

Different colonization patterns of *Aspergillus terreus* in patients with cystic fibrosis

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Abstract

Aspergillus terreus is a common soil saprophyte. After *Aspergillus fumigatus* and *Scedosporium apiospermum* it ranks third amongst the filamentous fungi colonizing the airways of patients with cystic fibrosis. In this context, the clinical presentation of *A. terreus* infection mainly corresponds to allergic broncho-pulmonary aspergillosis. In the work presented here, we studied colonization patterns of *A. terreus* in CF patients by genotyping using nine short tandem repeat markers. A total of 115 clinical isolates from respiratory secretions collected from five French CF patients were studied. The number of isolates varied from 15 to 39 per patient, and the duration of the follow-up period ranged from 2 months to 7.5 years. Seventeen genotypes were identified, corresponding to three distinct colonization patterns. The first colonization pattern consisted of a chronic colonization by one dominant genotype associated with few other genotypes found only incidentally. The second colonization pattern consisted of a prolonged colonization by two distinct genotypes detected simultaneously. The last pattern was characterized by multiple different genotypes that were present only transiently. These results demonstrate the importance of genotyping clinical isolates before making conclusions about chronic colonization of the airways in CF patients in the case of repeated isolation of the fungus.

Keywords: *Aspergillus terreus*, colonization pattern, cystic fibrosis, microsatellite typing

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Introduction

Patients with cystic fibrosis (CF) are at high risk of colonization of the airways by various microorganisms. This may lead to true infections, which are the major cause of morbidity and mortality in these patients [1]. Bacteria, particularly *Staphylococcus aureus* and *Pseudomonas aeruginosa*, cause acute pulmonary exacerbations. Nevertheless, fungal colonization of the airways, which is rarely found in young

children, is increasingly gaining attention. Bronchoalveolar epithelial lesions resulting from bacterial infections seem to be required for the establishment of fungi in the respiratory tract of CF patients [2]. *Aspergillus fumigatus* is by far the most common filamentous fungus colonizing the airways of CF patients [3], but the fungal biota is obviously much more complex. The *Scedosporium apiospermum* species complex and *Exophiala dermatitidis* are among the most clinically relevant other fungi recovered from respiratory secretions of CF patients [4,5] but other *Aspergillus* species may also be encountered in this context. Whereas *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus nidulans* were usually found transiently, *Aspergillus terreus* is also reported as a chronic colonizer of the airways of CF patients. This fungus, the prevalence of which ranges from 1.9 to 6.2%, ranks third among the filamentous fungi colonizing the airways of CF patients [6,7]. Clinical signs and symptoms associated with

A. terreus colonization are mainly associated with allergic broncho-pulmonary aspergillosis.

Aspergillus terreus is a common soil saprophyte, previously described in desert soils and grasslands as well as a contaminant of stored corn. Its relative paucity in the environment compared with other fungi such as *A. fumigatus* contrasts with its relatively high occurrence in the CF context. Little is known about the epidemiology of this fungus. Several genotyping methods have been used before, including random amplified polymorphic DNA (RAPD) [7–10], repetitive-sequence-based PCR (rep-PCR) [11] and Inter-Simple Sequence Repeat (ISSR) PCR [12], which demonstrated extensive genotype diversity in *A. terreus* species. However, these techniques, based on complex banding pattern analysis, often have poor inter-laboratory reproducibility and do not allow exchange of results between laboratories [13]. Balajee [14] applied limited multi-locus sequencing typing (MLST) based on three genes to study *A. terreus* diversity and defined a new genotypically distinct species, *Aspergillus alabamensis*. Disadvantages of this sequencing approach are its moderate ease of use, its limited discriminatory power and its higher cost. Microsatellite-based typing, also referred to as short tandem repeat (STR)-based typing, involves analysis of the number of tandem repetitions of short repetitive sequences at specific loci yielding a numerical and exact typing result. Such assays are already well-established for other *Aspergillus* species such as *A. fumigatus* and *A. flavus* [15–17] and offer multiple advantages such as a high discriminatory power, high reproducibility and easy exchange of results. Whereas genus-wide microsatellite markers seem to contain only limited allelic variation [18], species-specific microsatellite markers often show extensive allelic variation and offer high discriminatory power. Here, we report for the first time on the use of a microsatellite-based typing method designed for *A. terreus* to investigate its molecular epidemiology by studying colonization patterns of this fungus in CF patients.

Materials and methods

Isolates and culture conditions

One hundred and fifteen clinical isolates of *A. terreus* were recovered from 45 respiratory secretions (sputum or bronchial aspiration samples) collected from five CF patients followed-up in hospitals in Angers (patients A, B, C and E) and Giens (patient D). All *A. terreus* isolates recovered from the samples were preserved by freeze-drying in our culture collection after confirmation of the identification. The number of available isolates for genotyping varied from 15 to 39 per patient, and the duration of the follow-up ranged from

2 months to 7.5 years (Table S1). Twenty-five additional clinical samples from the same patients were collected for mycological analysis. Eight of them were culture-negative for *A. terreus*. Four out of these eight samples were culture-positive for *A. fumigatus*. Consequently, due to its more rapid and more extensive growth, we cannot rule out that *A. fumigatus* may have masked the presence of more slowly growing fungi such as *A. terreus*. For the other samples, *A. terreus* isolates were not available for microsatellite analysis, mainly because of the simultaneous presence of *A. fumigatus*, which impeded isolation of pure *A. terreus* colonies.

Isolates were maintained on yeast extract-peptone-dextrose agar (YPDA) plates supplemented with 0.5% chloramphenicol. Cultures were incubated at 37°C. Identification was established by cultural features according to standard description and microscopic investigation after mounting in lactic blue stain. For DNA extraction, isolates were grown in yeast extract-peptone-dextrose (YPD) broth.

Genomic DNA extraction

Mycelium was harvested from 7-day-old cultures in YPD broth (10 mL) and ground in liquid nitrogen with a mortar and pestle. DNA was recovered using the DNeasy plant mini kit (Qiagen Inc., Valencia, CA, USA) protocol with minor modifications as described previously [19]. The DNA concentration was estimated using a Nano-Drop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) at 260 nm.

Microsatellite typing

Nine short tandem repeat (STR) markers were selected from the available genomic sequence of *A. terreus* NIH 2624 using selection criteria previously reported for *A. fumigatus* [16]. Three multiplex PCRs, identified as M2, M3 and M4, were performed amplifying three di-, tri- or tetranucleotide repeat markers, respectively. For each marker, one of the primers was labelled at the 5' end with either carboxyfluorescein (FAM), hexachlorofluorescein (HEX) or tetramethylrhodamine (TAMRA). Primer concentrations were optimized (Table 1) and PCR conditions were carried out using reaction conditions as described previously [16]. The amplification programme consisted of 10 min of denaturation at 95°C, followed by 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. After an additional incubation for 10 min at 72°C, reaction mixtures were cooled to room temperature. PCR products were diluted 30-fold with distilled water. One microlitre of diluted products was combined with 0.25 µL of ET-ROX 400 marker (GE Healthcare, Diegem, Belgium). Amplicons were then separated by size and detected on a MegaBACE 500 automated DNA analysis platform equipped with a 48-capillary

TABLE 1. Basic features of the selected microsatellite markers and primers used

Multiplex panel	Marker	Primer sequences	Repeat unit	Allelic range ^a	Primer concentration (μM)
M2	STRat-2A	FAM- TGGTACTGCTCCGAGTTTCC GACGAGACGAAGAGACCAAGG	GT	4–34	0.2 0.2
	STRat-2B ^b	HEX- CGCAAGACCATCCGAAGTAT GCTGGATAGGCGATGGAG GCTGGATAGGCGAGGG	TC	6–43	0.1 0.1 0.1
	STRat-2C	TAMRA- GCGGTGAGGAGAAGAAGATG GGCTTCCCTCTGTGTACTG	TC	7–40	1 1
M3	STRat-3A	FAM- CCTGTATCGTCGGGAAAGAA GATCGCTGGAGGAGATGGTA	AGT	3–21	1 1
	STRat-3B	HEX- TTTCTTCTGGCCTCGCTCTA GCGGAGCGACTCTCTAGTGA	ATG	3–24	0.1 0.1
	STRat-3C	TAMRA- TCGGCGACGTAGCAAATTAC GAGTAGGGAATACCCGCACA	GTA	5–67	1 1
M4	STRat-4A	FAM- GATGCAGGTGGACATAAGCA GGGGTAAGTTGCGTTCCATA	ACAT	3–11	0.1 0.1
	STRat-4B	HEX- TGGCTGGAATAGGGGTACA GCATGCATTGGGCTATTTA	TTTC	3–42	0.5 0.5
	STRat-4C	TAMRA- ATCCTAGTGCATGGGTCCAG GAACGCGAGAGAGATGGAAGA	TCTT	3–15	1 1

^aThe allelic range for each marker was determined using a larger panel of ~400 *A. terreus* isolates (CHW Klaassen et al., unpublished observations).

^bDue to sequence variations in the binding site of the unlabelled primer for this marker, initially a significant fraction of isolates did not yield an amplification product. Therefore a third amplification primer was included for this marker to cover this variation.

array (GE Healthcare), as recommended by the manufacturer. Repeat numbers in each marker were assigned using Genetic Profiler v2.2 (GE Healthcare) using *A. terreus* NIH 2624 as a reference. The genotype of NIH 2624 is 19-19-24-15-21-18-9-9-8 for markers 2A-2B-2C-3A-3B-3C-4A-4B-4C, respectively. Genotyping data were imported and analysed using BioNumerics v6.0 (Applied Math, St Martens-Latum, Belgium).

Definitions

Isolates were considered to be (i) identical when all nine markers yielded corresponding repeat numbers, (ii) genetically closely related when their genotypes differed in only one marker and (iii) unrelated if their genotypes differed in more than one marker. A patient was considered to be chronically colonized when identical or closely related genotypes were recovered from at least three distinct clinical samples collected over a period of more than 6 months. For a shorter period (ranging from 2 to 6 months) the term 'prolonged colonization' was used.

Results

A total of 115 isolates collected from five CF patients were analysed using the nine microsatellite marker panel. Seventeen distinct genotypes were identified (Table S2 and Fig. 1). Among them, five genotypes (2, 3, 6, 9 and 17) were found only once, and three genotypes (8, 10 and 15) were shared between isolates collected from distinct patients (patients C and E, patients A and E and patients A and C, respectively). In addition, two small clusters were observed of closely related genotypes that differed from each other in a single marker.

Isolates contained in these clusters were recovered from clinical specimens collected from the same patient (cluster of genotypes 4, 5 and 6) or not (cluster of genotypes 14 and 15).

The five patients showed three different colonization patterns. First, a chronic colonization by largely one dominant genotype was observed in patients A and B (Fig. 2). Regarding patient A, 35 out of the 39 isolates of *A. terreus* studied were of the same genotype (Fig. 2a). These isolates were recovered over a 2-year period (from March 2004 to March 2006) and a 6-month period (from June to December 2006). Between these two periods, two other sputum samples were culture-positive for *A. terreus* on 21 March 2006 and 13 April 2006, but corresponding isolates were not available for microsatellite analysis. Therefore, one may speculate for this patient that there was a chronic colonization of the airways by a specific genotype over a 3-year period. Three other genotypes were found only once (genotypes 2 and 15) or twice (genotype 11) over the study period. For patient B, 27 sputum samples were collected over a period of 7.5 years. Of them, 22 were culture-positive for *A. terreus* and 24 isolates were available for genotyping study (Fig. 2b). Only two different genotypes were identified (genotypes 1 and 13). While recurrent isolation occurred over a 7.5-year period for genotype 1 and a 22-month period for genotype 13 (from January 2008 to October 2009), only one episode of chronic colonization of the airways was clearly demonstrated for genotype 1 from November 2006 to January 2007. However, seven other sputum samples were also culture-positive for *A. terreus*, but these isolates were not available for microsatellite analysis. In addition, three of the five *A. terreus* culture-negative samples grew *A. fumigatus*, which could have masked the presence of *A. terreus* in primary plates. The possibility of a longer duration of chronic colonization by

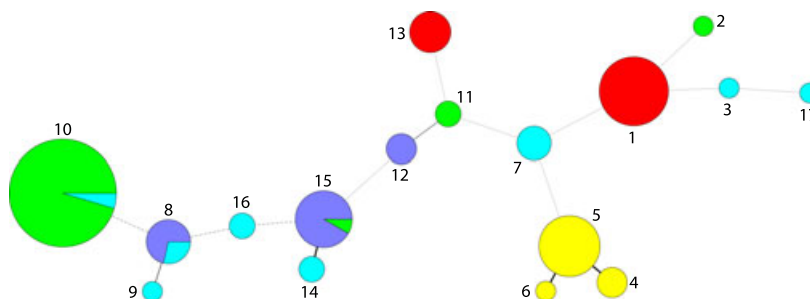


FIG. 1. Minimal spanning tree representing the observed genotypic diversity. Each colour represents a different patient. Patient A, green; patient B, red; patient C, blue; patient D, yellow; patient E, cyan. Each circle corresponds to a unique genotype. The size of each circle corresponds to the number of isolates with the same genotype. Connecting lines correspond to the number of different markers between the genotypes: short thick line, 1 marker difference (out of nine); regular line, 2 markers difference; dashed line, 3 markers difference; dotted line, 4 or more markers difference. For instance, in patient D (yellow) three different genotypes were observed that differed in only 1 marker. Numbers correspond to genotype numbers mentioned in the text.

genotype 1, from May 2005 to November 2008, followed by a chronic colonization with genotype 13 from January 2008 to October 2009, therefore cannot be ruled out.

The second colonization pattern consisted of a prolonged colonization by distinct (patient C) or closely related (patient D) genotypes (Fig. 3). The 19 isolates recovered from 12 sputum samples collected from patient C belonged to three different genotypes (Fig. 3a). Genotype 12 was found only once while genotypes 8 and 15 were isolated three times over a 4-month period and five times over a 3-month period, respectively. Co-colonization by these two genotypes was observed in April and May 2005. As some isolates were not available for microsatellite analysis, genotype 15 and/or genotype 8 may have been responsible for a chronic colonization over a longer period. Regarding patient D, three closely related genotypes that differed in only one marker as a possible result of micro-evolutionary changes were identified over the 2-month collection period among the 18 isolates recovered from four sputum samples (Fig. 3b). Genotypes 4 and 5 were recovered from four and three sputum samples, respectively, thus suggesting for patient D a prolonged colonization of the airways by these two related genotypes.

Finally, patient E demonstrated a totally different pattern of colonization with *A. terreus* because none of the genotypes identified for this patient seemed to be involved in a chronic or prolonged colonization as defined in the Materials and Methods section (Fig. 4). Eight genetically distinct genotypes were identified among the 15 isolates obtained from five sputum samples over a 4-year collection period. Only genotypes 7 and 14 were found twice, but separated in time by a culture-negative sample. *Aspergillus fumigatus* was found in only one of the three *A. terreus* culture samples. These results suggest carriage of various genotypes found transiently in the

respiratory secretions and not a real chronic colonization of the airways.

Discussion

A panel of nine short tandem repeat markers was used to study the epidemiology of *A. terreus* in cystic fibrosis patients. The STR analysis divided our 115 isolates recovered from 45 sputum samples into 17 genotypes. In agreement with previous studies [9,20], the isolates from the different patients showed a considerable genetic diversity. Nevertheless, two clusters of isolates with almost identical genotypes were observed, suggesting clonal expansion and/or micro-evolutionary events. Micro-evolution has already been suggested for *A. fumigatus* isolates in the CF context [21] and also for *A. flavus* in Indian clinical isolates [22].

Whereas previous studies in the CF context described colonization of the respiratory tract of the patients by a single genotype of *A. terreus* [7,10], we reported here three distinct colonization patterns: (i) a chronic colonization by a predominant genotype, (ii) a prolonged colonization by two genetically distinct or closely related genotypes and (iii) transient carriage of different genotypes. The discrepancy with regard to previous studies may be due to the limited number of isolates analysed in previous studies and/or lack of sufficient discriminatory power of previously used methods. Our results highlight the need to collect multiple isolates per sample and use highly discriminatory typing methods during mycological analysis for a better knowledge of the epidemiology. Isolates included in this retrospective study were recovered from 45 sputum samples and two or three genotypes were found simultaneously from 20% to 7% of these samples, respectively.

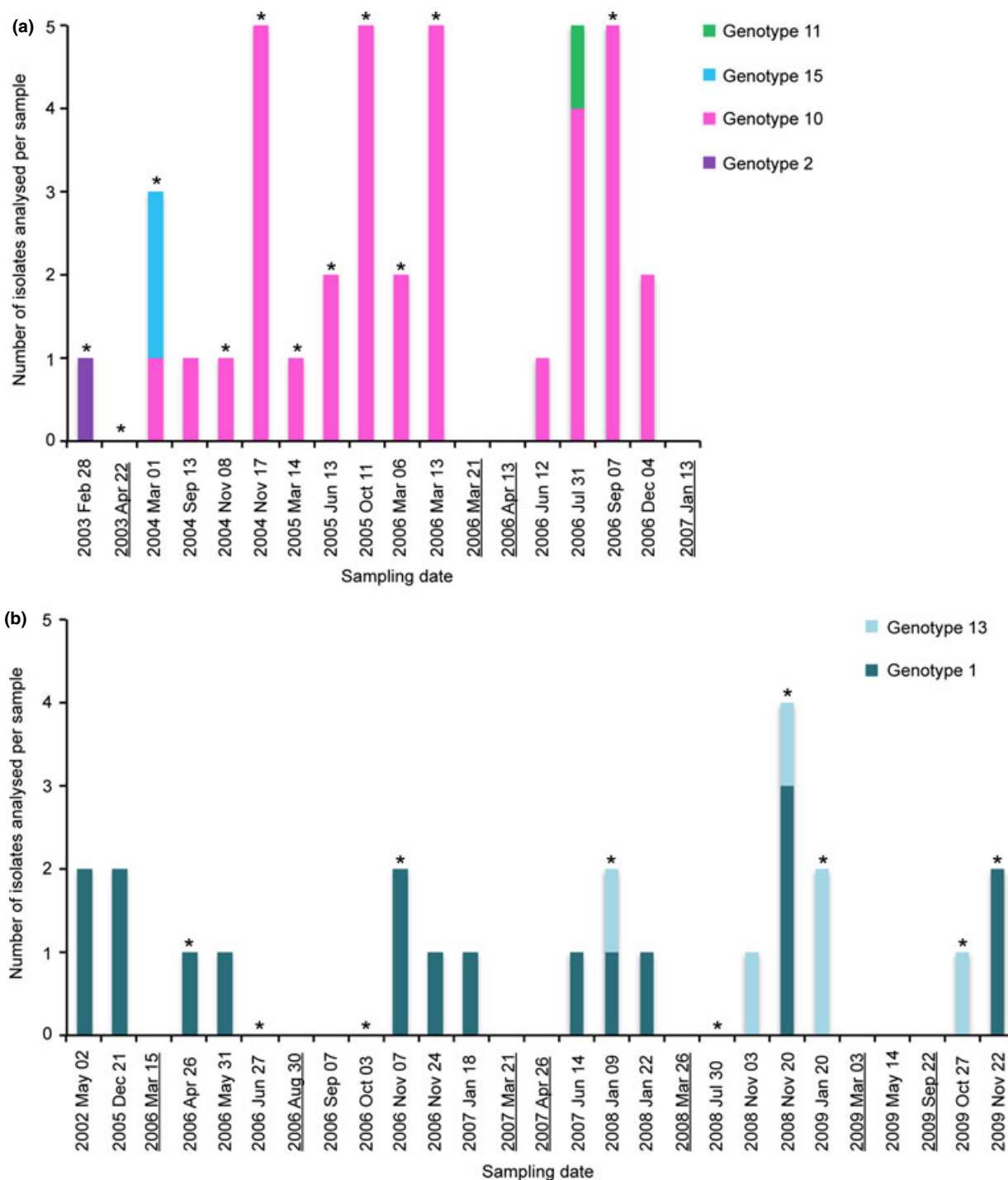


FIG. 2. Chronic colonization by a largely dominant genotype observed in patient A (a) and patient B (b). Genotypes are represented by distinct colours. Underlined samples were culture-positive for *Aspergillus terreus*, but the isolates were not available for microsatellite analysis. An asterisk indicates a sample positive for *Aspergillus fumigatus*.

This phenomenon was previously described in CF patients colonized by *A. fumigatus* [23], and in a few patients with an invasive aspergillosis due to *A. terreus* [9]. Moreover, this study points out that multiple and sequential isolates from respiratory

secretions of the patients should be analysed. Only such analyses may differentiate a transient carriage of genotypes always different from one sample to another (not clinically relevant) from a real chronic colonization of the airways by the

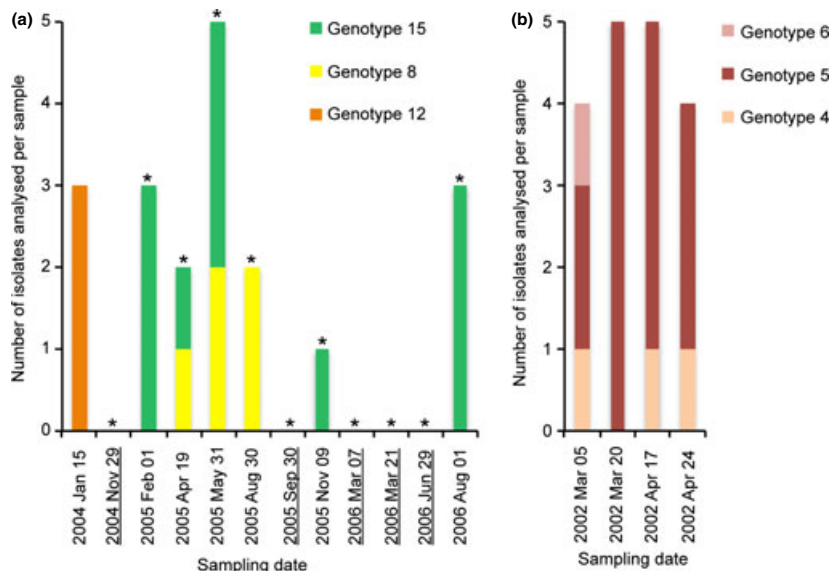


FIG. 3. Prolonged colonization by two distinct (patient C) (a) or closely related (patient D) (b) genotypes. Genotypes are represented by distinct colours. Underlined samples were culture-positive for *Aspergillus terreus*, but the isolates were not available for microsatellite analysis. An asterisk indicates a sample positive for *Aspergillus fumigatus*.

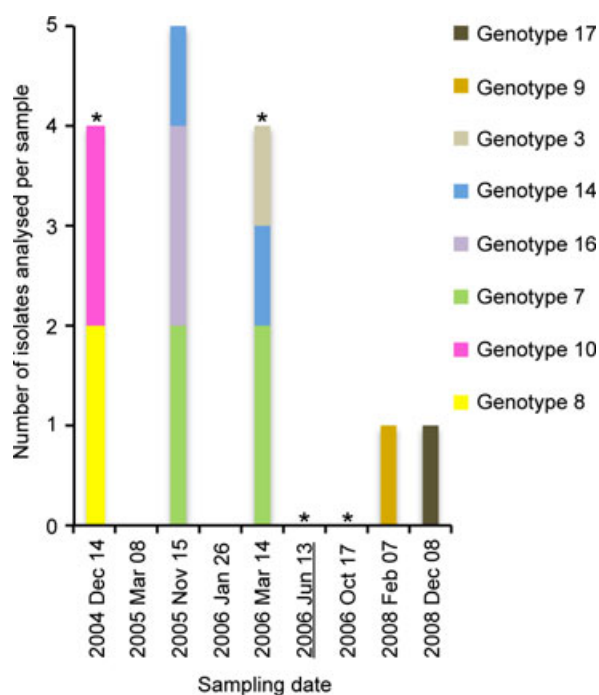


FIG. 4. Regular carriage of distinct genotypes found only transiently in patient E. Genotypes are represented by distinct colours. Underlined samples were culture-positive for *Aspergillus terreus*, but the isolates were not available for microsatellite analysis. An asterisk indicates a sample positive for *Aspergillus fumigatus*.

same genotype, which will contribute to the inflammatory reaction and to the progressive deterioration of lung function. Therefore, in addition to detection and precise identification of the fungal species colonizing the airways, genotyping may help

clinicians in their decision regarding whether or not to treat a patient, which may improve the quality of life of these patients.

In this study, seven genotypes were responsible for a prolonged or chronic colonization of the respiratory tract of CF patients, whereas 10 genotypes were found only transiently. Additional studies may further clarify whether certain *A. terreus* genotypes have inherent capacities to establish chronically in CF lungs. Moreover, two genotypes were involved in a chronic colonization of two patients in this study (i.e. genotype 8 in patient C and genotype 10 in patient A), whereas they were found only transiently in patient E. Involvement of some host factors in the establishment of a chronic colonization could be hypothesized, as suggested by Vanhee *et al.* [21] for colonization by *A. fumigatus* in the CF context. For instance, whereas all patients chronically colonized by *A. terreus* exhibited homozygosity for the F508del mutation, patient E harboured two moderate mutations (R709X/W79X) [24,25] that may have limited chronic colonization of the airways by filamentous fungi.

The large diversity of genotypes identified for patient E raises the question regarding the origin of colonization by this fungus [14,20]. In an epidemiological study performed at Hospital Renée Sabran (Giens, France) of air, surface and soil (from the hospital park) samples, *A. terreus* was detected exclusively in soil [7]. Additional analyses are warranted, particularly at patients' homes, to investigate a community-acquired colonization and to improve our knowledge on the environmental origin of this fungus. Given the frequent co-cultivation with the much more prevalent *A. fumigatus*, this requires the prior development of a selective culture medium and/or a molecular detection method specific to *A. terreus*.

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Transparency Declaration

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Patient and sampling data.

Table S2. Genotypes of the isolates used in this study. Blank values indicate a lack of amplification product.

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Table S1. Patient and sampling data

Patient (date of birth, CFTR genotype)	Sampling date	<i>A. terreus</i>	Isolates no (IHEM no)	Other fungal species detected
Patient A (F508del/F508del)	2003 Feb 28	+	40302278	<i>C. albicans</i> , <i>A. fumigatus</i>
	2003 Apr 22	+	NP	<i>C. albicans</i> , <i>A. fumigatus</i>
	2004 Mar 01	+	40402302/1, 40402302/2, 40402302/3	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>A. flavus</i>
	2004 Sep 13	+	40407483	<i>C. albicans</i>
	2004 Nov 08	+	40408882	<i>C. albicans</i> , <i>A. fumigatus</i>
	2004 Nov 17	+	40409010/1, 40409010/2, 40409010/3, 40409010/4, 40409010/5	<i>C. albicans</i> , <i>A. fumigatus</i>
	2005 Mar 14	+	40501891	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>A. flavus</i>
	2005 Jun 13	+	40503840/1, 40503840/4	<i>A. fumigatus</i>
	2005 Oct 11	+	40506495/1, 40506495/2, 40506495/3, 40506495/4, 40506495/5	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>A. flavus</i>
	2006 Mar 06	+	40601603/1, 40601603/2	<i>A. fumigatus</i> , <i>A. flavus</i>
	2006 Mar 13	+	40601821/1, 40601821/2, 40601821/3, 40601821/4, 40601821/5	<i>A. fumigatus</i> , <i>A. flavus</i>
	2006 Mar 21	+	NP	<i>C. albicans</i>
	2006 Apr 13	+	NP	None
	2006 Jun 12	+	40603924	None
	2006 Jul 31	+	40605209/1, 40605209/2, 40605209/3, 40605209/4, 40605209/5	None
	2006 Sep 07	+	40606071/1, 40606071/2, 40606071/3, 40606071/4, 40606071/5	<i>A. fumigatus</i>
	2006 Dec 04	+	40608628/1, 40608628/2	<i>A. flavus</i>
	2007 Jan 13	+	NP	<i>A. flavus</i>
	2002 May 02	+	40203953/3 (19402), 40203953/5 (19404)	None
	2005 Dec 21	+	40508264/1, 40508264/2	<i>C. parapsilosis</i>
	2006 Mar 15	+	NP	<i>C. parapsilosis</i>
	2006 Apr 26	+	40602848	<i>C. parapsilosis</i> , <i>A. fumigatus</i>
	2006 May 31	+	40603540	None
	2006 Jun 27	-		<i>C. parapsilosis</i> , <i>A. fumigatus</i>
	2006 Aug 30	+	NP	None
	2006 Sep 07	-		<i>C. albicans</i>
	2006 Oct 03	-		<i>A. fumigatus</i>
	2006 Nov 07	+	40607728/1, 40607728/3	<i>C. parapsilosis</i> , <i>A. fumigatus</i> , <i>A. nidulans</i>
	2006 Nov 24	+	40608374	None
	2007 Jan 18	+	40700605	<i>A. flavus</i>
	2007 Mar 21	+	NP	None
	2007 Apr 26	+	NP	<i>C. parapsilosis</i>
	2007 Jun 14	+	40705052	<i>C. parapsilosis</i> , <i>P. variotii</i>
	2008 Jan 09	+	080018337-01, 080021744-01	<i>C. parapsilosis</i> , <i>A. fumigatus</i>
	2008 Jan 22	+	080049083-01	None
	2008 Mar 26	+	NP	<i>C. parapsilosis</i>
	2008 Jul 30	-		<i>C. parapsilosis</i> , <i>A. fumigatus</i> , <i>Rasamsonia argillacea</i> complex
	2008 Nov 03	+	080621791-01	<i>C. parapsilosis</i>
	2008 Nov 20	+	080652857-01/1, 080652857-01/2, 080652857-01/3, 080652857-01/4	<i>C. parapsilosis</i> , <i>A. fumigatus</i>
Patient B (F508del/F508del)	2009 Jan 20	+	090045944-01/1, 090045944-01/2	<i>C. parapsilosis</i> , <i>A. fumigatus</i>
	2009 Mar 03	+	NP	None
	2009 May 14	-		None
	2009 Sep 22	+	NP	None
	2009 Oct 27	+	090635488-01	<i>C. parapsilosis</i> , <i>A. fumigatus</i>
	2009 Nov 22	+	090689217-01/1, 090689217-01/3	<i>C. parapsilosis</i> , <i>A. fumigatus</i>
Patient C (delF508/delF508)	2004 Jan 15	+	40400705, 40400549/1, 40400549/3	None
	2004 Nov 29	+	NP	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>S. prolificans</i>
	2005 Feb 01	+	40500790/1, 40500790/2, 40500790/3	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>Geotrichum</i> sp.
	2005 Apr 19	+	40502679/1, 40502679/2	<i>C. albicans</i> , <i>A. fumigatus</i>
	2005 May 31	+	40503521/1, 40503521/2, 40503521/3, 40503521/4, 40503521/5	<i>C. albicans</i> , <i>A. fumigatus</i>
	2005 Aug 30	+	40505504/1, 40505504/2	<i>C. albicans</i> , <i>A. fumigatus</i>
	2005 Sep 30	+	NP	<i>C. albicans</i> , <i>A. fumigatus</i>

Patient D (NA, NA)	2005 Nov 09	+	40507318	<i>C. albicans</i> , <i>A. fumigatus</i>
	2006 Mar 07	+	NP	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>S. prolificans</i>
	2006 Mar 21	+	NP	<i>C. albicans</i> , <i>A. fumigatus</i>
	2006 Jun 29	+	NP	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>A. flavus</i>
	2006 Aug 01	+	40605233/1, 40605233/2, 40605233/3	<i>C. albicans</i> , <i>A. fumigatus</i> , <i>A. flavus</i> , <i>S. prolificans</i>
	2002 Mar 05	+	40202399/1, 40202399/2 (21140), 40202399/3 (21141), 40202399/4 (21143)	NA
	2002 Mar 20	+	40202894/1 (21144), 40202894/2 (21145), 40202894/3 (21146), 40202894/4 (21147), 40202894/5 (21148)	NA
	2002 Apr 17	+	40203704/1 (19291), 40203704/2 (19292), 40203704/3 (19293), 40203704/4 (19294), 40203704/5 (19295)	NA
	2002 Apr 24	+	40203974/1 (19361), 40203974/2 (19362), 40203974/3 (19363), 40203974/4 (19364)	NA
	2004 Dec 14	+	40409633/1, 40409633/2, 40409633/3, 40409633/4	<i>A. fumigatus</i>
Patient E (R709X/W79X)	2005 Mar 08	-		None
	2005 Nov 15	+	40507426/1, 40507426/2, 40507426/3, 40507426/4, 40507426/5	None
	2006 Jan 26	-		None
	2006 Mar 14	+	40601936, 40601888/1, 40601888/2, 40601888/3	<i>A. fumigatus</i>
	2006 Jun 13	+	NP	<i>A. fumigatus</i>
	2006 Oct 17	-		<i>A. fumigatus</i>
	2008 Feb 07	+	080079458-01	None
	2008 Dec 08	+	080698908-01	None

* IHEM, Institute of Hygiene and Epidemiology-Mycolology section ; NP, Not preserved; NA, Not available ; +, culture-positive ; -, culture-negative

Table S2. Genotypes of the isolates used in this study. Blank values indicate a lack of amplification product.

Patient	Isolate nr.	Genotype	2A	2B	2C	3A	3B	3C	4A	4B	4C
A	40302278	2	10.1	20.0	19.0		10.0	17.0	10.0	9.0	5.0
A	40402302/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40407483	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40408882	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409010/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409010/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409010/3	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409010/4	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409010/5	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409022/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409022/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409022/3	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409022/4	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40409022/5	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40501891	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40503840/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40503840/4	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40506495/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40506495/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40506495/3	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40506495/4	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40506495/5	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601603/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601603/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601773	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601821/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601821/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601821/3	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601821/4	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601821/5	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40601868	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40603924	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40605209/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40605209/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40605209/4	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40605209/5	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40606071/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40606071/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40606071/3	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40606071/4	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40606071/5	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40608628/1	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0

A	40608628/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
A	40402302/1	11	18.0	21.0	21.0		9.0		9.0	5.0	7.0
A	40402302/3	11	18.0	21.0	21.0		9.0		9.0	5.0	7.0
A	40605209/3	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
B	080018337-01	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	080049083-01	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	090689217-01/1	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	090689217-01/3	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40203953/3	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40203953/5	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40508264/1	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40508264/2	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40602848	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40603540	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40607728/1	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40607728/3	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40608374	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40700605	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	40705052	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	80652857/1	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	80652857/2	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	80652857/3	1	11.1	16.0	20.0		9.0	15.0	10.0	9.0	5.0
B	080021744-01	13	20.0	25.0	21.0		7.0		5.0	5.0	13.0
B	090635488-01	13	20.0	25.0	21.0		7.0		5.0	5.0	13.0
B	80621791	13	20.0	25.0	21.0		7.0		5.0	5.0	13.0
B	80652857/4	13	20.0	25.0	21.0		7.0		5.0	5.0	13.0
B	90045944/1	13	20.0	25.0	21.0		7.0		5.0	5.0	13.0
B	90045944/2	13	20.0	25.0	21.0		7.0		5.0	5.0	13.0
C	40502679/2	8	8.0	9.0	20.0		7.0	29.0	5.0	8.0	7.0
C	40503521/3	8	8.0	9.0	20.0		7.0	29.0	5.0	8.0	7.0
C	40503521/5	8	8.0	9.0	20.0		7.0	29.0	5.0	8.0	7.0
C	40505504/1	8	8.0	9.0	20.0		7.0	29.0	5.0	8.0	7.0
C	40505504/2	8	8.0	9.0	20.0		7.0	29.0	5.0	8.0	7.0
C	40400549/1	12	18.0	24.0	12.0	8.0	9.0		9.0	5.0	7.0
C	40400549/3	12	18.0	24.0	12.0	8.0	9.0		9.0	5.0	7.0
C	40400705	12	18.0	24.0	12.0	8.0	9.0		9.0	5.0	7.0
C	40500790/1	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40500790/2	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40500790/3	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40502679/1	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40503521/1	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40503521/2	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40503521/4	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40507318	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40605233/1	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
C	40605233/2	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0

C	40605233/3	15	21.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
D	40202399/3	4	9.0	16.0	19.0	5.0	7.0	25.0	9.0	16.0	8.0
D	40203704/5	4	9.0	16.0	19.0	5.0	7.0	25.0	9.0	16.0	8.0
D	40203974/1	4	9.0	16.0	19.0	5.0	7.0	25.0	9.0	16.0	8.0
D	40202399/2	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40202399/5	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40202894/1	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40202894/2	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40202894/3	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40202894/4	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40202894/5	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40203704/1	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40203704/2	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40203704/3	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40203704/4	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40203974/2	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40203974/3	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40203974/4	5	9.0	16.0	19.0	5.0	7.0	25.0	9.0	14.0	8.0
D	40202399/1	6	9.0	16.0	19.0	5.0	7.0	25.0	9.0	18.0	8.0
E	40601888/2	3	12.1	22.0	19.0		13.0	15.0	8.0	9.0	5.0
E	40507426/1	7	19.0	16.0	19.0		12.0		9.0	7.0	5.0
E	40507426/3	7	19.0	16.0	19.0		12.0		9.0	7.0	5.0
E	40601888/1	7	19.0	16.0	19.0		12.0		9.0	7.0	5.0
E	40601888/3	7	19.0	16.0	19.0		12.0		9.0	7.0	5.0
E	40409633/1	8	8.0	9.0	20.0		7.0	29.0	5.0	8.0	7.0
E	40409633/4	8	8.0	9.0	20.0		7.0	29.0	5.0	8.0	7.0
E	080079458.01	9	8.0	9.0	24.0		7.0	30.0	5.0	8.0	7.0
E	40409633/2	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
E	40409633/3	10	25.0	9.0	21.0	8.0	9.0	29.0	5.0	8.0	7.0
E	40507426/5	14	22.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
E	40601936	14	22.0	18.0	20.0	8.0	7.0	45.0	9.0	7.0	7.0
E	40507426/2	16	23.0	25.0	20.0	8.0	7.0	29.0	5.0	7.0	7.0
E	40507426/4	16	23.0	25.0	20.0	8.0	7.0	29.0	5.0	7.0	7.0
E	80698908	17	11.0	24.0	11.0		8.0	38.0	8.0	4.0	5.0