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Original Article

Intra-species Variation of Genotypes of *Exophiala jeanselmei* Isolated from Patients in Japan

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Abstract

Isolates of *Exophiala jeanselmei* have been classified into 15 types based on their mitochondrial DNA (mtDNA). Thirteen of the 15 types and *E. spinifera*, which has been classified as *E. jeanselmei* Type 14, were confirmed to be also clearly differentiated by restriction fragment length polymorphism (RFLP) in internal transcribed spacer (ITS) regions of ribosomal RNA genes in their nuclear DNA (nDNA). Twenty strains of *E. jeanselmei*, newly identified or isolated from patients in Japan, were examined for mtDNA-RFLP and ITS-RFLP. The twenty isolates were comprised of: 11 *E. jeanselmei* Type 5, 6 *E. jeanselmei* Type 6, 2 Type 10, and 1 Type 8. *E. jeanselmei* Type 6 was the second most common strain in Japan after Type 5. Type 5 was definitely identified as *E. jeanselmei* var. *jeanselmei* and Type 8 was identified as *E. jeanselmei* var. *lecanii-corni* based on the genotypes of type strains of these species. However, two other types were still designated as *E. jeanselmei* Type 6 and *E. jeanselmei* Type 10.

Key words: *Exophiala jeanselmei*, internal transcribed spacer region, restriction fragment length polymorphism

Introduction

Identification of *Exophiala jeanselmei*, the etiological agent of chromomycosis, is very difficult even for skilled mycologists[1]. Consequently, the results of genotype analyses of *E. jeanselmei* strains identified and kept in the canonical institutes for fungal collection revealed that many groups of morphologically similar but genetically different strains are included in *E. jeanselmei*[2]. These difficulties have also appeared in the literature, with an isolate (CBS 638.91) finally reported as *E. jeanselmei* var. *lecanii-corni*[3].

Previously, we reported that 45 *E. jeanselmei* isolates were comprised of 15 genetic types based on restriction fragment length polymorphisms (RFLP) in mitochondrial DNA (mtDNA) using the restriction enzymes, *Hae*III and *Msp*I, while 31 *E. dermatitidis* isolates showed genetic homogeneity[2]. Seventeen *E. moniliae* isolates were comprised of 10 types[4] and 36 isolates of *E. spinifera* were divided into 6 types[5]. Among the 15 types of *E. jeanselmei*, mtDNA-RFLP patterns of Type 1 strains were identical to those of *E. moniliae* Type 5, patterns of *E. jeanselmei* Type 6 were identical to those of *E. moniliae* Type 7[4], *E. jeanselmei* Type 3 was identical to *E. dermatitidis*[2], *E. jeanselmei* Type 7 was identical to *Fonsecaea pedrosoi* Type 1[6], and *E. jeanselmei* Type 14 was identical to *E. spinifera* Type 4[5].

Thus, despite extensive investigation, many problems remain to be resolved in identifica-

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tion of *Exophiala* species[7-9].

Since 1990, when the heterogeneity of *E. jeanselmei* was reported, we have investigated the genotypes of 20 *E. jeanselmei* isolated from patients in Japan. Here, we present the genetic variations among these 20 *E. jeanselmei* isolates along with the problems in molecular identification of this agent.

Materials and Methods

First, the nuclear DNA (nDNA) types of the representative strains of 13 of 15 mtDNA types of *E. jeanselmei*, which were still alive, and *E. spinifera* Type 4 were examined. The species and strains used in the present study are shown in Table 1.

Table 1. Representative strains of 13 *E. jeanselmei* mtDNA types and *E. spinifera* Type 4 used in the present study as references

mtDNA type	KMU no.	Origin	nDNA (ITS) type	Revised name
E. jeanselmei Type 1	2719	IFM 4863 = KUM 1881 (Toyama)	E1	
E. jeanselmei Type 2	2711	IFM 41494 = DCU 419	E2	
E. jeanselmei Type 3	2703	IFM 4876 = CBS 577.76	E3 = Ed	*E. dermatitidis*
E. jeanselmei Type 4	2716	IFM 4983	E4	
E. jeanselmei Type 5-1	2714	IFM 4852 = ATCC 34128	E5	*E. jeanselmei* var. *jeanselmei*
E. jeanselmei Type 5-2	2723	IFM 4866 = KUM 2034 (Aichi)	E5	*E. jeanselmei* var. *jeanselmei*
E. jeanselmei Type 6	2720	IFM 41496 = KUM 1940 (Osaka)	E6	?
E. jeanselmei Type 7	2712	IFM 4856 = Nippon Med School #80001	E7 = Fp3	*F. pedrosoi*
E. jeanselmei Type 8	2691	IFM 4970	E8 = Ej lecanii-corni	*E. jeanselmei* var. *lecanii-corni*
E. jeanselmei Type 8	2701	IFM 4981	E8 = Ej lecanii-corni	*E. jeanselmei* var. *lecanii-corni*
E. jeanselmei Type 9	2694	IFM 4973	E9	
E. jeanselmei Type 10-1	2689	IFM 4854 = KUM 2041 = Duke 2858	E10	?
E. jeanselmei Type 10-2	2722	IFM 4865 = KUM 1913 (Hiroshima)	E10	?
E. jeanselmei Type 11	2692	IFM 4971	E11	
E. jeanselmei Type 12	2705	IFM 4857 = CDC B1003	E12	
E. jeanselmei Type 13	2710	IFM 41493 = DCU 418	E13	
E. spinifera Type 4	3220	IFM 48830 = ATCC 18218	Es	

ATCC: American Type Culture Collection, Rockville, Maryland, USA.
CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
CDC: Communicable Disease Centers (Centers for Disease Control), United States Public Health Services, Atlanta, Georgia, USA.
DCU: Department of Dermatology, School of Medicine, Chiba University, Chiba, Japan.
Duke: Department of Microbiology and Immunology, Duke University, Durham, North Carolina, USA.
IFM: Research Center for Pathogenic Fungi & Microbial Toxicoses, Chiba University, Chiba, Japan.
KMU: Kanazawa Medical University, Department of Dermatology, Uchinada, Ishikawa, Japan.
KUM: Department of Dermatology, School of Medicine, Kanazawa University, Kanazawa, Japan

Table 2. mtDNA types and nDNA types of 20 clinical isolates of *E. jeanselmei*

Case*	Strain no. (KMU)	Place of isolation	mtDNA type	nDNA (ITS) type	Reference**
1	3116	Ishikawa	6	E6	13
2	3179	Toyama	5	E5	
3	3180	Toyama	5	E5	14
4	3370	Tokyo	5	E5	KO 2010: 15
4	3419	Tokyo	5	E5	KO 2011: 15
5	3378	Fukuoka	8	E8	3
6	3420	Shizuoka	5	E5	KO 2115: 16
6	3421	Shizuoka	5	E5	KO 2116: 16
7	3852	Tokyo	10	E10	MTU 22033
8	4076	Tottori	5	E5	
9	4258	Fukui	5	E5	
10	4391	Shizuoka	6	E6'***	17
10	4396	Shizuoka	6	E6	17
10	4428	Shizuoka	6	E6	17
11	4477	Okayama	5	E5	
12	4651	Kanagawa	10	E10	18
13	4671	Yamagata	ND	E6	
14	4789	Ishikawa	5	E5	
14	4799	Ishikawa	5	E5	
15	4803	Mie	6	E6	

*: Cases with the same number indicate that the strains were isolated from the same patient.
**: Original strain number and/or number of the reference.
***: Differences within a type were detected.

Among the black fungi isolated from patients and sent to our laboratory for molecular biological analyses, the mtDNA and nDNA types of 20 isolates identified as *E. jeanselmei* are summarized in Table 2. Identification of *E. jeanselmei* was performed based on the microscopic characteristics of an elongated tip of conidiogenous cells, lack of characteristics of *E. spinifera*, and genotype of one of the 15 *E. jeanselmei* types[2].

mtDNA type was analyzed as described previously[2]. Briefly, the mitochondrial fraction was isolated by centrifugation from 10 m*l* of disrupted cell suspension. Then, DNA was isolated by dissolution of mitochondria with sodium dodecyl sulfate, phenol-chloroform extraction, and ethanol precipitation. mtDNA was digested with the restriction enzyme, *Hae*III, and electrophoresis patterns of fragments (RFLP pattern) were compared on 0.8% agarose gels.

nDNA type was analyzed as described previously[10]. After extraction of total DNA, the internal transcribed spacer regions of ribosomal RNA genes (ITS) were amplified using the primers, ITS1 and ITS4, according to the method of White *et al.*[11]. Amplified ITS fragments were digested with *Dde*I or *Msp*I, and electrophoresis patterns of digested fragments were compared on 6% polyacrylamide gels.

Results

PCR products showed variations in size from 600 to 700 bp implying differences in nucleotide sequences of the ITS regions among types (Fig. 1). All the ITS-RFLP patterns yielded three or more bands of approximately 300 base pairs or less with *Dde*I digestion (Fig. 2), and approximately 500 base pairs or less with *Msp*I digestion (Fig. 3). Although some showed very similar patterns with one enzyme, fourteen types could be discriminated clearly as well as by mtDNA-RFLP based on the combination of their ITS-RFLP patterns with two enzymes. These were designated as nDNA Types E1~E13 and Es (Table 1). On the other hand, ITS-RFLP patterns of strains of the same mtDNA type were identical and intra-type differences could not be detected in Type 5 or Type 10, while Types 5-1 and 5-2, or Types 10-1 and 10-2 were differentiated by mtDNA-RFLP with *Hind*III[2]. There were no disagreements between the results of the two typing methods.

Eleven of twenty isolates were *E. jeanselmei* mtDNA Type 5 and nDNA Type E5. Five were of mtDNA Type 6 and nDNA Type E6. One was of mtDNA Type 8 and nDNA Type E8. Two were *E. jeanselmei* of mtDNA Type 10 and nDNA Type E10 (Table 2). Among the 11 isolates of *E. jeanselmei* Type 5, 6 comprised three cases, of which two strains were isolated from each patient.

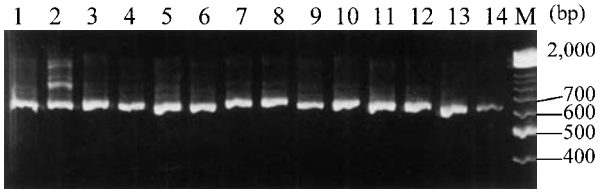

Fig. 1. PCR products from representative strains of 13 mtDNA types of *E. jeanselmei* and *E. spinifera* Type 4 amplified with the primer set, ITS1 and ITS4. Lanes 1–14, strains of E1–E13 and Es (Table 1).

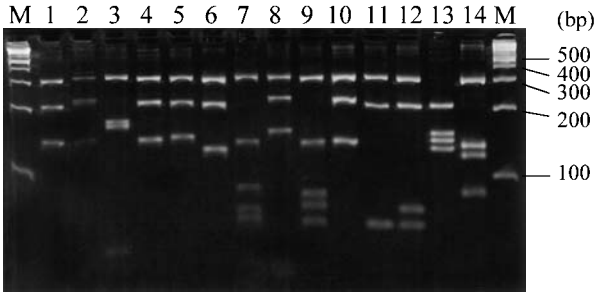

Fig. 2. ITS-RFLP patterns with *Dde*I. Lane M, size marker. Lanes 1–14, strains of E1–E13 and Es (Table 1).

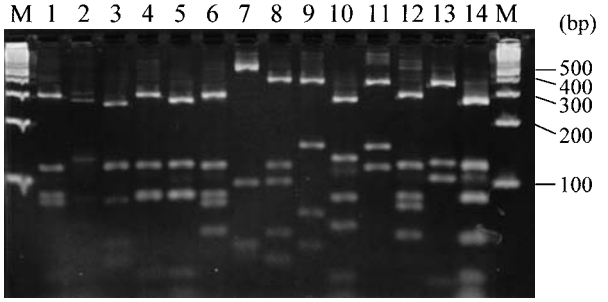

Fig. 3. ITS-RFLP patterns with *Msp*I. Lane M, size marker. Lanes 1–14, strains of E1–E13 and Es (Table 1).

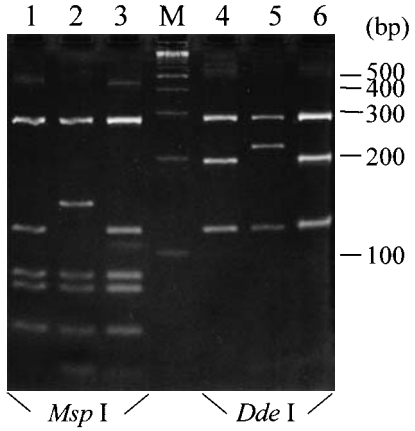

Fig. 4. ITS-RFLP patterns of *E. jeanselmei* Type E6 strains. Lane M, size marker. Lanes 1 and 4, KMU 2720 (E6). Lanes 2 and 5, KMU4391 (E6'). Lanes 3 and 6, KMU4396 (E6).

These were isolated from two different tissues, at different times, or from morphologically different colonies. Of the six isolates of *E. jeanselmei* Type 6, 3 were isolated from the same patient at different times or from different tissues. In addition, minor differences in the ITS-RFLP patterns were found between one (KMU 4391) and two other isolates (KMU 4396 and KMU 4428) (Fig. 4). KMU 4391 gave ITS-RFLP patterns with a fragment longer than the corresponding fragment of the other. The remaining 4 fragments produced by *Msp*I digestion and the remaining 2 fragments produced by *Dde*I digestion were shared. Thus, the differences were considered intra- rather than inter-type differences. The strain was designated as nDNA Type E6'. Therefore, this was a case of double infection with two genetically different *E. jeanselmei* strains.

In summary, 20 *E. jeanselmei* isolates comprised 8 cases of *E. jeanselmei* Type 5, 3 of *E. jeanselmei* Type 6, 1 of *E. jeanselmei* Type 6', 2 of *E. jeanselmei* Type 10, and 1 of *E. jeanselmei* Type 8.

Discussion

ITS-RFLP with *Dde*I and *Msp*I were shown to be more useful for typing *E. jeanselmei* strains than mtDNA-RFLP because there was no disagreement between the results of these two methods and the former is superior with regard to the clear band patterns, rapidity (one day *vs.* five days), requirement of only a small fungal colony (a tiny colony on a slant *vs.* 200 m*l* of liquid culture), and ease of processing.

More than half of the 20 isolates were *E. jeanselmei* Type 5. The Type strain of *E. jeanselmei*, ATCC34128, showed the genotype of *E. jeanselmei* Type 5 and Type E5. Therefore, most Japanese clinical isolates were shown to be *E. jeanselmei* var. *jeanselmei*.

mtDNA-RFLP patterns of *E. jeanselmei* Type 8 were identical to those of *E. jeanselmei* var. *lecanii-corni* Type strain ATCC 12734[9]. This was in agreement with the results of Uijthof[8] and it seemed appropriate to understand *E. jeanselmei* Type 8 as *E. jeanselmei* var. *lecanii-corni*.

mtDNA-RFLP patterns of *E. jeanselmei* Type 10 were compared with those of 8 *E. jeanselmei* var. *heteromorpha*[7]. Four of the 8 *E. jeanselmei* var. *heteromorpha* isolates showed mtDNA-RFLP patterns identical to those of *E. jeanselmei* Type 10. Nevertheless, three of the remaining four, including the Type strain of *E. jeanselmei* var. *heteromorpha*, showed unique patterns. In addition, two others showed the same patterns as *E. dermatitidis*. Therefore, *E. jeanselmei* Type 10 could not be identified as *E. jeanselmei* var. *heteromorpha*. This was not in conflict with the results of Uijthof[8], who placed strains of *E. jeanselmei* Type 10 into unknown groups. mtDNA-RFLP patterns of *E. jeanselmei* Type 6 were identical to those of *E. moniliae* Type 7. However, *E. moniliae* seemed also to be a complex species, and the differences between the Type strain, *E. moniliae* Type 10 and *E. moniliae* Type 7 were as great as that between two species[4]. Therefore, *E. jeanselmei* Type 6 could not be identified as *E. moniliae*.

All 8 strains of *E. jeanselmei* Type 6, including the strain of *E. moniliae* Type 7, were isolated from patients in Japan. At least 5 cases are known and this type is the second most common *E. jeanselmei* in Japan following *E. jeanselmei* var. *jeanselmei*.

When types of identical genotypes with those of *E. dermatitidis*, *E. spinifera*, *F. pedrosoi* or *E. jeanselmei* var. *lecanii-corni* were excluded from 15 *E. jeanselmei* types[2], *E. jeanselmei* was still comprised of 11 genetically different types. While environmental isolates are heterogeneous despite their morphological similarity, clinical isolates may be homogeneous because of the filtration effect of the human body in clearing non-pathogenic fungi. This phenomenon was also reported previously in a study involving genotype analyses of environmental isolates of *Sporothrix schenckii*[12]. In *E. jeanselmei*, however, all but one type -*E. jeanselmei* Type 9- seemed to be pathogenic, including clinical isolates.

In the present study, the names *E. jeanselmei* Type 6 and *E. jeanselmei* Type 10 were used. However, these groups must be designated as two new *Exophiala* species as soon as possible, because there are large problems in identification of *Exophiala* species using ITS-sequences.

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