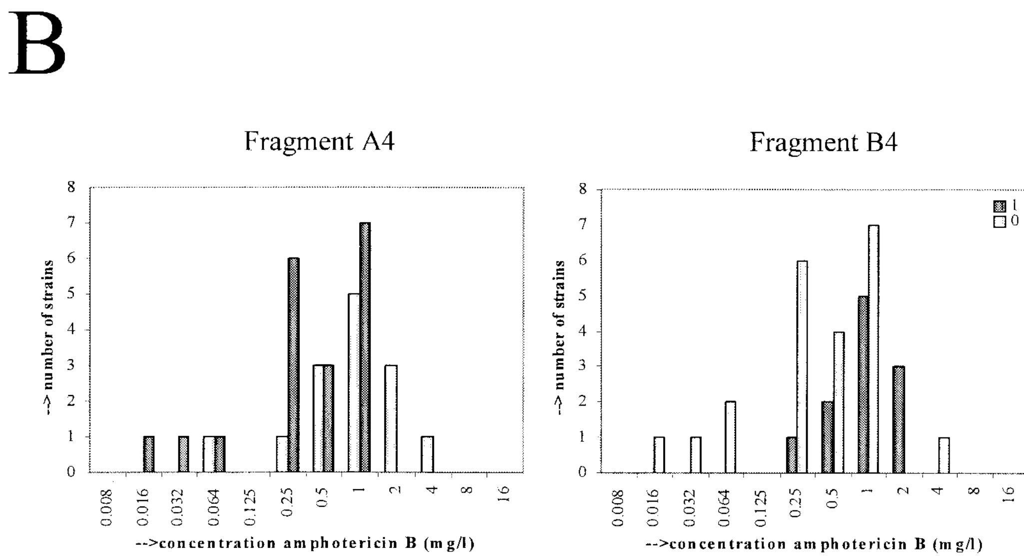


FIG. 2. (A) Distributions of susceptibility to the following antifungal agents for strains in both clusters I and II: amphotericin B (AMB), fluconazole (FLZ), itraconazole (ITZ), ketoconazole (KTZ), voriconazole (VCZ), and flucytosine (FC). Each bar represents the number of strains (indicated at the top of the bar) with an MIC at the concentration listed on the *x* axis for the antifungal shown on the *y* axis. For example, in cluster I there are three strains with an MIC of 1 mg/liter for amphotericin B. (B) MIC distributions for amphotericin B for fragments A4 and B4. The numbers of strains for a certain MIC are shown for the two fragments. The strains for which a fragment is present are indicated with black bars; the strains for which this fragment is absent are indicated with white bars.

polymorphic markers, namely, A4 and B4, a correlation with susceptibility to amphotericin B was found. These data are shown in Fig. 2B. As shown in Fig. 2B, strains positive for A4 had lower MICs for amphotericin B (median MIC, 0.5 mg/liter) than strains in which this fragment was not present (median MIC, 1 mg/liter) (Mann-Whitney, $P = 0.02$). Strains for which fragment B4 could be amplified had higher MICs for amphotericin B (median MIC, 1.0 mg/liter) than strains without B4 (median MIC, 0.5 mg/liter) ($P = 0.02$). This difference is very small. However, all four strains with an MIC for amphotericin B of 2 μg/ml or higher (usually implicated in therapy failure) were found in the group without fragment A4. Absolutely no association between the other markers and susceptibility to any of the antifungals tested was found.

DISCUSSION

Not much is known about the genetic diversity of *M. mycetomatis*. Until now, only two reports in which the genetic heterogeneity of *M. mycetomatis* was investigated have appeared (2, 14). Lopes et al. collected 17 isolates from countries all over the world, ranging from The Netherlands Antilles to Argentina and from Djibouti to Morocco (14). By using both restriction endonuclease assays and RAPD analysis, it appeared that the 17 strains could be divided into 10 different groups (14). In contrast, the 38 strains from Sudan and the 2 strains from Mali used in high-throughput RAPD analysis by Ahmed et al. appeared to be highly clonal, and all attempts to identify genetic markers failed (2). In the study performed by Lopes et al., the isolates obtained from Sudan also could not be discriminated from each other, confirming regional clonality (14).

In the past AFLP was demonstrated to be a valuable technique for the typing of various fungal species, such as *Aspergillus fumigatus* and *Cryptococcus neoformans* (12, 22). For *M. mycetomatis* we showed that AFLP is also valuable for the differentiation of this species. Despite the remarkable clonality found before by RAPD analysis (2), AFLP was able to discriminate these same strains into three clusters. This suggests that AFLP is a much stronger technique than RAPD analysis for the discrimination of *M. mycetomatis* strains. With AFLP the *M. mycetomatis* isolates could be divided into two main clusters and one minor cluster. Cluster I mainly consisted of strains that originated from central Sudan and that caused moderate or large lesions, while cluster II was more heterogeneous. Although there was an association with the lesion size and the duration of the disease, the association found between cluster I and larger lesions still remained intact after adjustment for disease duration. As a matter of fact, the lesion size is probably defined on the basis of individual host-pathogen interactions. With the AFLP conditions used, it was also possible to discriminate between two *M. mycetomatis* isolates obtained from the same patient but from different lesions.

At least 4 of the 12 polymorphic markers were actually part of coding regions, and 2 of these (A12 and B3) were not previously identified. Marker A7 was homologous to the gene for endo-1,4-beta-glucanase, an important enzyme involved in cellulose degradation (11, 18). Polymorphic marker B4, homologous to casein kinase 1 delta, was primarily detected in strains isolated from Central Sudanese patients. Casein kinase 1 is thought to play a physiological role in activating transcription of various DNA repair genes, in intracellular trafficking, and in normal cell cycle progression (10, 13, 16, 23).

In the present study, MICs were determined by using the Sensititre YeastOne system instead of using the modified CLSI (formerly NCCLS) method for *M. mycetomatis*. It has been demonstrated that the results obtained with this system are in good agreement with those obtained by the modified NCCLS method. The rates of agreement ranged from 91.2 to 100.0%, depending on the antifungal tested (20). The reproducibility of this test system was also satisfactory, ranging from 88.2 to 97.1% (20). We found an association between susceptibility to amphotericin B and both markers B4 and A4. The distribution of MICs of strains with and without the B4 and A4 markers differed significantly ($P = 0.02$), but the difference was very small. The median for the groups differed only by a single twofold dilution, which is allowed as background variation in antifungal susceptibility testing. Furthermore, the median MICs for each group were in the treatable range and are therefore probably not clinically relevant. The association found in this study could be a chance finding and not reproducible in other strain collections. Further study of the relevance of this finding is needed.

In conclusion, the AFLP method differentiates clinical isolates of *M. mycetomatis*. AFLP clusters I and II are associated with different clinical presentations, with cluster I strains apparently causing larger lesions. An AFLP marker sequence with casein kinase 1 homology seemed to be associated with the geographical origin of the fungal isolate. We present the first pathogenicity markers for a fungal species that still has a devastating socioeconomic effect on small communities in rural Sudan.

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AFLP is a registered trademark of Keygene NV, and the AFLP technology is covered by patents (US 6,045,994 and EP 0 534 858 B1) and patent applications owned by Keygene NV.

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