

## Comparison of Two Highly Discriminatory Molecular Fingerprinting Assays for Analysis of Multiple *Aspergillus fumigatus* Isolates from Patients with Invasive Aspergillosis<sup>∇</sup>

Hanneke A. de Valk,<sup>1</sup> Jacques F. G. M. Meis,<sup>1</sup> Ben E. de Pauw,<sup>2,3</sup>  
Peter J. Donnelly,<sup>3</sup> and Corné H. W. Klaassen<sup>1\*</sup>

Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands,<sup>1</sup> and  
Department of Blood Transfusion and Clinical Immunology<sup>2</sup> and Department of Hematology,<sup>3</sup>  
Academic Medical Center Nijmegen St. Radboud, Nijmegen, The Netherlands

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**Two highly discriminatory fingerprinting assays, short tandem repeat typing and amplified fragment length polymorphism (AFLP), were compared to determine the genetic relatedness between 55 isolates of *Aspergillus fumigatus* obtained from 15 different patients suffering from proven invasive aspergillosis. Both techniques showed that interpatient isolates belonged to different genotypes and that inpatient isolates from deep sites were all of the same genotype. By contrast, multiple genotypes were found among isolates originating from respiratory samples. Both techniques have specific advantages and disadvantages. AFLP is more universally applicable, but short tandem repeat analysis offers better discriminatory power and should be the preferred method for standardizing typing of clinical isolates of *Aspergillus fumigatus*.**

Invasive aspergillosis (IA) is a life-threatening disease of patients who are immunocompromised by hematopoietic stem cell or solid organ transplantation, intensive chemotherapy, or treatment with corticosteroids (11). *Aspergillus fumigatus* is most frequently involved in causing this life-threatening disease. IA is a growing problem in hospitals because of the inexorable rise in the number of severely immunocompromised patients (12, 14). A better understanding of the epidemiology of this disease requires molecular techniques to investigate interstrain relatedness and strain dissemination (12).

Fingerprinting methods with high discriminatory power have to be applied to discriminate between unrelated isolates because of the wide genetic variability of *A. fumigatus*, (5) and several typing techniques have been described previously. Pattern-based techniques, such as random amplified polymorphic DNA (RAPD) analysis (2, 13), restriction fragment length polymorphism (RFLP) analysis (12, 19), and amplified fragment length polymorphism (AFLP) (20), have been employed but suffer from poor interlaboratory reproducibility and subjective interpretation of the fingerprinting data. Typing methods based on short tandem repeats (STRs), such as microsatellite length polymorphism (3) and STR $Af$  typing (8), yield unambiguous typing data, as do those based on sequence-based typing schemes, such as multilocus sequence typing. These methods may be preferable to other typing methods, provided that they possess sufficient discriminatory power, and may have the characteristics necessary for the standardization of *A. fumigatus* typing.

STR $Af$  typing is a recently described highly discriminatory

and potentially reproducible fingerprinting assay for *A. fumigatus* (8, 9), but its utility in epidemiological surveys has not yet been established. In this study, we compared AFLP and STR $Af$  typing to study the genetic relatedness between isolates of *A. fumigatus* from respiratory and tissue samples that had been obtained from 15 patients with proven IA (1). Previous studies have shown that the genotypes of isolates from inpatient samples of deep sites were identical (12), so these samples could be considered internal controls. We therefore set out to evaluate the advantages and disadvantages of both techniques in terms of their discriminatory power and applicability as well as technical aspects and computerized data processing.

### MATERIALS AND METHODS

**Isolates.** Fifty-five isolates of *A. fumigatus* were obtained from ante- and postmortem specimens of 15 patients with proven IA who had been nursed in the hematology ward of the Radboud University Nijmegen Medical Center in the period from 1992 to 1998 (Table 1). Isolates were stored as spore suspensions in regular microbial freezing broth containing 12.5% (vol/vol) glycerol at  $-80^{\circ}\text{C}$ . The isolates were revived by scraping off part of the sample, plating on Sabouraud's agar, and cultivation at  $30^{\circ}\text{C}$ . Fungal isolates were identified at the time of collection and again after revival by their macroscopic or microscopic appearance and their ability to grow at  $48^{\circ}\text{C}$ . Every isolate displayed normal growth characteristics after revival.

**DNA isolation.** Isolates were grown on Sabouraud's agar plates at  $30^{\circ}\text{C}$  until sporulation occurred. Spores were first mechanically lysed using the following procedures. A prewetted cotton swab was saturated with conidia from the sporulating culture. These spores were then suspended in a vial of MagNA Lyser green beads containing 350  $\mu\text{l}$  lysis buffer and 50  $\mu\text{l}$  proteinase K (all from Roche Diagnostics, Almere, The Netherlands). Lysis was performed in a MagNA Lyser instrument (Roche Diagnostics) for 30 s at 6,500 rpm. Next, the DNA was extracted and purified using a MagNA Pure LC instrument (Roche Diagnostics) in combination with MagNA Pure DNA isolation kit III according to the manufacturer's instructions. DNA yield and purity were estimated by UV absorbance measurements.

**STR $Af$  typing.** Nine STR markers specific for *A. fumigatus* were analyzed using the STR $Af$  primers and reaction conditions as previously described (8). Briefly, three multiplex PCRs each amplified three STR markers. The obtained PCR products were separated by size and detected on a MegaBACE 500 platform equipped with a 48-capillary array (GE Healthcare, Diegem, Belgium). The

\* Corresponding author. Mailing address: Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Weg door Jonkerbos 100, 6532 SZ Nijmegen, The Netherlands. Phone: 31-24-3657514. Fax: 31-24-3657516. E-mail: c.klaassen@cwz.nl.

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TABLE 1. Origin of *A. fumigatus* isolates from patients with IA

Patient	Origin	Date of isolation (day-mo-yr)	AFLP type	STR <i>Af</i> type	Sample
A	Bronchial secretion	22-07-1992	1	1	A1
	Tissue	25-07-1992	1	1	A2
B	Bronchial secretion	26-08-1992	2	2	B1
	Bronchial secretion	28-08-1992	2	2	B2
	Pleural abscess	31-08-1992	2	2	B3
C	Bronchial secretion	15-01-1993	3	3	C1
	Lung biopsy sample	19-01-1993	3	3	C2
	BAL fluid	19-01-1993	3	3	C3
D	Left lung	28-12-1993	4	4	D1
	Right kidney	28-12-1993	4	4	D2
	Left kidney	28-12-1993	4	4	D3
E	Feces	01-06-1994	5	5	E1
	Pharynx swab	06-06-1994	5	5	E2
	Feces	09-06-1994	5	5	E3
	Pharynx swab	13-06-1994	5	5	E4
	Sputum	20-06-1994	5	5	E5
	Lung	13-07-1994	5	5	E6
F	Pharynx swab	04-08-1994	6	6	F1
	Right lung	18-08-1994	6	6	F2
	Left kidney	18-08-1994	6	6	F3
	Left lung	18-08-1994	6	6	F4
G	BAL fluid	28-10-1994	7	7	G1
	Lung	04-11-1994	8	8	G2
H	Sputum	18-04-1995	9	9	H1
	Left lung	02-05-1995	10	10	H2
I	Pus	29-08-1995	11	11	I1
	Feces	31-08-1995	11	11	I2
	Pharynx swab	04-09-1995	11	11	I3
	Feces	14-09-1995	11	11	I4
J	BAL fluid	16-10-1995	12	12	J1
	Right lung	25-10-1995	12	12	J2
	Left lung	25-10-1995	12	12	J3
K	Feces	29-03-1996	13	13	K1
	Left lung	01-04-1996	13	13	K2
	Right kidney	01-04-1996	13	13	K3
	Heart	01-04-1996	13	13	K4
	Left kidney	01-04-1996	13	13	K5
	Right lung	01-04-1996	13	13	K6
	Spleen	01-04-1996	13	13	K7
	Liver	01-04-1996	13	13	K8
L	Bronchial secretion	02-01-1997	14	14	L1
	Bronchial secretion	03-01-1997	14	14	L2
	Bronchial secretion	09-01-1997	14	14	L3
	Right lung	13-01-1997	14	14	L4
M	Lung biopsy sample	26-03-1997	15	15	M1
	BAL fluid	26-03-1997	16	16	M2
	BAL fluid	26-03-1997	17	17	M3
	Lung biopsy sample	26-03-1997	18	15	M4
N	Pharynx swab	11-12-1997	13	18	N1
	Left lung	17-12-1997	13	18	N2
	Spleen	17-12-1997	13	18	N3
	Liver	17-12-1997	13	18	N4
O	BAL fluid	19-01-1998	19	19	O1
	BAL fluid	19-01-1998	20	20	O2
	Left lung	23-01-1998	19	19	O3

assignment of repeat numbers in each marker was performed using Fragment Profiler 1.2 software (GE Healthcare).

**AFLP.** Approximately 50 ng of genomic DNA was subjected to a combined restriction-ligation procedure containing 50 pmol of HpyCH4 IV adapter, 50 pmol MseI adapter, 2 U of HpyCH4 IV (New England Biolabs, Beverly, MA), 2 U of MseI (New England Biolabs), and 1 U of T4 DNA ligase (Promega, Leiden, The Netherlands) in a total volume of 20  $\mu$ l of 1 $\times$  reaction buffer for 1 h at 20°C. Next, the mixture was diluted five times with 10 mM Tris-HCl (pH 8.3) buffer. Adapters were made by mixing equimolar amounts of complementary oligonucleotides (5'-CTCGTAGACTGCGTACC-3' and 5'-CGGGTACGCAGTC-3' for HpyCH4 IV; 5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTC AT-3' for MseI) and heating to 95°C, subsequently cooling slowly to ambient temperature. One microliter of the diluted restriction-ligation mixture was used for amplification in a volume of 25  $\mu$ l under the following conditions: 1  $\mu$ M HpyCH4 IV primer with one selective residue (underlined) (5'-Flu-GTAGACT GCGTACCGTC-3'), 1  $\mu$ M MseI primer with four selective residues (underlined) (5'-GATGAGTCCTGACTAATGAA-3'), 0.2 mM of each deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase (Roche Diagnostics) in 1 $\times$  reaction buffer containing 1.5 mM MgCl<sub>2</sub>.

Amplification was done as follows. After an initial denaturation step for 4 min at 94°C in the first 20 cycles, a touchdown procedure was applied: 15 s of denaturation at 94°C, 15 s of annealing at 66°C, with the temperature for each successive cycle lowered by 0.5°C, and 1 min of extension at 72°C. Cycling was then continued for a further 30 cycles with an annealing temperature of 56°C. After completion of the cycles, an incubation at 72°C for 10 min was performed before the reaction mixtures were cooled to room temperature. The amplicons were then combined with the ET400-R size standard (GE Healthcare) and analyzed on a MegaBACE 500 automated DNA platform (GE Healthcare) according to the manufacturer's instructions.

**Data analysis.** All data were analyzed with the BioNumerics 4.5 unweighted-pair group method using average linkage clustering (Applied Maths, Sint-Martens-Latem, Belgium), employing the multistate categorical similarity coefficient for STR data and the Pearson correlation coefficient for DNA fragments between 60 and 250 bases for AFLP analysis.

## RESULTS

All AFLP fingerprints obtained with MseI and HpyCH4 IV contained multiple bands in the range of 50 to 250 bp (Fig. 1), and strong and weak bands were visible. When all samples were compared to each other, the fingerprints consisted of some 30 invariable bands present in all samples and around 20 variable bands which were present only in certain samples. Based on the variable bands, the different fingerprints can be distinguished from each other. In total, 20 visually recognizable different patterns were obtained. The calculated similarity for visually identical patterns ranges from 97 to 99%. The calculated similarity for patterns with visually recognizable differences ranges from 89 to 99%. Originally, the collection consisted of 56 isolates. However, one isolate yielded a fingerprint without resemblance to any of the other isolates. The reidentification of this isolate by internal transcribed spacer sequencing showed that this particular isolate proved to be *Neosartorya fischeri* (results not shown). This isolate was excluded from further analysis.

Using STR*Af* typing, all *A. fumigatus* isolates yielded a numerical typing result (Fig. 1). A total of 20 different genotypes were obtained with this technique. The *N. fischeri* isolate did not yield PCR products with the STR*Af* assay.

In general, there is an excellent agreement between the two typing methods, with only two tissue isolates (M1 and M4) of patient M that were differentiated by AFLP but not by STR*Af* typing, which yielded the same genotype. AFLP yielded the same genotype for all isolates of patients K and N, but these could be clearly distinguished from each other using STR*Af* typing. When all inpatient isolates were compared to each

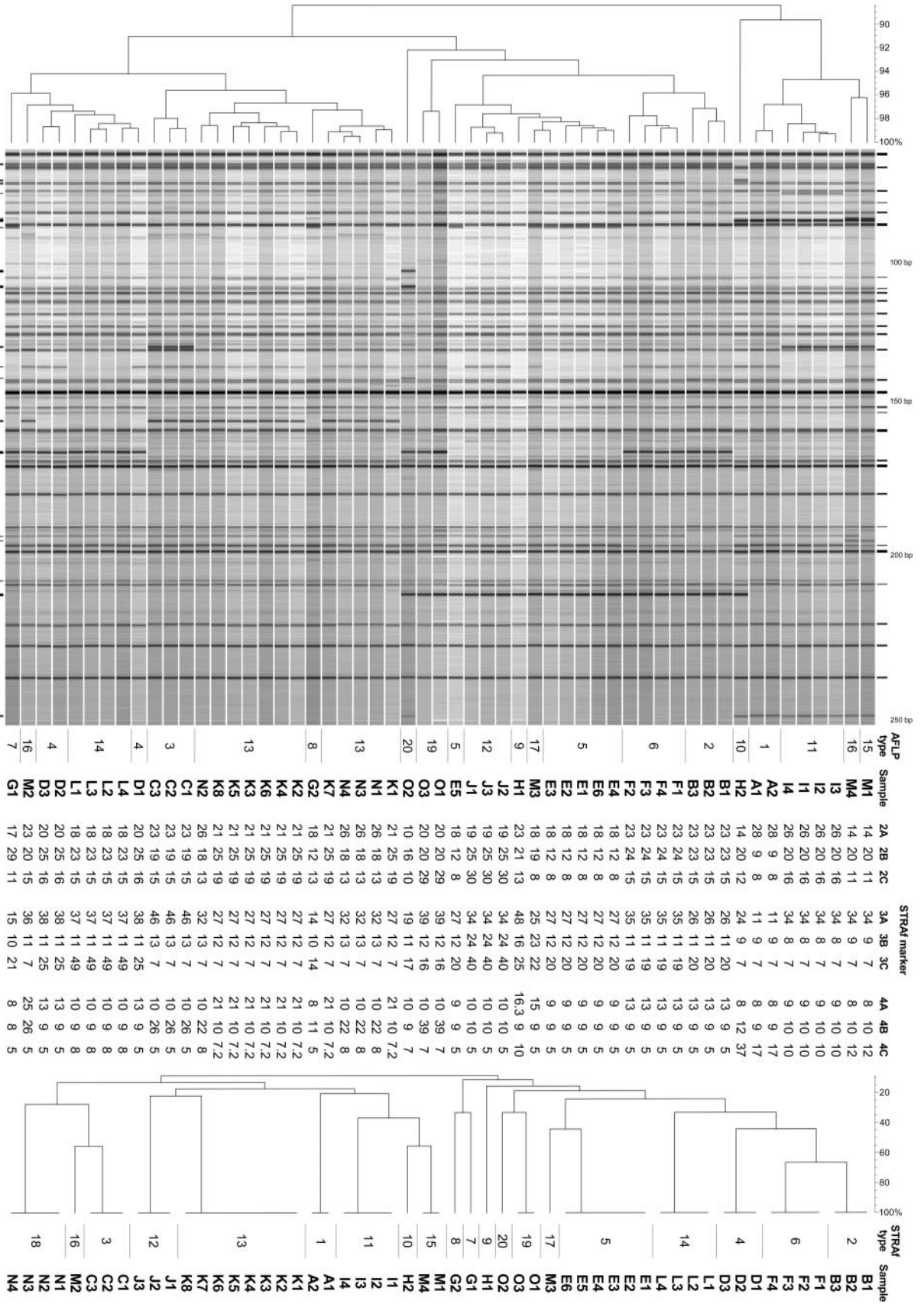


FIG. 1. Results of both AFLP and STR4f typing on a collection of 55 clinical *A. fumigatus* isolates. The dendrogram on the left is based on the AFLP fingerprints. The indicator lines above the traces represent invariable bands, whereas the indicator lines below the traces represent variable bands in the AFLP fingerprints. After each fingerprint are the typing results for the nine markers of the STR4f panel. The dendrogram on the right is based on the STR4f results. Types were assigned arbitrarily, starting with the oldest isolate. The scale bars indicate the percentages of similarity. Sample numbers correspond to those in Table 1.

other, samples of nonrespiratory origin belonged to the same genotype. By contrast, multiple genotypes were detected in isolates originating from respiratory samples, such as sputum and bronchoalveolar lavage (BAL) fluid samples, from the same patient. For instance, all eight isolates from the lung, kidney, heart, spleen, and liver of patient K shared the same STRA*f* genotype. An identical unique genotype was found in isolates from BAL fluid and lung samples obtained from patient O, whereas another BAL fluid sample yielded a different genotype. A similar observation was made for patients G, H, and M, as interpatient isolates belonged to different genotypes as determined by STRA*f* typing.

## DISCUSSION

We compared two high-resolution PCR fingerprinting assays for analyzing the epidemiological relationships between multiple *A. fumigatus* isolates from patients with proven IA. Both AFLP analysis and STRA*f* analysis provided high-resolution data allowing discrimination between clinical isolates from different patients and between different genotypes in multiple samples from a patient. In general, interpatient isolates belonged to different genotypes whereas inpatient isolates were of the same genotype. Cross-infections of respiratory origin or common-source infections were highly unlikely since the patients were nursed at different times in the ward.

The use of molecular epidemiological tools such as RAPD, multilocus enzyme electrophoresis, and sequence-specific DNA primer analyses (10) has led to suggestions that *A. fumigatus* isolates from several IA patients nursed in the same ward may originate from a single environmental source. However, these techniques lack discriminatory power and are not considered by us to be sufficiently reproducible to employ for this study. Rather, our results confirm the results reported by Girardin et al. (12) and Denning et al. (7), who investigated the genetic diversity of patients with IA by using RFLP and REA analyses, respectively. Our results are consistent with a scenario in which the respiratory tract may be colonized by several different genotypes, as inhalation of fungal conidia is assumed to be the primary means of acquiring IA (11), but a single genotype gains supremacy and invades the surrounding tissue, subsequently disseminating to the lung tissue or other organs. Thus, an isolate obtained from respiratory samples may not necessarily represent the same genotype as that found in deep sites, as appeared to be the case for patients G, H, M, and O. This also confirms that individuals may well be constantly exposed to a large variety of different *Aspergillus* genotypes from the environment (5, 15).

Both AFLP and the STRA*f* assay are seen as high-resolution and robust molecular fingerprinting assays for *A. fumigatus* isolates (8, 9, 20). As shown here, both techniques are suitable for unraveling the genetic relationship between clinical *A. fumigatus* isolates, with good correlation between the results of the two assays. Yet, compared to each other, both assays have their advantages and disadvantages. A clear advantage of the STRA*f* assay over AFLP is its ability to identify mixtures of strains. Mixed genotypes are recognized instantaneously by the presence of multiple peaks in each of the nine markers analyzed (8). Mixed AFLP genotypes are extremely difficult, if not impossible, to detect. Thus, when employing AFLP analysis for

epidemiological analysis, it is important that pure cultures be obtained and analyzed. Assuming that individuals are, in fact, likely to be colonized by more than one genotype, it is important to subculture and analyze each colony separately when several are obtained from a respiratory sample in order to prevent false conclusions from being drawn (4, 6).

DNA fragments are amplified in AFLP from random locations throughout the entire genome and, although in a single AFLP experiment only a very small fraction of the entire genome is analyzed (<0.1%), the amount analyzed by the STRA*f* assay is even smaller. In addition, unlike the AFLP assay, the STRA*f* approach is highly biased toward nine arbitrarily selected loci, so any genomic change outside these loci will remain undetected, as was the case for isolates M1 and M4. The AFLP technique is basically applicable to any organism's genome without the need for prior sequence information. However, depending on the genome size and GC content, there may be a need for optimizing the choice of restriction enzymes and selective residues. By contrast, the STRA*f* assay is specific for *A. fumigatus* and requires specific sequence information for the detection of STR loci and flanking sequences to be used as primer binding sites. However, both techniques do permit improperly identified isolates to be recognized. The nonvariable bands that are obtained with *A. fumigatus* isolates may represent species-specific markers. Upon analysis of isolates from species other than *A. fumigatus*, most of these invariable bands will not show up and a banding pattern will be obtained that is usually less than 30% similar to the fingerprint of *A. fumigatus* isolates (as was the case for the *N. fischeri* isolate). Likewise, using the STRA*f* approach, improperly identified isolates can be recognized by the absence of typical amplification products, provided that technical issues that may lead to PCR failure can be ruled out.

In order to compare the different AFLP fingerprints to each other and to determine the genetic relatedness between multiple isolates, one has to rely on specialized computer software. AFLP fingerprints are usually analyzed using a pattern-based algorithm. However, repeat analyses may show small differences in the AFLP fingerprint due to small variations occurring during restriction/ligation, PCR amplification, and fragment analysis that may affect the final peak intensity. For the same reasons, the long-term reproducibility and interlaboratory comparisons of AFLP fingerprints may be quite challenging. Thus, identical samples are seldom identical using a pattern-based approach, although they may appear to be so by visual examination. In the pattern-based dendrogram shown in Fig. 1, visually identical fingerprints were calculated to be 97 to 99% similar (i.e., all samples of patient K). However, in the same analysis, samples with visually recognizable differences were also calculated to be up to 99% similar (i.e., samples D1 and L4) (Fig. 1). Consequently, a gray zone was obtained during interpretation of the results of a pattern-based approach in which the wrong conclusions may be drawn when relying solely on the computer-based dendrogram. This requires a visual inspection of the fingerprints to be included in the final interpretation. Similar limitations apply to computer-based analysis of other pattern-based fingerprinting methods, such as RAPD and RFLP analyses. By contrast, due to the more exact nature of STRA*f* typing, intralaboratory reproducibility is virtually 100% and the typing result is unambiguous.

Due to the large number of available restriction enzymes, an almost indefinite number of restriction enzyme combinations can be used to generate an AFLP fingerprint. The success of a particular combination is dictated primarily by the size and composition (i.e., GC content and the presence of multicopy elements) of the genome to be investigated. The number of fragments to be obtained can be modulated by changing the number of selective residues. In previous studies, other restriction enzyme combinations such as EcoRI and MseI (16, 20) and EcoRI and BfaI (17) have been used to generate AFLP fingerprints for *Aspergillus* spp. To rule out the possibility that the results of this study may have been influenced by the specific use of the combination of HpyCH4 IV and MseI, all isolates were also tested with the combination of EcoRI and MseI. Although obviously completely different fingerprints were obtained, the same interstrain relatedness was observed (data not shown).

A problem with any typing method is the subjective interpretation of a "genotype." The assignment of a genotype is user dependent using AFLP, whereas the genotype resulting from STR*Af* typing is unambiguous. Nonetheless, two different but highly similar fingerprints could originate from two closely related isolates. Unfortunately, at present there are no established guidelines for defining a genotype for these methods (such as the criteria of Tenover et al. for the interpretation of pulsed-field gel electrophoresis fingerprints [18]). However, since there is an excellent correlation between the groupings of genotypes by using AFLP and STR*Af* typing, we believe that the differences between the fingerprints of the isolates in this study, although minor, are substantial enough to validate our assignment of genotypes. For instance, isolates E3 and M3 differed by only one band in the AFLP fingerprint but by five markers in the STR*Af* assay. This means that even a single band difference between two AFLP fingerprints could be interpreted as constituting different genotypes. Likewise, with the STR*Af* assay, any two different genotypes differ by a minimum of three markers. For instance, isolates from patients B and F differed by one repeat in marker 2B, nine repeats in marker 3A, and one repeat in marker 3C. In the AFLP fingerprint, there were at least two visually recognizable differences between these isolates. At present, it is unclear whether fewer than three differences in an STR*Af* genotype should also be interpreted as different genotypes, so more closely related isolates need to be analyzed.

In conclusion, both AFLP and STR*Af* typing are excellent techniques for analyzing the interstrain relatedness of *A. fumigatus* isolates. Each of the two techniques has its specific advantages and disadvantages. Therefore, the choice should be based primarily on the specific goal for analyzing collections of isolates. STR*Af* typing offers the most benefits for the standardization of typing *A. fumigatus* isolates.

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