MYCOLOGY

Typing of *Scedosporium apiospermum* by multilocus enzyme electrophoresis and random amplification of polymorphic DNA

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The genetic diversity among epidemiologically unrelated strains of the human pathogenic fungus *Scedosporium apiospermum* or its teleomorph, *Pseudallescheria boydii*, from different areas in Europe, was investigated by multilocus enzyme electrophoresis (MLEE) and random amplification of polymorphic DNA (RAPD). Fourteen enzyme activities were analysed by starch gel electrophoresis, corresponding to 27 polymorphic loci and 43 iso-enzymes. Among the enzymes studied, propionate esterase, carboxyl esterase, superoxide dismutase, carbonate dehydratase and malate dehydrogenase were the most polymorphic, allowing the classification of the strains into 6–11 groups each. Combination of the data obtained for the different enzyme activities studied allowed differentiation of the strains. Similarly, a high polymorphism was also revealed by each of the 20 RAPD primers tested, but no single primer was able to differentiate all the strains. The most efficient primers were GC70, UBC-701 and UBC-703, which revealed 17 distinct genotypes each, and combination of the results obtained with this three-primer set allowed complete discrimination of the strains. The dendrograms obtained from MLEE or RAPD by the unweighted pair-group method using arithmetic average cluster analysis did not reveal any clustering according to the geographic origin of the strains or their pathogenicity.

Introduction

The filamentous fungus *Scedosporium apiospermum* and its teleomorph, *Pseudallescheria boydii*, are responsible for a wide variety of mycoses in man [1–4] resulting from traumatic inoculation. These are mainly white-grain mycetoma, but also cutaneous and subcutaneous hyalohyphomycosis, keratitis and endophthalmitis, arthritis, osteomyelitis, meningitis and endocarditis. Other clinical forms thought to be due to inhalation of airborne spores have also been described, ranging from localised infections such as sinusitis to deep-seated infections, particularly pulmonary myceto-

ma, allergic bronchopulmonary mycoses and disseminated fatal infections in severely immunocompromised patients, all diseases that are comparable to those reported for the opportunist fungus *Aspergillus fumigatus*.

More recently, attention has been paid to the particular occurrence of this fungus in patients with cystic fibrosis (CF), which is the most common recessively inherited genetic disease among the Caucasian population [5, 6]. In these patients, mutations in the gene encoding the chloride channel transmembrane regulator result in an abnormal viscosity of the bronchial secretions, leading to colonisation of the bronchial tubes by various bacteria and the entrapment of some airborne mould spores in the viscous mucus. In a prospective study including 128 CF patients from two different care units in France, *S. apiospermum* was isolated from bronchial secretions in 8.6% of the patients, ranking it the second most frequent filamen-

Received 24 Jan. 2001; revised version accepted 19 March 2001.

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tous fungus identified [6]. In CF, previous alterations of the bronchopulmonary epithelial cells due to chronic bacterial infections and the host inflammatory response seem to be required for the establishment of *S. apiospermum* in the airways, the fungus being isolated usually from patients already infected by *Pseudomonas aeruginosa* and colonised by *A. fumigatus* [5]. However, *S. apiospermum* is rarely reported from the patients' environment, and the origin of their colonisation remains in question. This fungus, which has a world-wide distribution, has been isolated from soil and polluted water [1, 2, 7], as well as from bird feathers [8], but exceptionally from the air and surfaces in the patients' indoor and outdoor environment [9]. Moreover, its relatively high frequency in CF patients contrasts with its occasional isolation from pulmonary samples outside CF. It is intriguing to note that strains isolated from sputum samples of CF patients always present in the anamorph state, whereas sexual conidiation is usually obtained from strains isolated from soil. Therefore, questions arise about the uniqueness of the *S. apiospermum*/*P. boydii* complex and the possibility of clusters of strains associated with a particular disease or clinical context.

To gain a better understanding of the epidemiology of the fungus in CF, epidemiologically unrelated strains of *S. apiospermum* and *P. boydii* were typed by multilocus enzyme electrophoresis (MLEE) and random amplification of polymorphic DNA (RAPD). These methods have demonstrated their efficiency for differentiating strains in numerous pathogenic yeasts [10–17] and filamentous fungi [18–24] and have largely been used in epidemiological studies. The genetic relatedness of the strains tested was investigated by searching for clusters of strains according to their pathogenicity or geographic origin.

Materials and methods

Strains

This study was performed with 17 strains of *S. apiospermum* and two of its teleomorph *P. boydii*. Most were clinical isolates from patients investigated in different hospitals in Europe, two were obtained from feathers of European starlings and one from sludge in a purification station. All strains were referenced at the Scientific Institute of Public Health, IHEM Culture Collection (Brussels, Belgium) and are publically available. The origin of the strains, date of isolation and, for human strains, the clinical form or underlying disease are indicated in Table 1.

Multilocus enzyme electrophoresis (MLEE)

The strains were cultivated in 500-ml flasks each containing 150 ml of YEPD liquid medium, consisting of (g/L) yeast extract (10), pancreatic peptone (10), chloramphenicol (0.5) and glucose (20). After incubation for 2 weeks at 37°C without agitation, the mycelium was harvested by centrifugation and washed twice with sterile distilled water. Cells were mechanically disrupted for 2 min with glass beads (0.25 mm diameter; Braun Melsungen, Melsungen, Germany). Debris were removed by centrifugation at 12 000 *g* for 30 min at 4°C, and the supernate was stored in small volumes at –80°C until use.

Fourteen enzyme activities which were shown to be discriminant in a preliminary study [19] were analysed: malate dehydrogenase (MDH; EC 1.1.1.37), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), phosphoglucomutase (PGM; EC 5.4.2.2), malic enzyme (ME; EC 1.1.1.40), superoxide dismutase (SOD; EC 1.15.1.1), alcohol dehydrogenase (ADH; EC 1.1.1.1), aspartate

Table 1. Isolation data of the strains studied

Strain no.	IHEM number	Origin	Clinical sample	Isolation date*	Geographic location
<i>S. apiospermum</i>					
1	3817	Human	Cephalorachidian fluid	1986	Rotterdam, The Netherlands
2	7657	Human	Ocular lesion	1989	Lyon, France
3	6908	Human	Cutaneous lesion	1992	Huy, Belgium
4	7656	Human	Cutaneous lesion	1989	Lyon, France
5	13952	Human	Cutaneous lesion	20/05/97	Angers, France
6	13945	Human	Cutaneous lesion	03/12/93	Angers, France
7	4177	Human	Lung biopsy	1986	Milano, Italy
8	10209	Human, CF patient	Sputum	1996	Besançon, France
9	13951	Human, CF patient	Sputum	31/01/97	Toulouse, France
10	13939	Human, CF patient	Sputum	02/10/91	Angers, France
11	13940	Human, CF patient	Sputum	23/12/91	Angers, France
12	13944	Human, CF patient	Sputum	29/05/92	Angers, France
13	13946	Human, CF patient	Sputum	10/11/93	Angers, France
14	13942	Human, CF patient	Sputum	15/06/92	Giens, France
15	13943	Human, CF patient	Sputum	23/04/92	Giens, France
16	13950	Human, CF patient	Sputum	27/02/95	Giens, France
17	13947	European starling	Feathers	27/12/93	Locarn, France
<i>P. boydii</i>					
18	13948	European starling	Feathers	27/12/93	Locarn, France
19	13938	Purification station	Sludge	14/10/91	Pornic, France

*Year or day/month/year.

aminotransferase (AAT; EC 2.6.1.1), glucose-6-phosphate-1-dehydrogenase (G6PDH; EC 1.1.1.49), carboxylesterase (carboxyl EST; EC 3.1.1.1), propionate esterase (propionate EST; EC 3.1.1.1), carbonate dehydratase (CD; EC 4.2.1.1), acid phosphatase (ACP; EC 3.1.3.2), alkaline phosphatase (ALP; 3.1.3.1) and glucokinase (GK; EC 2.7.1.2). Electrophoresis was performed in 14% starch gels for 4 h at 220 V and gels were stained for enzyme activities according to Pasteur *et al.* [25]. Alleles were numbered in order of increasing anodal mobility. Each strain was characterised by its combination of alleles at the different polymorphic enzyme loci encountered.

DNA extraction

After incubation for 2 weeks at 37°C in YEPD broth, the mycelium was collected, washed with sterile distilled water and then ground with a mortar and pestle in liquid nitrogen. Total genomic DNA was extracted with a DNeasy Plant Mini Prep Kit (QIAGEN, Courtaboeuf, France) as recommended by the manufacturer. DNA concentration was quantified in a TKO 100 fluorimeter (Hoefer Scientific Instruments, San Francisco, CA, USA).

RAPD

Fourteen RAPD primers (10 mers) obtained from Genset (Paris, France) and previously tested to discriminate clinical isolates of *S. inflatum* [22, 23] were used in this study. Thirty-five other 10-mer RAPD primers from Operon Technologies (Alameda, CA, USA) were also tested in an initial screening and from them, five were selected for their ability to produce polymorphic and unambiguous markers. Reaction mixtures (25 µl final volume) consisted of 50 mM KCl, Triton X-100 0.1%, gelatin 0.2 mg/ml, 10 mM Tris-HCl buffer, pH 9.0, containing 1.5 mM MgCl₂, 200 µM of each deoxyribonucleotide triphosphate, 5 pmoles of primer, 25 ng of DNA and 1 unit of Taq DNA polymerase (Quantum-Appligène, Illkirch, France).

Samples were overlaid with 25 µl of autoclaved mineral oil to prevent evaporation and the thermocycler (Perkin Elmer 480) was programmed for 40 cycles consisting of denaturation (1 min at 94°C), annealing (1 min at 36°C) and DNA chain extension (2 min at 72°C), preceded by an initial denaturation of 4 min at 94°C. Amplification with the core sequence of phage M13 (Genset) was performed in 25-µl reaction mixtures as above, with 35 cycles of 30 s at 94°C, 50 s at 50°C and 1 min at 72°C [26].

The amplification products were separated by agarose 1.5% gel electrophoresis in 40 mM Tris-acetate buffer containing 0.2 mM EDTA. Mol. wt standards (100-bp ladder; Amersham Pharmacia-Biotech, Saclay, France) were run in parallel and amplicons were visualised by UV transillumination after ethidium bromide staining of the gels.

Data analysis

Data were recorded as the presence or absence of each allele detected at the different enzyme loci or amplification band, and analysed with the NT Sys-pc version 1.80 program [27]. The similarity was calculated for each couple of accessions according to the Jaccard coefficient [28]. For clustering, the unweighted pair-group method using arithmetic averages (UPGMA) was used.

Results

MLEE polymorphism analysis

Fourteen enzyme activities were analysed by starch gel electrophoresis, corresponding to 43 iso-enzymes (Table 2). One locus was genetically interpretable for PGM, ME, ADH, AAT, ACP, ALP and GK; two loci for MDH, GPI and G6PDH; three for SOD and CD; four for carboxyl EST and five for propionate EST. Thus data were obtained for 28 loci, but one was monomorphic.

Table 2. Iso-enzyme electrophoretic patterns of the different strains studied

Enzyme activity	Electrophoretic patterns of strains																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
MDH	A	B	C	D	D	A	A	C	B	B	B	A	E	D	B	D	B	F	A
GPI	A	A	B	B	A	A	A	A	A	A	A	A	A	C	A	A	B	D	A
PGM	A	A	A	B	A	B	B	A	A	A	A	A	A	A	A	B	A	A	A
ME	A	A	A	B	A	A	B	A	A	A	A	A	A	A	A	C	A	A	A
SOD	A	B	C	A	C	B	C	D	C	C	A	E	F	A	G	H	G	I	A
ADH	A	B	B	B	B	B	B	C	B	A	B	A	B	A	A	B	C	B	A
AAT	A	A	A	A	B	A	A	A	B	A	A	B	B	B	A	C	B	B	B
G6PDH	A	A	A	A	A	B	C	A	A	A	A	B	B	A	A	A	A	A	A
Carboxyl EST	A	B	C	D	C	E	F	G	G	C	G	H	G	I	G	C	G	J	G
Propionate EST	A	B	C	D	E	F	G	B	E	B	B	H	B	I	J	E	B	K	B
CD	A	B	C	A	D	E	F	B	B	C	G	G	B	B	B	D	B	B	B
ACP	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	A	A	A
ALP	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	A	A	A
GK	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

The letters (A, B, C, etc.) correspond to the different electrophoretic patterns obtained for each enzyme activity; the electrophoretic patterns were different from one enzyme activity to another even if the same letters were used. For abbreviations, see *Materials and methods*.

Among the enzymes studied, propionate EST and carboxyl EST were the most polymorphic, allowing the classification of the strains into 11 and 10 groups, respectively. Nine electrophoretic patterns were observed for SOD, seven for CD and six for MDH. GK was highly conserved and the other enzyme activities presented only limited variations. The combination of the different iso-enzyme data allowed an accurate differentiation of all the strains studied, as each strain differed from the others by at least four iso-enzymes.

RAPD analysis

Amplification of the fungal DNAs with the 20 selected primers, whose sequences are shown in Table 3, led to numerous PCR products of 250–2400 bp. The selection of amplicons as markers was based on their reproducibility, size and intensity (bands >2000 bp and weak bands were discarded). The 322 remaining bands were highly polymorphic. Among them, the low-frequency bands, present only in $\leq 25\%$ of the strains, constituted the larger part (57%), confirming the important degree of polymorphism (Table 3). Of these bands, 44 were strain-specific, and most of them were exhibited by strain 19. However, some of the strains were closely related – such as *S. apiospermum* 13 and *P. boydii* 18, which presented the same electrophoretic patterns with 14 primers (Fig. 1). Identical patterns were also observed with numerous primers for strains 2, 9, 10 and 12, as well as for strains 1, 4 and 8. Moreover no single primer allowed discrimination of all the strains and, on average, *c.* 13 electrophoretic patterns were generated by a single primer (Table 3). Three RAPD primers, GC70, UBC-701 and UBC-703, gave the most discriminatory results, generating 17 distinct electrophoretic patterns each (Fig. 1a–c).

All the strains could be differentiated by combining the results obtained with GC70, AB1.18 and UBC-703 or GC70, GC80 and UBC-701. However, the association of primers GC70, UBC-701 and UBC-703, which generated the highest numbers of bands and electrophoretic patterns, was the most efficient, as the differentiation obtained with one primer was confirmed by at least one of the others.

Cluster analysis of the strains

The phylogenetic tree constructed by the UPGMA method using the Jaccard coefficient from the data obtained for the 14 enzyme activities is shown in Fig. 2a. The dendrogram did not reveal any cluster, but showed a relatively gradual scale of branches which isolated only one or two strains from the main group. The basal node ($SD = 0.09$) separated strains 13 and 18 from the rest of the test set. These two strains, which were differentiated at level 0.30, were not related by their origin or geographic location. The most closely related strains by UPGMA were strains 3 and 10 ($SD = 0.63$) and 16 and 5 ($SD = 0.60$), which were

unrelated according to their origin, isolation date and geographic location (Table 1).

In contrast to MLEE, cluster analysis of the results obtained with this three-primer set revealed two clearly differentiated groups with a degree of similarity of 0.09 (Fig. 2b). The first group comprised five strains, among which strain 13 isolated from a patient with CF and strain 18 from feathers of a European starling were closely related ($SD = 0.89$). The second group subdivided into two subgroups ($SD = 0.11$), each composed of seven strains. In the first subgroup, strain 7 was clearly separated ($SD = 0.27$) from the others. This subgroup also included four strains isolated from sputum samples in CF patients, two of them (10 and 12) being closely related ($SD = 0.81$). However, three strains from CF patients were also present in the last subgroup, which comprised three further strains from subcutaneous lesions and one from cephalorachidian fluid. The dendrogram obtained by combining the results from MLEE and RAPD experiments (Fig. 2c) retained the basic pattern, although a few minor changes were seen, particularly in the positions of strains 3 and 8, and 4 and 11 in the last subgroup.

Discussion

Epidemiological investigation of *S. apiospermum*/*P. boydii* diseases requires the development of effective typing methods. With this goal in mind, two typing systems commonly used for strain delineation in pathogenic fungi, MLEE and RAPD, were applied to 19 epidemiologically unrelated strains. A high degree of polymorphism was revealed by MLEE, as 27 polymorphic loci were identified for the 14 enzyme activities analysed, and the combination of the different iso-enzyme data permitted accurate differentiation of the strains. Similarly, none of the 20 individual RAPD primers allowed the differentiation of all the strains. Primers GC70, UBC-701 and UBC-703, which identified 17 types each, were the most discriminatory. Complete discrimination of the strains was possible only by combining the patterns obtained with three primers, GC70, AB 1.18 and UBC-703, or GC70, GC80 and UBC-701, but the best results were provided by the primers GC70, UBC-701 and UBC-703. Interestingly, similar findings have been reported for *S. inflatum*, a closely related species responsible for systemic infections in severely immunocompromised patients. For this fungus, no single primer allowed the discrimination of all the epidemiologically unrelated isolates studied. This was also the case in studies by Ruiz-Diez *et al.* [22], that required the combination of the primers GC70, GC80 and M13, and San Millan *et al.* [23] that required different three-primer sets, all including UBC-701 which also demonstrated the best discriminatory power in the present study.

RAPD performed with the three-primer set GC70,

Table 3. RAPD primers used and characteristics of the electrophoretic patterns generated

Primer	Sequence (5' → 3')	Number of bands generated	Number of low frequency bands	RAPD patterns of strains																			Number of patterns
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
OP-A02	TGCCGAGCTG	14	8	A	B	C	A	D	E	F	A	B	B	C	B	G	A	H	D	I	G	J	10
OP-A03	AGTCAGCCAC	13	9	A	B	C	D	E	C	F	G	H	B	I	H	J	C	K	E	L	J	M	13
OP-A10	GTGATCGCAG	16	8	A	B	A	A	C	A	D	A	B	E	A	E	F	A	B	G	H	F	I	9
OP-A11	CAATCGCCGT	15	7	A	B	A	A	C	A	A	A	B	B	A	D	E	F	G	C	H	E	I	9
OP-A13	CAGCACCCAC	16	12	A	B	A	A	C	D	E	A	B	B	A	B	F	G	H	I	J	K	L	12
AB1.01	GTTTCGCTCC	14	5	A	B	A	C	D	E	F	A	B	B	E	B	G	H	I	D	J	K	L	12
AB1.08	GTCCACACGG	14	9	A	B	A	A	C	A	D	A	B	E	A	B	F	A	G	C	H	F	I	9
AB1.11	GTAGACCCGT	15	9	A	B	A	A	C	A	D	A	B	E	A	E	F	A	G	C	H	I	J	10
AB1.18	CCACAGCAGT	12	7	A	B	A	A	C	A	D	A	B	B	A	B	E	F	G	C	H	I	J	10
AB1.20	GGACCCCTTAC	14	11	A	B	C	C	D	C	E	C	F	F	C	F	G	C	H	D	I	G	J	10
GC70	CGGCCACTGT	26	14	A	B	C	D	E	A	F	G	H	I	J	K	L	M	N	O	P	L	Q	17
GC80	CGGCCCTGT	18	7	A	B	C	D	E	F	G	H	B	B	I	J	K	L	M	E	N	K	O	15
UBC-701	CCCACAACCC	20	12	A	B	C	D	E	F	G	H	I	J	K	J	L	A	M	N	O	P	Q	17
UBC-703	CCAACCACCC	22	10	A	B	C	D	E	F	G	H	I	J	K	J	L	M	N	O	P	L	Q	17
UBC-706	GGTGGTTGGG	16	10	A	B	A	A	C	A	D	A	E	F	A	E	G	A	H	C	I	J	K	11
UBC-707	CCCAACACCC	14	8	A	B	C	D	E	F	G	H	B	B	H	B	I	H	J	E	K	I	L	12
UBC-717	CCCACACCCA	15	7	A	B	C	D	E	A	F	A	G	G	H	G	I	A	J	E	K	I	L	12
UBC-721	CCCACCCACA	18	12	A	B	C	A	D	A	E	F	G	B	A	G	H	F	I	D	J	H	K	11
UBC-722	CCTCTCCCTC	12	7	A	B	C	A	D	E	F	C	B	B	C	B	G	C	H	D	I	G	J	10
M13	GAGGGTGGCGGTTCT	18	12	A	B	C	D	E	F	G	H	B	B	I	B	J	D	K	E	L	J	M	13

The letters (A, B, C, etc.) correspond to the different electrophoretic patterns generated by each RAPD primer; the electrophoretic patterns were different from one primer to another even if the same letters were used.

Fig. 1. RAPD patterns of the strains of *S. apiospermum* (lanes 1–17) and *P. boydii* (18 and 19) generated by primers GC70 (a), UBC-701 (b) or UBC-703 (c). M, DNA size markers (100-bp ladder from Amersham Pharmacia-Biotech).

UBC-701 and UBC-703, and MLEE were revealed to be equally efficient for strain delineation. However, the results of these typing systems differed greatly, as strains which appeared to be closely related by one of these methods were usually genetically different by the other. For example, strains 3 and 10 presented similar iso-enzyme electrophoretic patterns, but they were <15% similar by RAPD analysis. Conversely, MLEE easily differentiated strains 10 and 12 and strains 13 and 18, which were closely related by RAPD. Nevertheless, both methods led to similar overall conclusions. As shown in the composite dendrogram prepared from data obtained by MLEE and RAPD, no clustering of the strains was seen according to pathogenicity or clinical syndromes. The three strains isolated from soil or feathers of European starlings were not distinguishable from human strains, suggesting that all strains present the same pathogenic potency. Strains isolated from sputum samples in CF patients were distributed

Fig. 2. Dendrograms showing the genetic relationships between strains of *S. apiospermum* and *P. boydii* evidenced by MLEE (a) and RAPD (b) or by combining the results from the two methods (c). For MLEE, the dendrogram was generated by the UPGMA method of clustering from a matrix of pairwise coefficients of genetic distance based on the analysis of the 14 enzyme activities. For RAPD, the low frequency bands obtained with the primers GC70, UBC-701 and UBC-703 were scored. Genetic distances were calculated between each pair of strains and UPGMA cluster analysis was based on Jaccard similarity index. The scale at the top of the figure shows the degree of similarity.

all along the phylogenetic tree with the strains isolated from cutaneous/subcutaneous hyalohyphomycosis or mycetoma or from other clinical entities. Thus the present study did not reveal the clustering described in *S. apiospermum* by Guého and de Hoog [29] and de Hoog *et al.* [7]. In these studies, strains of *S. apiospermum*/*P. boydii* could be classified by DNA-DNA re-association experiments into three groups corresponding mainly to airway colonisation in CF

patients or pulmonary mycoses in immunocompromised patients, sinusitis and otitis, and soil isolates.

Moreover, the present study did not find any clustering of the strains according to their geographic origin. For example, among the six strains isolated in Angers (in Western France), only two strains (10 and 12) were closely related. The other strains were distributed all along the phylogenetic tree, such as strains 5 and 6 close to strain 16 isolated in Giens in the south of France and to strain 1 from the Netherlands, respectively. Similar epidemiological and pathological features have also been reported for the other filamentous fungi associated with CF, *A. fumigatus* [30] and *A. terreus* [24]. Thus, in a large-scale study including isolates from CF patients, from pulmonary invasive aspergillosis in patients with haematological disorders, and environmental isolates, no clustering was seen according to pathogenicity [30]. Similarly, whereas strains of *Paracoccidioides brasiliensis* [31] or *Candida albicans* [11] could be classified by RAPD into clusters related to geographic origin, this was not possible for *A. fumigatus* [30] and *A. terreus* [24].

In agreement with a previous study by Issakainen *et al.* [32], the two strains of *P. boydii* were not separable from those of *S. apiospermum* either by iso-enzyme analysis or by RAPD. Although the strains studied by these authors belonged to the different groups defined by Guého and de Hoog [29] on the basis of their DNA-DNA re-association values, they were identical to each other once a 380-bp long insertion had been removed from the sequence of the gene encoding the small subunit rDNA for the strains of *S. apiospermum* [32]. Therefore, all molecular data yet available confirm the uniqueness of the complex *S. apiospermum/P. boydii* and there is no experimental evidence to explain the absence of sexual conidiation in primary cultures for clinical isolates from sputum samples in CF patients.

In conclusion, this work demonstrated important genetic diversity inside the complex *S. apiospermum/P. boydii*. An accurate differentiation of the strains studied was provided by MLEE. However, this method is time-consuming as it requires the electrophoretic analysis of 13 enzyme activities. Also, like other phenotypic methods, MLEE is dependent on gene expression and differences in culture conditions may affect the electrophoretic patterns. Therefore, RAPD analysis with the three-primer set GC70, UBC-701 and UBC-703, which overcomes these limitations, may constitute a valuable tool for epidemiological studies and we are now applying this method to sequential and multiple isolates of *S. apiospermum* from CF patients to assess the evolution of the fungus during the colonisation of the airways in these patients.

This work was supported by a grant from the Programme Hospitalier de Recherche Clinique du Ministère des Affaires Sociales, de la Santé et de la Ville.

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