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Original Article

Species Identification and Strain Typing of *Fonsecaea pedrosoi* Using Ribosomal RNA Gene Internal Transcribed Spacer Regions

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Abstract

The restriction fragment length polymorphism (RFLP) in the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (rDNA) was analyzed on *Fonsecaea pedrosoi* isolates kept in the Department of Dermatology, Kanazawa Medical University, Japan.

On the bases of the RFLP patterns with *Dde* I and *Msp* I, 131 isolates were classified into 5 types (D1-D5) and 4 types (M1-M4), respectively.

Combining the RFLP patterns with *Dde* I and *Msp* I, the isolates were further classified into 6 rDNA-types which corresponded to the 6 mtDNA-types reported by Kawasaki *et al*. based on the mtDNA-RFLP patterns, except for a single strain of mtDNA-type 7, which was indistinguishable from mtDNA-type 2.

The strains of each rDNA-type formed a clade on the phylogenetic tree constructed from sequences of the ITS regions. ITS-RFLP analysis discriminated *F. pedrosoi* from 11 other species of pathogenic phaeoid fungi except *F. compacta*. These results strongly suggest that the typing based on ITS-RFLP is reliable and that *F. pedrosoi* and *F. compacta* are conspecific.

Compared with mtDNA-RFLP analysis, ITS-RFLP analysis is less tedious, permits simultaneous analysis of many samples and gives equivalent results rapidly. This analysis is therefore useful for typing or epidemiologically investigating *F. pedrosoi* and for differentiating it from other dematiaceous fungi.

Key words: *Fonsecaea pedrosoi*, strain typing, ribosomal RNA gene, restriction fragment length polymorphism, nucleotide sequence, phylogeny

Introduction

The fungus *Fonsecaea pedrosoi* is a major cause of chromoblastomycosis and phaeohyphomycosis. Kawasaki *et al*.[1] classified 120 *F. pedrosoi* strains into 7 types on the basis of restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) using the restriction enzyme *Hae* III. They reported a link between the types and their geographical distribution and commented on the usefulness of the method for identifying and typing the species. On the other hand, Untereiner and Naveau[2] described the phylogenetic relations of several species of pathogenic black fungi including Herpotrichiellaceae, based on the internal transcribed spacer (ITS) 1, 5.8S rDNA, ITS 2, and 28S rDNA of their nuclear ribosomal RNA gene (rDNA). In their study, they investigated one strain of *F. pedrosoi* (CBS 271.37), but they neither investigated the intra-species variations nor typed the strain.

In 1999, Caligiorne *et al*.[3] performed RFLP analysis on small-subunit rRNA gene regions and ITS regions of 7 black fungi. They studied 12 strains, including 3 strains of *F. pedrosoi* and 2 of *F. compacta*, and reported that the inter- and intra-species variations were large in the ITS regions.

In this study, we applied ITS-RFLP analysis to type *F. pedrosoi*, which had already been typed by mtDNA analysis. We then investigated the base sequences of the ITS regions of

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Table 1. A *F. compacta* and 131 *F. pedrosoi* strains used in this study. (1)

KMU no.	*Dde* I*	*Msp* I**	rDNA type	Species	mtDNA type	Origin	Country
3537	D2	M2	3	*Foncecaea pedrosoi*	Fp2	RV 4004	Zaire
3538	D2	M2	3	*F. pedrosoi*	Fp2	RV 4478	Zaire
3539	D2	M2	3	*F. pedrosoi*	Fp2	RV 5146	Zaire
3540	D2	M2	3	*F. pedrosoi*	Fp2	RV 9268	Zaire
3541	D2	M2	3	*F. pedrosoi*	Fp2	RV 14661	Zaire
3542	D2	M2	3	*F. pedrosoi*	Fp2	RV 25295	Mozambique
3543	D2	M2	3	*F. pedrosoi*	Fp2	RV 48561	Zaire
3544	D2	M2	3	*F. pedrosoi*	Fp2	RV 49786	Zaire
3545	D2	M2	3	*F. pedrosoi*	Fp2	RV 58100	Zaire
3648	D1	M1	1	*F. pedrosoi*	Fp4	9087	Venezuela
3649	D1	M1	1	*F. pedrosoi*	Fp4	7109	Venezuela
3650	D1	M1	1	*F. pedrosoi*	Fp4	5720	Venezuela
3651	D1	M1	1	*F. pedrosoi*	Fp4	1975	Venezuela
3665	D1	M1	1	*F. pedrosoi*	Fp4	806	Argentina
3666	D2	M1	2	*F. pedrosoi*	Fp1	13409/160	Argentina
3669	D1	M1	1	*F. pedrosoi*	Fp4	85014/97	Argentina
3670	D1	M1	1	*F. pedrosoi*	Fp4	Durand	Argentina
3671	D1	M1	1	*F. pedrosoi*	Fp4	13408/160	Argentina
3672	D2	M1	2	*F. pedrosoi*	Fp1	10240/97	Argentina
3673	D1	M1	1	*F. pedrosoi*	Fp4	2078	Argentina
3709	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46418	Costa Rica
3710	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46419	Costa Rica
3711	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46420	Costa Rica
3712	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46421	Costa Rica
3713	D2	M1	2	*F. pedrosoi*	Fp1	IFM 46422	Costa Rica
3714	D2	M1	2	*F. pedrosoi*	Fp1	IFM 46423	Costa Rica
3715	D2	M1	2	*F. pedrosoi*	Fp1	IFM 46424	Costa Rica
3716	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46425	Costa Rica
3717	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46426	Costa Rica
3718	D2	M1	2	*F. pedrosoi*	Fp1	IFM 46427	Costa Rica
3719	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46428	Costa Rica
3720	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46429	Costa Rica
3721	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46430	Costa Rica
3722	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46431	Costa Rica
3723	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46432	Costa Rica
3724	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46433	Costa Rica
3725	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46434	Costa Rica
3726	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46435	Costa Rica
3727	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46436	Costa Rica
3728	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46437	Costa Rica
3730	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46439	Costa Rica
3732	D1	M1	1	*F. pedrosoi*	Fp4	IFM 46441	Costa Rica
3772	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 530	Japan
3773	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 533	Japan
3774	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 606	Japan
3775	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 622	Japan
3776	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 634	Japan
3777	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 637	Japan
3778	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 639	Japan
3779	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 642	Japan
3780	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 656	Japan
3781	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 661	Japan
3782	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 664	Japan
3783	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 666	Japan
3784	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 669	Japan
3785	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 671	Japan
3786	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 676	Japan
3787	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 677	Japan
3788	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 683	Japan
3789	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 684	Japan
3790	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 685	Japan
3791	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 686	Japan
3792	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 687	Japan
3793	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 688	Japan
3794	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 689	Japan
3795	D2	M1	2	*F. pedrosoi*	Fp1	TIMM 690	Japan
3796	D2	M1	2	*F. pedrosoi*	Fp1	Clinical isolate	Japan
3797	D1	M1	1	*F. pedrosoi*	Fp4	TIMM 532	USA
3798	D1	M1	1	*F. pedrosoi*	Fp4	IFM 4915	Mexico
3799	D1	M1	1	*F. pedrosoi*	Fp4	IFM 4916	Mexico

Table 1. A *F. compacta* and 131 *F. pedrosoi* strains used in this study. (2)

KMU no.	*Dde* I*	*Msp* I**	rDNA type	Species	mtDNA type	Origin	Country
3800	D1	M1	1	*F. pedrosoi*	Fp4	IFM 4917	Venezuela
3801	D1	M1	1	*F. pedrosoi*	Fp4	IFM 4918	Venezuela
3802	D3	M1	4	*F. pedrosoi*	Fp3	IFM 41517	Venezuela
3803	D3	M1	4	*F. pedrosoi*	Fp3	IFM 41518	Venezuela
3804	D3	M1	4	*F. pedrosoi*	Fp3	IFM 41519	Venezuela
3805	D1	M1	1	*F. pedrosoi*	Fp4	IFM 41520	Venezuela
3806	D1	M1	1	*F. pedrosoi*	Fp4	IFM 1415A	Colombia
3807	D4	M3	5	*F. pedrosoi*	Fp5	IFM 41867	Colombia
3808	D5	M4	6	*F. pedrosoi*	Fp6	IFM 41868	Colombia
3809	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41705	China
3810	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41706	China
3811	D2	M2	3	*F. pedrosoi*	Fp2	MMC 42	Thailand
3812	D2	M2	3	*F. pedrosoi*	Fp2	IP A1	Madagascar
3813	D2	M2	3	*F. pedrosoi*	Fp2	IP A2	Madagascar
3815	D2	M2	3	*F. pedrosoi*	Fp2	IP A7	Madagascar
3816	D2	M2	3	*F. pedrosoi*	Fp2	IP A10	Madagascar
3817	D2	M2	3	*F. pedrosoi*	Fp2	IP A11	Madagascar
3818	D2	M2	3	*F. pedrosoi*	Fp2	IP A13	Madagascar
3819	D2	M2	3	*F. pedrosoi*	Fp2	IP A15	Madagascar
3820	D2	M2	3	*F. pedrosoi*	Fp2	IP A16	Madagascar
3821	D2	M2	3	*F. pedrosoi*	Fp2	IP A17	Madagascar
3822	D2	M2	3	*F. pedrosoi*	Fp7	IP A18	Madagascar
3823	D2	M2	3	*F. pedrosoi*	Fp2	IP A19	Madagascar
3824	D2	M2	3	*F. pedrosoi*	Fp2	IP A20	Madagascar
3825	D2	M2	3	*F. pedrosoi*	Fp2	IP A21	Madagascar
3827	D2	M2	3	*F. pedrosoi*	Fp2	IP A30	Madagascar
3828	D2	M2	3	*F. pedrosoi*	Fp2	IP A32	Madagascar
3829	D2	M2	3	*F. pedrosoi*	Fp2	IP A33	Madagascar
3831	D2	M2	3	*F. pedrosoi*	Fp2	IP A37	Madagascar
3842	D2	M1	2	*F. pedrosoi*	Fp1	Clinical isolate	Japan
3846	D1	M1	1	*F. pedrosoi***	Fp4	IFM 4887	Argentina
3847	D2	M1	2	*F. pedrosoi*	Fp1	IFM 4889	Japan
3848	D2	M1	2	*F. pedrosoi*	Fp1	IFM 4912	Japan
3849	D2	M1	2	*F. pedrosoi*	Fp1	IFM 4913	Japan
3850	D1	M1	1	*F. pedrosoi*	Fp4	IFM 4914	Venezuela
3851	D2	M1	2	*F. pedrosoi*	Fp1	IFM 5070	Japan
3853	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41521	Japan
3854	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41522	Japan
3855	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41523	Japan
3856	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41524	Japan
3857	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41525	Japan
3858	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41526	Japan
3859	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41527	Japan
3860	D2	M1	2	*F. pedrosoi*	Fp1	IFM 41528	Japan
3861	D2	M1	2	*F. pedrosoi*	Fp1	IFM 45991	Japan
3862	D2	M1	2	*F. pedrosoi*	Fp1	IFM 45992	Japan
3863	D2	M1	2	*F. pedrosoi*	Fp1	IFM 45993	Japan
3864	D2	M1	2	*F. pedrosoi*	Fp1	IFM 45994	Japan
3927	D2	M1	2	*F. pedrosoi*	Fp1	AMMRL 116.5	Australia
3928	D2	M1	2	*F. pedrosoi*	Fp1	AMMRL 116.7	Australia
3929	D2	M1	2	*F. pedrosoi*	Fp1	AMMRL 116.8	Australia
3930	D2	M2	3	*F. pedrosoi*	Fp2	AMMRL 116.9	Australia
4015	D1	M1	1	*F. pedrosoi*	Fp4	220	Mexico
4016	D1	M1	1	*F. pedrosoi*	Fp4	238	Mexico
4017	D2	M1	2	*F. pedrosoi*	Fp1	278	Mexico
4043	D1	M1	1	*F. pedrosoi*	Fp4	Clinical isolate	Brazil
4044	D1	M1	1	*F. pedrosoi*	Fp4	Clinical isolate	Brazil
4067	D2	M1	2	*F. pedrosoi*	Fp1	Clinical isolate	Japan
4088	D2	M1	2	*F. pedrosoi*	Fp1	Clinical isolate	Brazil
4114	D2	M1	2	*F. pedrosoi*	Fp1	Clinical isolate	Japan
4191	D2	M1	2	*F. pedrosoi*	Fp1	Clinical isolate	Japan
3769	D1	M1	1	*F. compacta*	Fp4	IFM 4886	USA

* indicates RFLP pattern with *Dde* I. D1 - D5 correspond to D1 - D5 in Figure 1.
** indicates RFLP pattern with *Msp* I. M1 - M4 correspond to M1 - M4 in Figure 1.
*** indicates the type strain of this species.
KMU: Department of Dermatology, Kanazawa Medical University, Ishikawa, Japan.
IFM: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan.
MMC: School of Medicine, Chiang Mai University, Chiang Mai, Thailand.
RV: Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium.
TIMM: Research Center for Medical Mycology, Teikyo University, Tokyo, Japan.
IP: Institut Pasteur, Paris, France.
AMMRL: Australian National Reference Laboratory in Medical Mycology, Sydney, Australia.

Table 2. Eleven dematiaceous fungi used in this study for comparison

Species	KMU no.	Origin
Cladophialophora carrionii	3408	ATCC 16264
Exophiala dermatitidis	3000	ATCC 28869
E. jeanselmei	2714	ATCC 34123
E. spinifera	3220	ATCC 18218
Hortaea werneckii	3443	IFM 4885
Phialophora parasitica	3536	IFM 4924
P. repens	3535	IFM 4925
P. richardsiae	3427	NHL 2924
P. verrucosa	3471	ATCC 38561
Rhinocladiella aquaspersa	3533	ATCC 24410
R. atrovirens	3532	IFM 4931

ATCC: American Type Culture Collection, Rockville, USA.
IFM: same as in Table 1.
NHL: National Institute of Hygienic Sciences, Tokyo, Japan.

representatives of each type and compared the differences that exist within each type and those between types. We used the information to construct a phylogenetic tree and compared it with a corresponding tree based on mtDNA sequences.

Further, to investigate whether ITS-RFLP can be used for identifying *F. pedrosoi*, we compared the results for *F. pedrosoi* with those for representative strains of 11 other pathogenic species of dematiaceous fungus.

Materials and Methods

Species and strains

One hundred thirty-one strains of *F. pedrosoi* kept in the Department of Dermatology, Kanazawa Medical University (Table 1) were used and, for comparison, representative strains of 11 other species of dematiaceous fungi (Table 2). The type classification based on mtDNA-RFLP of the *F. pedrosoi* strains and the strains' geographical origins are shown in Table 1.

Experimental methods

The base sequence of the ITS region in *F. pedrosoi* (IFM 4887 = CBS 271.37: type strain) were obtained from GenBank. The computer software GENETYX-MAC version 10.1 (Software Development Co., Ltd., Tokyo, Japan) was used to select 2 restriction enzymes, *Dde* I and *Msp* I, which are capable of cutting the ITS region into fragments of appropriate length and number. Total DNA was extracted from each strain and the ITS regions of the rDNA was amplified by polymerase chain reaction (PCR) using the pair of primers ITS 1 and ITS 4[4]. PCR products were digested with the restriction enzymes *Dde* I or *Msp* I, and then subjected to electrophoresis. The *F. pedrosoi* were classified into types according to their RFLP patterns.

1. Total DNA extraction for PCR

Total DNA were extracted by the modified Makimura's method[5]. Fungi were picked up from each colony cultured on Sabouraud's slant agar and soaked in 70% ethanol for 24 hours. Fungi, about the size of a pin head, were transferred to a sample tube and washed once in buffer (200 mM Tris-HCl, 0.5% W/V SDS, 25 mM EDTA, 250 mM NaCl) to lyse the filamentous fungus. Next, 300 μl of the buffer was added and the cells were disrupted with a micro-mixer for approximately 15 seconds. The mixture was kept at 100°C for 5 minutes and then 150 μl of 3 M sodium acetate was added. The mixture was agitated, and allowed to stand at −20°C for 10 minutes. After centrifuging at 12,000 × *g* for 5 minutes, the supernatant was transferred to a new tube, to which 400 μl of phenol/chloroform was added. The mixture was centrifuged at 19,000 × *g* for 15 minutes, and the supernatant was transferred to another tube to which 400 μl of chloroform was added. This mixture was centrifuged at 19,000 × *g* for 20 minutes, and the supernatant was transferred to a new tube, to which 350 μl of propanol was added. After mixing, the solution was centrifuged at 19,000 × *g* for 20 minutes. The propanol was then discarded, 200 μl of 70% ethanol was added, and the mixture was centrifuged at 19,000 × *g* for 20 minutes. Then ethanol was removed. After allowing it to stand at room temperature for 10 minutes, 30 μl of ultra-pure water was added to dissolve the DNA. The solution was refrigerated and 2 μl used as a template for PCR.

2. PCR

For PCR, a TaqDNA Master Mix Kit (QIAGEN GmbH, 40724 Hilden, Germany) was used. PCR was performed using a 20 μl mixture consisting of 2 μl of the template, 0.2 μl of Taq DNA polymerase, 2 μl of × 10 buffer, 4 μl of Q solution, 0.2 μl of dNTP (10 mM), 0.2 μl of primer ITS1 (50 μM), 0.2 μl of primer ITS4 (50 μM), and 11.2 μl of dH_2O. The thermal cycler was set at 94°C for 4 minutes, 35 cycles of 94°C for 1 minute, 58°C for 2 minutes and then 72°C for 1.5 minutes, after which it was maintained at 4°C.

3. Digestion of PCR products with restriction enzymes

Five microliters of PCR products was mixed with 1 μl of either the restriction enzyme *Dde* I or *Msp* I (TOYOBO, Osaka, Japan), 1.5 μl of high or medium buffer (TOYOBO), respectively, and 7.5 μl of ultra-pure water, and incubated at 37°C for 1 hour.

4. Detection of RFLP patterns

Five microliters of the digested PCR products was mixed with 1 μl of loading dye, loaded on a 6% acrylamide gel, and subjected to electrophoresis at 100 V. After 45 min, the gel was stained with 5 μg/ml of ethidium bromide solution, and the electrophoretic patterns were photographed under a transilluminator. Steps 1 through 4 took about 8 hours.

5. Nucleotide sequence

The rDNA-ITS regions of representative strains that had been classified by Kawasaki *et al.*[1] into 7 types (Fp1 to Fp7) according to their mtDNA-RFLP, were amplified by PCR using the primers ITS1 and ITS4 together. After agarose gel electrophoresis, PCR products of approximately 0.6 kb were recovered from the agarose gel by a TOYOBO DNA purification kit (TOYOBO). Using the recovered DNA as a template and using the same primers, they were labeled by a cycle sequencing reaction with ABI Prism Big Dye™ Terminator Cycle Sequencing Ready Reaction Kits (PE Biosystems, USA). The base sequence was automatically read by an ABI PRISM TM 310 Genetic Analyzer (PE Biosystems). The sequencing reaction was repeated a total of 3 times for each strain using primers ITS1, ITS2, ITS3, and ITS4.

The base sequence alignment was done using gene analysis software, GENETYX-MAC 10.1 and then modified manually. The NJ tree was reconstructed by the software using the sequence of the ITS regions of *P. verrucosa* (NIH 8701), obtained from GenBank, as an out-group.

Results

Each of the 131 strains showed 1 of 5 ITS-RFLP band patterns (D1 - D5) when digested with *Dde* I. The D1 group had 4 bands of 300, 150, 130, and 80 bp, D2 had 5 bands of 300, 130, 80, 65 and 60 bp, D3 had 3 bands of 300, 210, and 130 bp, D4 had 3 bands of 300, 200, and 100 bp, and D5 had 3 bands of 300, 200, and 130 bp (Fig. 1). Compared with mtDNA-RFLP patterns (Fp1 to Fp7) reported by Kawasaki *et al.*[1], without exception, D1 strains corresponded to the Fp4 strains, D2 strains corresponded to the Fp1, Fp2, and Fp7 strains, D3 strains to the Fp3 strains, D4 strains to the Fp5 strains, and D5 strains to the Fp6 strains.

Four ITS-RFLP patterns (M1 - M4) were observed when digested with *Msp* I. M1 had three bands of 500, 100 and 50 bp, M2 had 4 bands of 400, 120, 100 and 50 bp, M3 had 4 bands of 300, 150, 120 and 60 bp, and M4 had 4 bands of 350, 120, 100 and 50 bp (Fig. 1). Compared with mtDNA-RFLP patterns (Fp1 - Fp7) reported by Kawasaki *et al.*[1], without exception, M1 strains corresponded to the Fp1, Fp3, and Fp4 strains, M2 strains to the Fp2 and Fp7 strains, M3 strains to the Fp5 strains, and M4 strains to the Fp6 strains. When the electrophoretic patterns of the *Dde* I digests and the *Msp* I digests were considered together, the 131 strains could be classified into 6 types: D1M1 (hereafter referred to as rDNA-type 1), D2M1 (rDNA-type 2), D2M2 (rDNA-type 3), D3M1 (rDNA-type 4), D4M3 (rDNA-type 5),

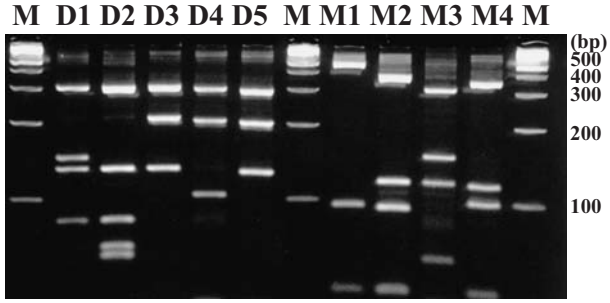

Fig. 1. rDNA-ITS-RFLP patterns with *Dde* I and *Msp* I

M: size marker, D1 - D5: RFLP patterns with *Dde* I, M1 - M4: RFLP patterns with *Msp* I.

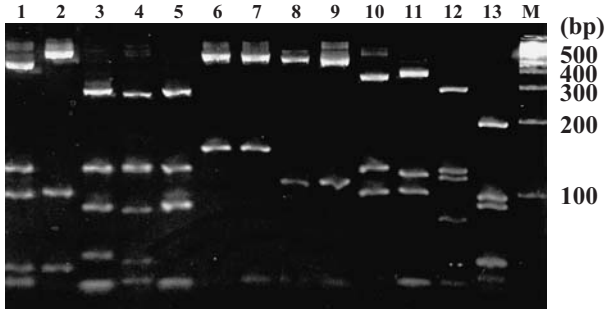

Fig. 2. rDNA-ITS-RFLP patterns of 11 other dematiaceous fungi with *Msp* I

Lane 1, *F. pedrosoi* rDNA-Type 3; lane 2, *F. pedrosoi* rDNA-Type 1; lane 3, *E. spinifera*; lane 4, *E. dermatitidis*; lane 5, *E. jeanselmei*; lane 6, *C. carrionii*; lane 7, *P. verrucosa*; lane 8, *P. repens*; lane 9, *P. parasitica*; lane 10, *P. richardsiae*; lane 11, *R. aquaspersa*; lane 12, *R. attrovirens*; lane 13, *H. werneckii*; lane M, size marker.

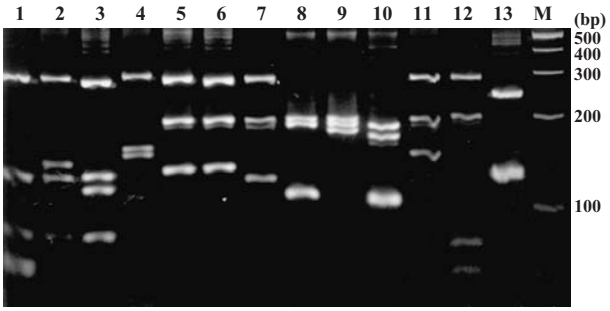

Fig. 3. rDNA-ITS-RFLP patterns of 11 other dematiaceous fungi with *Dde* I

Lane 1, *F. pedrosoi* rDNA-Type 3; lane 2, *F. pedrosoi* rDNA-Type 1; lane 3, *E. spinifera*; lane 4, *E. dermatitidis*; lane 5, *E. jeanselmei*; lane 6, *C. carrionii*; lane 7, *P. verrucosa*; lane 8, *P. repens*; lane 9, *P. parasitica*; lane 10, *P. richardsiae*; lane 11, *R. aquaspersa*; lane 12, *R. attrovirens*; lane 13, *H. werneckii*; lane M, size marker.

and D5M4 (rDNA-type 6). Compared with typing based on their mtDNA, rDNA-type 1 strains corresponded to the Fp4 strains, rDNA-type 2 to the Fp1, rDNA-type 3 to the Fp2 and Fp7, rDNA-type 4 to the Fp3, rDNA-type 5 to the Fp5, and rDNA-type 6 to the Fp6. Fp2 and Fp7 could not be differentiated by ITS-RFLP in the present study. These results are summarized in Table 1.

The ITS-RFLP of *F. compacta* had the rDNA-type 1 (D1 and M1) pattern. Furthermore, the ITS-RFLP patterns of the 11 species of phaeoid fungi were different from those of *F. pedrosoi* (Fig. 2, 3).

Gene analysis

The base sequences of the ITS regions of the 18 strains were registered in GenBank and their accession numbers are shown in Figure 4. The maximum base sequence differences were 6 out of 647 among the 4 rDNA-type 1 strains, 8 out of 646 among the 4 rDNA-type 2 strains, 3 out of 643 among the 5 rDNA-type 2 strains, and 0 out of 647 among the 3 rDNA-type 4 strains.

A tree for 17 strains of *F. pedrosoi* and 1 strain of *F. compacta* is shown in Figure 4. The tree is mainly divided into 3 branches, with rDNA-type 1 - 4 and *F. compacta* forming one, and rDNA-type 5 and rDNA-type 6 forming the other two. The branching closely matches that in the tree for the 7 types classified by their mtDNA, though the branch for Fp7 branches off further from Fp2 in the mtDNA classification.

Discussion

One-hundred-thirty-one strains of *F. pedrosoi* were classified into 6 types based on two sets of ITS-RFLP patterns obtained by two restriction enzymes. RFLP patterns of all 6 types differed from those of the 11 other species of dematiaceous fungi, indicating that *F. pedrosoi* can be clearly identified, typed and differentiated by ITS-RFLP analysis.

In the present study, however, the Fp2 and Fp7 strains of Kawasaki *et al.*[1] could not be

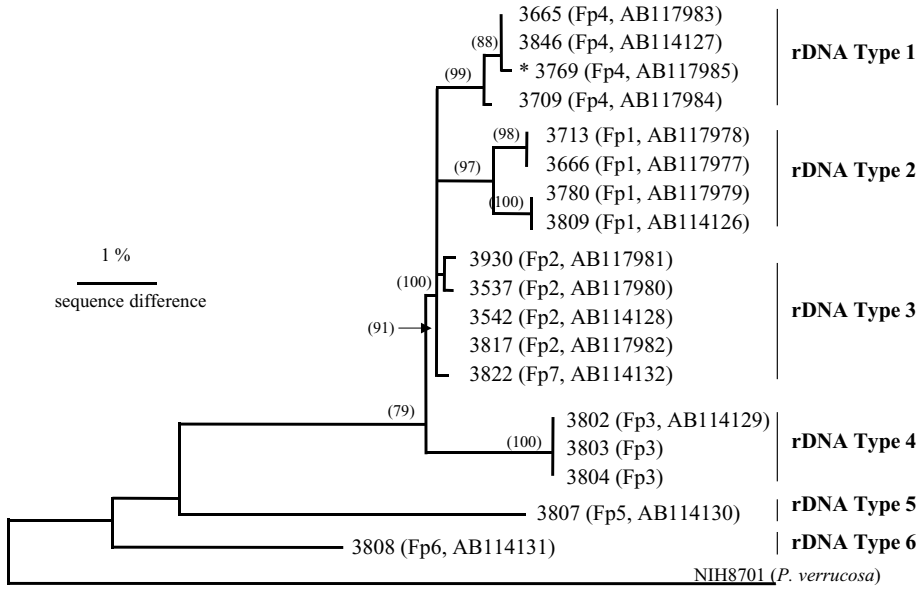

Fig. 4. A phylogenetic tree of 6 rDNA-types of *F. pedrosoi* and *F. compacta*, based on the nucleotide sequences of 5.8S rRNA gene and internal transcribed spacers

The mtDNA type and the accession number of sequence are shown in parenthesis after each KMU number. * indicates *F. compacta*. The sequence of *P. verrucosa* (NIH 8701) was from GenBank. Numbers in parentheses on the branches indicate bootstrap values of 1000 trials. Values over 70% are shown.

distinguished and they were both classed as rDNA-type 3. Except for a single strain of Fp7, which was indistinguishable from Fp2, our findings suggest that the type classification based on mtDNA and that based on rDNA are nearly identical. Therefore, these type classifications are reliable. Since the ITS-RFLP typing matches the mtDNA-RFLP typing, each rDNA-type shows geographical specificity as well as a mtDNA-type.

Comparison of the base sequences of ITS regions of 6 rDNA-types showed that the differences within each type (0 - 7 bases) were clearly smaller than those between types (13 - 72 bases), and the strains of each rDNA-type formed a clade on the phylogenetic tree. Therefore the classification of the *F. pedrosoi* strains based on ITS-RFLP with *Dde* I and *Msp* I is phylogenetically reliable.

Whether *F. pedrosoi* and *F. compacta* are actually separate species has been a topic of discussion[6-8]. In their molecular biology, however, mtDNA analysis has shown them to be conspecific[9]. Attili *et al.*[10] performed rDNA-RFLP analysis and determined the base sequence of the ITS 1 region in 13 strains of *F. pedrosoi* and 3 *F. compacta*; they reported that the two fungi could not be distinguished by genetic analysis. Furthermore, Caligiorne *et al.*[11] claimed that they could not be distinguished by the random amplification of polymorphic DNA (RAPD) analysis. Moreover, Abliz *et al.*[12] reported similar results using the species-specific primers for *F. pedrosoi*. Although the strains used were different from theirs, in the present study *F. compacta* showed the same RFLP patterns as those of rDNA-type 1 *F. pedrosoi*, strongly suggesting that *F. pedrosoi* and *F. compacta* are conspecific.

Furthermore, considering that recombination of the nuclear rDNA can occur during sexual reproduction and that mtDNA is inherited through the maternal cytoplasm, the fact that the typing based on the rDNA agrees with the typing based on the mtDNA seems to imply that *F. pedrosoi* has been reproducing only asexually for a very long time, and during this period differentiated into several types.

From a phylogenetic tree based on mtDNA analysis, Kawasaki *et al.* (1) suggested that Fp1 (rDNA-type 2), which is distributed extensively throughout Asia and Africa, might be a more ancient strain than Fp4 (rDNA-type 1), which is predominant in North and South America.

From the phylogenetic tree based on base sequences, we suggest that rDNA-types 5 and 6 diverged first in South America, followed later by the divergence of rDNA-types 1 - 4 diverging one after the other. It is hard to tell the sequence in which rDNA-types 1 - 4 diverged, however, because they are extremely closely related to each other.

Until now, *F. pedrosoi* has been identified by its morphological features. However, since the variety of morphological features of all the fungi is so vast, identification by morphology is not always possible. In comparison, ITS-RFLP analysis is superior in that it is much faster and less laborious. Moreover, ITS-RFLP analysis can be used reliably to type even a small amount of fungus taken from just a part of a cultured colony. The results are highly reproducible and a large number of samples can be analyzed at the same time. We consider ITS-RFLP analysis to be extremely useful for molecular epidemiological studies, as it reveals geographical specificity.

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