

Microsatellite Typing To Trace *Aspergillus flavus* Infections in a Hematology Unit[∇]

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Assessing the relatedness of strains isolated from patients and their environment is instrumental in documenting the source of preventable health care-associated life-threatening *Aspergillus flavus* human infection clusters. The present study aimed at identifying and selecting suitable microsatellite markers for *A. flavus* typing. This typing scheme was then applied to investigate the *A. flavus* epidemiology within a hematology unit in Sfax, Tunisia. Use of a combination of five markers made it possible to discern clusters of isolates and to substantiate the genetic diversity of *A. flavus* within clusters. Isolates from Tunisia and Marseille, France, displayed distinct haplotypes, indicating a highly significant geographical structuring of *A. flavus*. The typing of clinical and environmental *A. flavus* isolates in a hematology unit provided insights into its hospital epidemiology. From a heterogeneous genetic background, a cluster indicative of a clonal propagation episode within the unit could be identified. In two patients with invasive aspergillosis, the same genotype was found in clinical and environmental isolates, indicating hospital-acquired colonization and infection. In further studies, this novel microsatellite typing scheme might be instrumental in illuminating important epidemiological issues about *A. flavus* population genetics or epidemiology, including tracing the sources and routes of transmission.

Invasive aspergillosis is a life-threatening disease in hematological units. Typing studies aiming at assessing the relatedness of strains isolated from patients and their environment are instrumental in understanding the epidemiology of this mold and documenting the source of preventable health care-associated life-threatening human infections. Most of the studies have focused on *Aspergillus fumigatus*, the medically most important species. Briefly, typing studies showed that environmental outdoor *A. fumigatus* populations are genetically very heterogeneous (3, 4). However, aspergillosis outbreaks due to clonal, i.e., genotypically identical, *A. fumigatus* strains have been documented in hospitals (2). Very few data exist on non-*A. fumigatus* *Aspergillus* species that are also involved in human infections. In particular, *Aspergillus flavus* has been described to be an emerging species second only to the *A. fumigatus* pathogenic *Aspergillus* species (17). Furthermore, *A. flavus* has been described as the main etiological agent of human aspergillosis in Saudi Arabia and in Tunisia, Sudan, and other African countries (10, 12). Three molecular methods have been proposed for *A. flavus* typing: restriction fragment length polymorphism (15, 19), randomly amplified polymorphic DNA (20), and random amplified microsatellite polymorphism analysis (11). When it was applied to *A. fumigatus*, microsatellite typing (22) showed a higher discriminatory power than other typing methods (13, 15, 19), including the other popular exact typing method used to date: multilocus sequence typing (MLST) (1). Recently, Tran-Dinh and Carter have described seven polymorphic microsatellite markers that may be used to type aflatoxin-producing *A. flavus* and *A. para-*

siticus (21), but to our knowledge, this typing scheme has not been used either in population genetic or in epidemiological studies.

The study described here aimed at identifying and selecting microsatellite markers and at applying this typing scheme in order to investigate the epidemiology of *A. flavus* in one hematology unit.

MATERIALS AND METHODS

Genomic sequences of *Aspergillus flavus* were formed from published sequences (<http://www.Aspergillusflavus.org/>). The sequences were analyzed for the presence of short tandem repeats by using the Tandem Repeats Finder software (<http://tandem.bu.edu/trf/trf.html>). Only di-, tri-, and tetramicrosatellite loci that had perfect repeat sequences (having 100% identity between repeats units) and that were highly repeated (with a copy number of >12) were selected; imperfect repeats containing point mutations and/or insertions or deletions or having mismatches in their repeated units were excluded. This software made it possible to give a flanking sequence for these selected microsatellites. Then, primers were designed using Primer (version 3) software (<http://frodo.wi.mit.edu>) and verified for their *Aspergillus flavus* specificity using a BLASTn search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). We additionally used five markers described by Tran-Dinh and Carter (21).

The 63 *Aspergillus flavus* isolates used in this study originated from either Tunisia or France. Fifteen isolates were collected from three patients and their environment during the investigation of a single epidemic episode at Timone Hospital in Marseille, France. The characteristics of the 48 isolates collected from 14 patients with proven and probable invasive aspergillosis hospitalized in the hematology ward of the Hedi Chaker Hospital in Sfax, Tunisia, and their environment over a period of 3 years (2004 to 2007) are summarized in Table 1. This study design has been described elsewhere (12). Briefly, *A. flavus* infections were diagnosed in high-risk patients hospitalized in one hematology ward where neither high-efficiency particulate air filtration nor an aspergillosis prophylaxis policy was implemented. The 35 clinical and 13 environmental isolates sampled in the patients' rooms are detailed in Table 1. These *A. flavus* strains were identified on the basis of macroscopic and microscopic morphological characteristics. The morphological identification of the *Aspergillus flavus* isolates was verified by internal transcribed spacer 1 (ITS1), 5.8S, and ITS2 region rRNA sequence analysis, as described previously (5).

DNA was extracted by using a Nucleospin kit (Machery-Nagel, France), as indicated by the manufacturer's instructions, and eluted with 70 μ l of sterile

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TABLE 1. Origins of *A. flavus* isolates collected from patients with aspergillosis and their hospital environment in the hematology ward of the University Hospital of Sfax, Tunisia

Patient no.	Isolate no.	Date (day-mo-yr)	Sample source	
			Clinical	Environmental
Patient 1	0588	02-05-05	Sputum	
	0590	06-05-05	Sputum	
	0658	06-05-05		Air conditioner
	0704	15-05-05	Nasal	
	0870	15-05-05		Table
Patient 2	0601	15-05-05		Table
	0605	18-05-05	Nasal	
	0603	15-05-05	Sputum	
	0604	15-05-05	Nasal	
	0626	18-05-05	Sputum	
	0662	21-05-05		Air conditioner
	0948	25-05-05	Sputum	
	LBA2	21-05-05	BAL fluid	
Patient 4	1058	05-06-05	Sputum	
	1069	08-06-05	Nasal	
	1268	22-06-05	Sputum	
	1080	15-06-05		Bed
	LBA4	11-06-05	BAL fluid	
	1075	11-06-05		Air conditioner
Patient 5	1169	24-11-05	Nasal	
	1240	30-11-05	Nasal	
	1289	03-12-05	Sputum	
	1211	27-11-05		Bed
Patient 6	0019	05-05-06	Sputum	
	0016	09-05-06		Air conditioner
Patient 7	0922	22-03-06	Sputum	
	0944	25-03-06	Nasal	
	0938	29-03-06	Sputum	
Patient 8	1056	27-02-06		Bed
	1119	01-03-06	Nasal	
	1128	01-03-06	Sputum	
	1090	9-03-06	Sputum	
Patient 9	1403	23-04-07	Sputum	
	0811	27-04-07		Bed
Patient 10	1073	12-08-05		Table
	1076	16-08-05	Nasal	
	1368	16-08-05	Sputum	
Patient 11	1369	16-11-07	Sputum	
	0486	26-11-07	Lung biopsy	
	LBA33	19-11-07	BAL fluid	
Patient 12	LBA16	23-06-06	BAL fluid	
Patient 13	1331	27-03-07	Sputum	
	0887	24-03-07	Nasal	
	0890	24-03-07		Bed
Patient 14	0587	13-03-07	Sputum	
	0889	16-03-07		Table
	0855	21-03-07	Nasal	
Patient 15	LBA31	27-12-05	BAL fluid	

water. In each multiplex PCR, different fluorescent labels (6-carboxyfluorescein, PET [Applied Biosystems], NED [Applied Biosystems], VIC) were used for the different markers in order to distinguish the amplification products from each other. PCRs were performed in a final volume of 25 µl containing 1 ng of genomic DNA, 1 µM all amplification primers, 0.2 mM each deoxynucleoside triphosphate, 3 mM MgCl₂, and 1 U of Ampli Taq DNA polymerase (Applied Biosystems, Courtaboeuf, France) in 1× reaction buffer (Applied Biosystems). Thermocycling was performed in a T1 thermocycler (Biometra, Göttingen, Germany) using the following protocol: 5 min of initial denaturation at 94°C, followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 54°C, and 30 s of extension at 72°C, finally followed by 30 min at 72°C. The PCR products were diluted 10-fold with formamide. One microliter of the diluted PCR products was combined with 15 µl of formamide and 0.5 µl of LIZ 500 marker (Applied Biosystems Inc., CA). Following denaturation, the PCR products were resolved by capillary electrophoresis with polymer POP-7 in an ABI Prism 3130 genetic analyzer (Applied Biosystems Inc.). The injection and running parameters were those according to the recommendations of the manufacturer (Applied Biosystems Inc.). Analyses were performed with Gene Mapper software (Applied Biosystems Inc.). The repeatability of microsatellite typing was evaluated by using five different DNA preparations of the same isolate and by 10 repeated analyses of the same DNA preparation.

The Simpson index of diversity, *D* (14), was computed for each marker and each possible marker combination, with the aim of determining the most parsimonious combination yielding a *D* value of > 0.95, a sufficiently high discriminatory power recommended for typing experiments. The degree of similarity was calculated by applying the Dice coefficient test. This was performed using an NTSYS-PC numerical taxonomy and multivariate analysis system (version 2.1; Exeter Software, Setauket, NY). A dendrogram was generated using neighbor-joining methods. The fixation index (*F_{ST}*) on all loci was estimated from 1,000 bootstrap repetitions using the ARLEQUIN software package (9). Isolates possessing alleles with the same number of repeat units in all loci were defined as a clonal cluster.

RESULTS

Using the Tandem Repeats Finder software package, we screened the 6,972,320 bp of *A. flavus* genomic sequence available (www.Aspergillusflavus.org, accessed in June 2007). We found 338 microsatellites; in 60% repeat units, they ranged from 1 to 6 nucleotides, and in 40%, they were greater than 6 nucleotides (Fig. 1). We finally retained seven markers: two dinucleotides, four trinucleotides, and one tetranucleotide matched our selection criteria (>12 repeats and 100% match). Each marker was tested in five repeated assays with different DNA preparations of the same isolate as well as with the same DNA preparation. Each marker's reproducibility, i.e., the ability to assign an identical type to the same isolate, was 100%. These markers were specific to *A. flavus*. The primers used to amplify the microsatellite flanking regions (Table 2) were se-

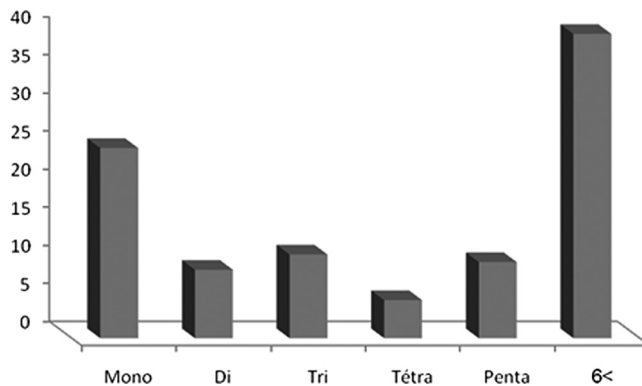


FIG. 1. Distribution frequency of microsatellites in *A. flavus* genome.

TABLE 2. Features of the 12 polymorphic microsatellite sequences of *A. flavus* upon analysis of 63 isolates

Primer name	Primer sequence (5' to 3')	Repeat unit	Fragment size (bp)	No. of alleles	<i>D</i>
AFLA1	CGTTGGCATGTTATCGTCAC CTACTGAATGGCGGGACCTA	AC	249–291	7	0.795
AFLA2	GAGCACGTGCGATTTAGTCA TATCTACTCCGCGCAACTCG	CTT	282–291	3	0.414
AFLA3	CTGAAAGGGTAAGGGGAAGG CACGCGAACTTATGGGACTT	TAGG	174–229	5	0.703
AFLA4	TGCTTAAGTGACCCCAATCC CAGTTGATTTAAGGGGCAACA	CT	212–242	4	0.554
AFLA5	GTGAGAGCAATTGGGAAACC TGGCATTGATCTCTTGCAG	GAG	197–209	3	0.531
AFLA6	CATAAGCGCTCCGAAGTCAT CCACCGATGATGGAAAAGAT	CTT	238–250	3	0.533
AFLA7	GCGGACACTGGATGAATAGC AACAAATCGGTGGTTGCTTC	TAG	261–354	7	0.738
AFPM2	CTGGACGGAGATCACGAC CCACGCTCCTCAAATACG	(AC)5T(CTC)4	206–266	5	0.455
AFPM3	CCTTTCGCACTCCGAGAC CACCACCAGTGATGAGGG	(AT)6AAGGGCG(GA)	199–217	5	0.740
AFPM4	AGCGATACAGTTTTAACACC TCTTGCTATACATATCTTCACC	CA	179–206	4	0.634
AFPM5	TCCTACCCGAGAGAGTCTG CCATTATGACATGTGGTTAAGAG	(AG)5AC(AG)2	210–338	2	0.493
AFPM7	TTGAGGCTGCTGTGGAACGC CAAATACCAATTACGTCCAACAAGGG	AC	215–276	7	0.633

lected on the basis of *in silico* specificity to *A. flavus*. *In vitro* PCR amplification was not observed either with *A. fumigatus* and *A. niger* or with *Penicillium* sp.

Upon the analysis of 63 isolates (48 from Sfax and 15 from Marseille), 2 to 7 distinct alleles were detected for each microsatellite marker (Fig. 2). The highest discriminatory power for a single locus was obtained with the AFLA1 marker, which had seven distinct alleles and a *D* value of 0.795 (Table 2). The combination of all 12 markers yielded 35 different haplotypes with a *D* value of 0.97. A five-marker combination (AFLA1, AFLA7, AFLA3, AFPM3, and AFPM7) yielded 27 different alleles with a *D* value of 0.952 (Table 3). This five-marker combination was selected, as it was the most parsimonious panel achieving a *D* value of >0.95.

Use of this microsatellite panel analysis revealed a marked genetic heterogeneity of *A. flavus* within each geographic area, with some alleles being present in the isolates from only one country (Fig. 2). A 0.424 F_{ST} index indicated a highly significant ($P < 10^{-5}$) geographical genetic structure in the *A. flavus* population studied.

From the 48 clinical and environmental *A. flavus* isolates sampled in the hematology ward in Sfax, 35 distinct genotypes could be identified. The dendrogram (Fig. 3) illustrates this genetic heterogeneity, where each isolate from one patient and that patient's room had distinct genotypes. For instance, all five

isolates sampled from patient 1 (P1) and the environment of P1 were genetically unrelated. However, in contrast to this very heterogeneous genetic background, we were able to identify a clonal cluster that included two out of three environmental isolates and all five clinical isolates of P2, who was hospitalized concomitantly with P1 (Table 1). The four remaining isolates of this cluster were concomitantly isolated from P4 or from two patients (P5 and P6) hospitalized up to 1 year after P2 was hospitalized (Table 1). Three out of four isolates from P8 formed another clonal cluster that included two clinical isolates (nasal and sputum) and one environmental isolate (from the patient's bed). This cluster also included one unrelated environmental isolate. In P11, two closely related isolates (recovered from a lung biopsy specimen and bronchoalveolar lavage [BAL] fluid) differed in only a single (AFPM3) marker genotype.

DISCUSSION

We present here a novel typing scheme that uses microsatellite markers highly polymorphic for *A. flavus*. Use of this five-microsatellite-marker combination yielded a high discriminatory power and could easily distinguish epidemiologically related *A. flavus* isolates within two distinct geographic areas (Tunisia and France). In addition to a high degree of discrim-

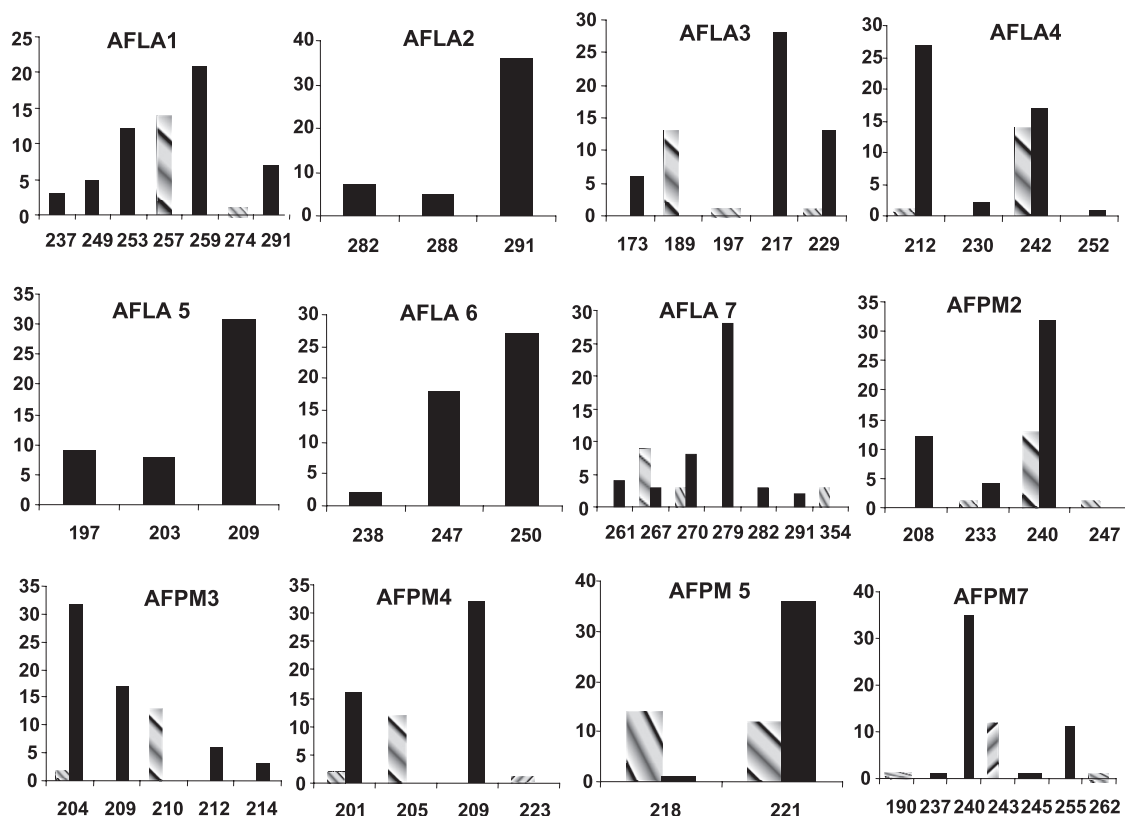


FIG. 2. Allele size distribution of *A. flavus* at 12 microsatellites upon analysis of 15 isolates from Marseille (hatched bars) and 48 isolates from Tunisia (black bars). x axis, number of isolates; y axis, allele size.

ination, reproducibility, and typeability, microsatellite analysis has several other advantages over other DNA-based typing assays. Because the assay is PCR based, microsatellite analysis requires relatively small amounts (~1 ng per reaction) of template DNA, and it can be simplified by using multiplex assays with primers labeled with different fluorescent dyes, resulting in higher throughput (6, 23). Finally, microsatellite analysis allows the detection of mixed cultures, which would appear as a PCR profile harboring two or more microsatellite alleles from a single DNA sample of the haploid genome of *A. flavus*. The strengths and pitfalls of microsatellite-based typing have been comprehensively described for *A. fumigatus* (8). With

respect to *A. fumigatus*, microsatellites have proven to offer the best available typing option by outperforming MLST in terms of speed, throughput, costs, and discriminatory power (16). The major pitfall is the interlaboratory reproducibility of PCR fragment sizing. However, this was overcome for *A. fumigatus* typing by using allelic ladders (7), which then make it possible to deposit the microsatellite allelic profiles in a global typing database similar to those developed for several pathogens using MLST (18).

This 3-year longitudinal genotyping survey provided insights into the *A. flavus* epidemiology in one hematology unit where patients who are at high risk of invasive aspergillosis are hospitalized. Microsatellite polymorphism analysis revealed a genetically very heterogeneous *A. flavus* population in this hospital ward. This finding is in line with the *A. fumigatus* data (3, 4). Indoor and outdoor aerocontamination are probably strongly correlated in the absence of air filtration systems, as was the case in this hospital. Therefore, the genetic heterogeneity of these isolates probably reflects the diversity of airborne *A. flavus* spores in the hospital area. In this setting it seems particularly challenging to trace patients' exposure to a particular clone disseminated in the hospital environment. However, outbreaks due to clonal *A. fumigatus* strains have been documented (2). In our survey, there was no cluster of aspergillosis and we did not identify an invasive aspergillosis outbreak. However, we could detect a temporally related clonal cluster of *A. flavus*. This situation, where over a short time period one genotype becomes predominant, indicates that clonal repro-

TABLE 3. Discriminatory indices of different microsatellite marker combinations

Locus combination	No. of profiles	D
AFLA3-AFLA7	12	0.840
AFLA1-AFLA3	14	0.845
AFLA3-AFPM3	10	0.853
AFLA7-AFPM3	10	0.858
AFLA1-AFLA7	15	0.882
AFLA1-AFPM3	15	0.892
AFLA1-AFLA3-AFPM3	18	0.911
AFLA1-AFLA7-AFPM3	18	0.920
AFLA1-AFLA7-AFLA3-AFPM3	22	0.927
AFLA1-AFLA7-AFLA3-AFPM3-AFPM7	27	0.952
12 microsatellites	35	0.970

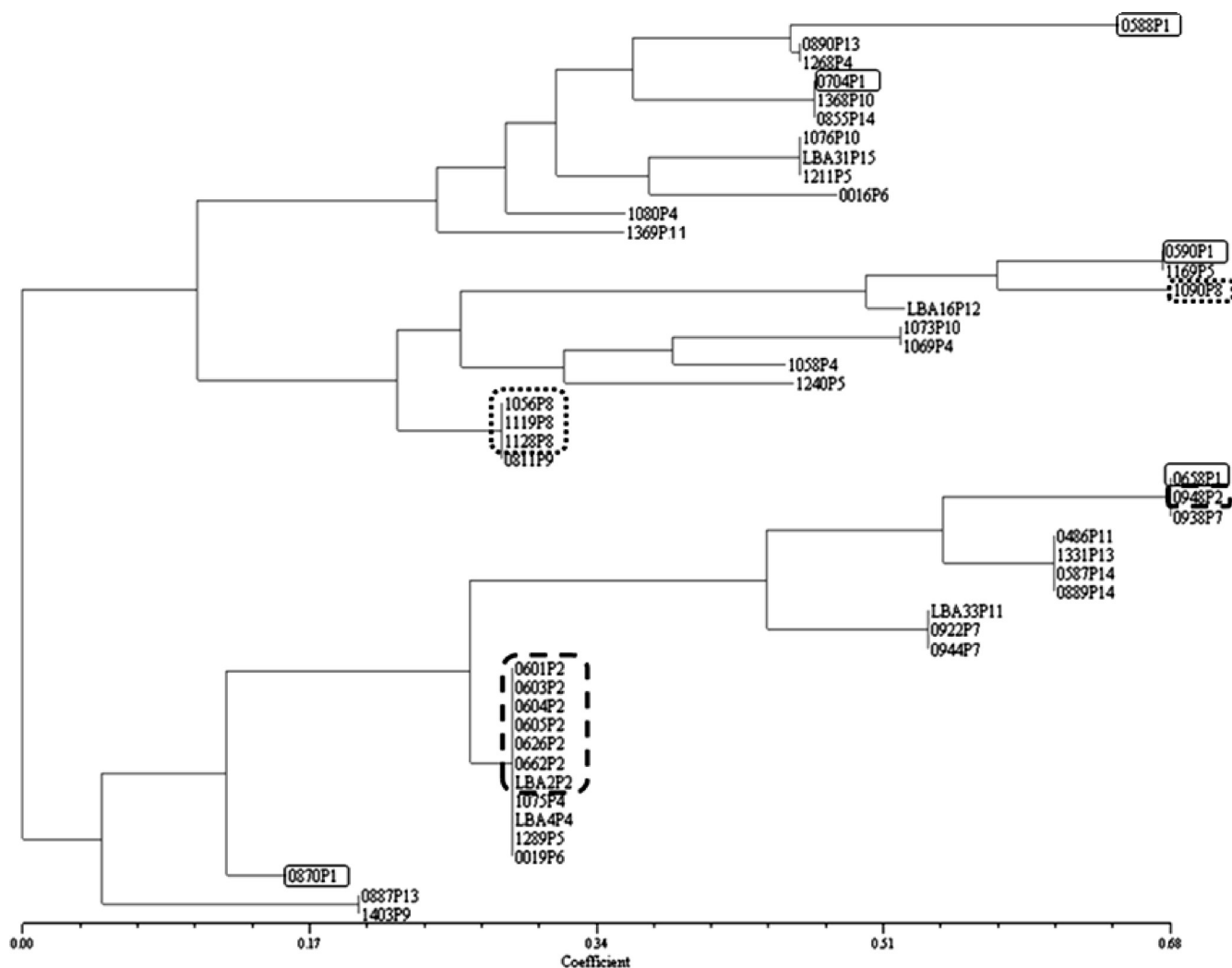


FIG. 3. Neighbor-joining dendrogram based on the Dice similarity coefficient upon analysis of five microsatellite makers in 48 *A. flavus* isolates from a hematology ward in Tunisia. The isolates are identified by the isolate number followed by the patient number (P1 to P15). The isolates are described in Table 1. The highlighted isolates from three patients and their environment illustrate two epidemiological scenarios: for P1 (square with solid-line border), each isolate (clinical and environmental) has a distinct genotype, indicating exposure to a genetically highly heterogeneous *A. flavus* population; for P2 (square with dashed-line border) and P8 (square with dotted-line border), clinical and environmental isolates share the same genotype, indicating exposure to a clonal *A. flavus* population.

duction, which we would like to refer to as a clonal burst, of the fungus occurred within the ward. Here this clonal burst was detected and probably occurred in P2's room. The genotype involved in this clonal burst was then occasionally identified in isolates sampled in the ward up to 1 year after this episode. Regrettably, aerobiological monitoring was lacking. Therefore, neither a concomitant increase in the number of airborne *A. flavus* spores in the ward nor the cause of this clonal burst could be documented to support this hypothesis.

Clinical and environmental isolates were independent in the majority of the patients. However, in P2 and P8 clinical and environmental isolates had identical genotypes, suggesting that *A. flavus* colonization and infection were hospital acquired in these two patients. Moreover, in P2 invasive aspergillosis can be traced to the above-mentioned clonal burst genotype. This finding supports the hypothesis that aerobiological monitoring might help promote actions to restrict, if not prevent, similar

clonal burst episodes inside the wards. From this perspective, highly discriminating typing methods such as the microsatellite typing scheme are advantageous in limiting false results and helping to locate the origin of outbreaks.

In conclusion, the novel microsatellite typing scheme described here demonstrated a significant geographical genetic structure in *A. flavus* and provided further insight into the hospital epidemiology of this emerging human pathogen. In the hematology unit described here, invasive aspergillosis could be traced to two main epidemiological scenarios. The first and most common scenario was colonization and infection by multiple genotypes, reflecting the high genetic diversity of *A. flavus* outdoors. The second was colonization and infection by a single genotype, most probably indicating a clonal burst episode. This clonal burst can be triggered by favorable local conditions, such as a water pipe leak in the patient's room. The first scenario points to the necessity of air filtration systems in

rooms where high-risk patients are hospitalized. The hypotheses underlying the second scenario point to the utility of implementing aerobiological monitoring in those rooms, but they need to be confirmed in further epidemiological studies.

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We declare that we have no conflict of interest.

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