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Original Article

RFLP Analysis of the Internal Transcribed Spacer Regions of *Sporothrix schenckii*

Seiji Watanabe, Masako Kawasaki, Takashi Mochizuki, Hiroshi Ishizaki

Department of Dermatology, Kanazawa Medical University,
1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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Abstract

Restriction fragment length polymorphism (RFLP) analysis was performed on the internal transcribed spacer regions of 204 *Sporothrix schenckii* isolates and on one strain each of the related fungi, *S. schenckii* var. *luriei*, *S. curviconia*, *S. inflata* and *Ceratocystis stenoceras*. *S. schenckii* isolates, which have been collected from around the world, have already been typed according to their mitochondrial DNA (mtDNA), and are kept in the Department of Dermatology, Kanazawa Medical University, Japan.

Approximately 600 bp of the internal transcribed spacer region 1 (ITS1) of their nuclear ribosomal RNA gene (rDNA), 5.8S rDNA and ITS2 was amplified by PCR. From ITS-RFLP analysis of the PCR products, *S. schenckii* isolates comprised 4 types, rDNA types I - IV. The rDNA type I - III strains corresponded to the Group A strains (mtDNA types 1 - 3, 11, 14 - 19, 22 and 23), while the rDNA type IV strains corresponded to the Group B strains (mtDNA types 4 - 10, 12, 13, 20 and 21), as previously categorized according to their mtDNA-RFLP. The ITS-RFLP patterns of the above 4 related fungi all differed from those of the 4 rDNA types of *S. schenckii*.

Furthermore, only 22 (3.5%) out of a sequence of about 620 bases of the ITS regions of the rDNA differed among representatives of the mtDNA types 1 - 5, 7, 11, 14 - 19, 22 and 23. This difference in the ITS region is smaller than the 10% difference among isolates when estimated by mtDNA-RFLP. From the phylogenetic tree based on the base sequences, rDNA type I - III strains belong to Group I, while rDNA type IV strains belong to Group II which correspond with Groups A and B based on their mtDNA. The Group I strains are predominant in South America and Africa, while Group II are predominant in Australia and Asia.

ITS-RFLP analysis is better than mtDNA-RFLP in allowing faster discrimination and identification, and for its ability to divide the 4 types into groups, which is useful in clinical diagnosis and epidemiological investigations of *S. schenckii*.

Key words: *Sporothrix schenckii*, strain typing, ITS region, restriction fragment length polymorphism

Introduction

Sporothrix schenckii, a causative agent of sporotrichosis, exists in soil in a wide range of regions from tropical to temperate[1, 2]. *S. schenckii* has been identified morphologically, but this conventional method is limited in the differentiation of closely related fungi. It takes a great deal of skill to discriminate between *S. curviconia*, *S. inflata*, *S. schenckii* var. *luriei* and *Ceratocystis stenoceras*, which are all related. In 1970, Taylor[3] asserted that morphological, serological and mouse virulence characteristics of a number of species of *Ceratocystis*, including *C. stenoceras*, cannot be discriminated from those of *S. schenckii*. These taxonomical problems have been resolved by molecular biological techniques[4-8].

Suzuki *et al.*[4] investigated mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) of *S. schenckii* and several related fungi. Based on their RFLP patterns, they reported 11 types of *S. schenckii*, 4 types of *C. stenoceras* out of 17 strains, 4 types of *S. inflata*

Correspondence author: Seiji Watanabe MD
1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan
Department of Dermatology, Kanazawa Medical University

out of 7 strains and 7 types of *C. minor* out of 7 strains. All of these had different RFLP patterns to those of *S. schenckii*, making it possible to discriminate *S. schenckii* from them.

Also based on mtDNA RFLP analysis, Takeda *et al.*[5] divided 258 Japanese isolates of *S. schenckii* into 10 types, and phylogenetically, the types were clustered into two groups, Group A and Group B.

Ishizaki and Kawasaki[6] investigated clinical isolates that had been identified morphologically as *S. schenckii*, and by mtDNA-RFLP analysis confirmed that they were all *S. schenckii*. They reported that the 24 mtDNA types of strains formed two large phylogenetic groups: Group A comprising types 1 - 3, 11, 14 - 19, 22 and 23, and Group B comprising types 4 - 10, 12, 13, 20, 21 and 24. They also reported that group A strains are predominant in South America and Africa, while Group B strains are predominant in Australia and Asia.

Later, Cabrera *et al.*[9] analyzed mtDNA-RFLP in *S. schenckii* isolated in Mexico, Guatemala and Columbia, and reported 6 new types. However, because the RFLP pattern of some of their new types matched the pattern of types already reported by Ishizaki *et al.*[10], reconfirmation of the typing of their isolates is now underway.

Tateishi *et al.*[7] applied karyotype analysis to 8 strains of *S. schenckii*, and divided them into 3 types. The two molecular biological techniques did not give conflicting results. Sugita[8] applied single-strand conformation polymorphism to a part of a putative membrane transporter protein gene of 28 strains of *S. schenckii* whose mtDNA types are clear, and divided them into 3 types. His findings were compatible with those based on mtDNA-RFLP. However, he investigated only a small number of strains and his findings are not geographically broad.

Recently, analysis of the internal transcribed spacer (ITS) region of the nuclear rRNA gene (rDNA)[11], which has been used to identify and clarify the phylogenetic relationship of Eumycetes, has been applied to dermatophytes[12-15] and black fungi[16, 17], amongst others. Mochizuki[15] has pointed out that ITS-RFLP analysis provided more detailed information than mtDNA-RFLP analysis of the *Trichophyton mentagrophytes* complex. Attili *et al.*[16] typed *Fonsecaea pedrosoi* by their ITS-RFLP. In this study, we used ITS-RFLP analysis to identify and type *S. schenckii*, to investigate its molecular epidemiology, and then we examined the phylogenetic relations between the types.

Materials and Methods

Fungi

Two hundred and four *S. schenckii* clinical isolates have been collected from Japan, China, Holland, Spain, North America, Costa Rica, Venezuela, Mexico, Brazil, Argentina, Australia and South Africa. They are kept in the Department of Dermatology, Kanazawa Medical University, Japan, where they have been typed and subtyped according to their mtDNA, *S. schenckii* var. *luriei*, *S. curviconia*, *S. inflata* and *C. stenoceras*, which are all related to *S. schenckii*, were also studied (Table 1).

DNA extraction

DNA was extracted by the method of Makimura *et al.*[12]. Each species of fungus was cultured on 4% Sabouraud's agar medium. A part of each colony was soaked in 70% ethanol. Ten mg of the filamentous fungus was transferred from the ethanol into 300 μl of buffer (200 mM Tris-HCl at pH 7.5, 0.5% w/v SDS, 25 mM EDTA, 250 mM NaCl) and disrupted by a Handy Grinder (Kontes Glass Company, New Jersey, USA) for 10-15 sec. The mixture was kept at 100°C for 5 minutes and, after addition of 150 μl of 3 M sodium acetate, allowed to stand at −20°C for 10 minutes. After centrifuging at 12,000 x g for 5 min, the supernatant was transferred to a new tube, to which 400 μl of phenol/chloroform was added. After agitation, the mixture was centrifuged at 19,000 x g for 15 min. Again, the supernatant was transferred to another tube, to which 400 μl of chloroform was added and, again after agitation, the mixture was centrifuged at 19,000 x g for 15 min. This time, after transferring the supernatant to a new tube, an equal volume of propanol was added and, after mixing, the solution was centrifuged at 19,000 x g for 20 min, and the precipitate harvested. The precipitate was washed in 200 μl of 70% ethanol, and dried at room temperature for 10 min. It was then dissolved in 30 μl of ultra-pure water. The resultant solution of DNA was refrigerated and 2 μl used as a template for PCR as described below.

Amplification of rDNA by PCR

PCR was performed using a Taq DNA polymerase kit (QIAGEN GmbH, Hilden, Germany) and the primer pair ITS1, ITS4[11]. The reaction mixture contained 2 μl of the template DNA, 0.2 μl of Taq DNA polymerase, 2 μl of buffer, 4 μl of Q solution, 0.2 μl of dNTP, 0.2 μl of primer ITS1, 0.2 μl of primer ITS4, 11.2 μl of

Table 1. *S. schenckii* clinical isolates and other species used in this study (1)

KMU no.	mtDNA type, Accession no.	Group	Country	Origin	rDNA Type	Group	ITS-RFLP pattern with		
							Hae II	*Apa* I	*Nla* III
975*	1, AB122038	A	USA	ATCC 10268	I	I	H1	A1	N1
2285*	1, AB127999	A	Japan	Clinical isolate	I	I	H1	A1	N1
2921	2a	A	Japan	Clinical isolate	I	I	H1	A1	N1
2924	2a	A	Japan	Clinical isolate	I	I	H1	A1	N1
2771	2a	A	Japan	Clinical isolate	I	I	H1	A1	N1
2286*	2a. AB122039	A	Japan	Clinical isolate	I	I	H1	A1	N1
3694	2a	A	Japan	TIMM 1019	I	I	H1	A1	N1
3505	2a	A	USA	TIMM 0966	I	I	H1	A1	N1
3114*	2a, AB128000	A	Netherlands	Clinical isolate	I	I	H1	A1	N1
4014*	2b, AB128004	A	Mexico	781	I	I	H1	A1	N1
3656	3a	A	Argentina	10. 226/207	I	I	H1	A1	N1
3660	3a	A	Argentina	1611/22	I	I	H1	A1	N1
2501	3a	A	Brazil	IFM 5050	I	I	H1	A1	N1
3587	3a	A	Costa Rica	IFM 46281	I	I	H1	A1	N1
2500*	3a, AB089139	A	Japan	IFM 5890	I	I	H1	A1	N1
3943	3a	A	South Africa	MRC 5375	I	I	H1	A1	N1
3944*	3a, AB128002	A	South Africa	MRC 5377	I	I	H1	A1	N1
3967	3a	A	South Africa	MRC 6868	I	I	H1	A1	N1
3970	3a	A	South Africa	MRC 6870	I	I	H1	A1	N1
3615	3b	A	Venezuela	9334	I	I	H1	A1	N1
3620*	3b, AB128001	A	Venezuela	7179	I	I	H1	A1	N1
3623	3b	A	Venezuela	7247	I	I	H1	A1	N1
3624	3b	A	Venezuela	254	I	I	H1	A1	N1
3626	3b	A	Venezuela	9862	I	I	H1	A1	N1
3629	3b	A	Venezuela	2888	I	I	H1	A1	N1
3638	3b	A	Venezuela	8888	I	I	H1	A1	N1
3639	3b	A	Venezuela	8775	I	I	H1	A1	N1
3643	3b	A	Venezuela	5000	I	I	H1	A1	N1
3644	3b	A	Venezuela	4702	I	I	H1	A1	N1
3893	3c	A	Australia	AMMR15. 1	I	I	H1	A1	N1
3907	3c	A	Australia	AMMR15. 23	I	I	H1	A1	N1
3898	3c	A	Australia	AMMR15. 13	I	I	H1	A1	N1
3910	3c	A	Australia	AMMR15. 26	I	I	H1	A1	N1
3902	3c	A	Australia	AMMR15. 18	I	I	H1	A1	N1
4039	3c	A	Brazil	Clinical isolate	I	I	H1	A1	N1
4040*	3c, AB128005	A	Brazil	Clinical isolate	I	I	H1	A1	N1
4012*	3d, AB128003	A	Mexico	234	I	I	H1	A1	N1
3657	4	B	Argentina	1611/18	IV	II	H2	A2	N2
3661	4	B	Argentina	1617/18	IV	II	H2	A2	N2
3663	4	B	Argentina	10777/205	IV	II	H2	A2	N2
3894	4	B	Australia	AMMR15. 9	IV	II	H2	A2	N2
3896	4	B	Australia	AMMR15. 11	IV	II	H2	A2	N2
3897	4	B	Australia	AMMR15. 12	IV	II	H2	A2	N2
3899	4	B	Australia	AMMR15. 14	IV	II	H2	A2	N2
3900	4	B	Australia	AMMR15. 15	IV	II	H2	A2	N2
3901	4	B	Australia	AMMR15. 16	IV	II	H2	A2	N2
3903	4	B	Australia	AMMR15. 19	IV	II	H2	A2	N2
3904	4	B	Australia	AMMR15. 20	IV	II	H2	A2	N2
3905	4	B	Australia	AMMR15. 21	IV	II	H2	A2	N2
3906	4	B	Australia	AMMR15. 22	IV	II	H2	A2	N2
3908	4	B	Australia	AMMR15. 24	IV	II	H2	A2	N2
4029	4	B	China	5 00077	IV	II	H2	A2	N2
4030	4	B	China	11 00069	IV	II	H2	A2	N2
4031	4	B	China	22 00071	IV	II	H2	A2	N2
2740	4	B	Japan	SM1207	IV	II	H2	A2	N2
2742	4	B	Japan	SM1209	IV	II	H2	A2	N2
2747	4	B	Japan	K1007	IV	II	H2	A2	N2
2749	4	B	Japan	K1009	IV	II	H2	A2	N2
2751	4	B	Japan	K1011	IV	II	H2	A2	N2
2757	4	B	Japan	K1019	IV	II	H2	A2	N2
2758	4	B	Japan	K1021	IV	II	H2	A2	N2
2766	4	B	Japan	K1073	IV	II	H2	A2	N2
2770	4	B	Japan	K1079	IV	II	H2	A2	N2
2773	4	B	Japan	K1094	IV	II	H2	A2	N2

(continued)

Table 1. *S. schenckii* clinical isolates and other species used in this study (2)

KMU no.	mtDNA type, Accession no.	Group	Country	Origin	rDNA Type	Group	ITS-RFLP pattern with		
							Hae II	*Apa* I	*Nla* III
2920	4	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3111	4	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3509*	4, AB128007	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3512	4	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3517	4	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3614	4	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3696	4	B	Japan	TIMM 1022	IV	II	H2	A2	N2
3993*	4, AB128006	B	South Africa	MRC7348	IV	II	H2	A2	N2
2052*	4, AB089138	B	USA	Duke #3751	IV	II	H2	A2	N2
3618	4	B	Venezuela	2461	IV	II	H2	A2	N2
3619	4	B	Venezuela	7045	IV	II	H2	A2	N2
3631	4	B	Venezuela	9682	IV	II	H2	A2	N2
3641	4	B	Venezuela	73	IV	II	H2	A2	N2
3642	4	B	Venezuela	8962	IV	II	H2	A2	N2
2746	5	B	Japan	K1004	IV	II	H2	A2	N2
2754	5	B	Japan	K1015	IV	II	H2	A2	N2
2761	5	B	Japan	K1024	IV	II	H2	A2	N2
2767	5	B	Japan	K1074	IV	II	H2	A2	N2
2777	5	B	Japan	K1098	IV	II	H2	A2	N2
3047	5	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3308	5	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3309	5	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3311*	5, AB122042	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3341	5	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3510	5	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3513	5	B	Japan	Tsukuba 1	IV	II	H2	A2	N2
3514	5	B	Japan	Tsukuba 2	IV	II	H2	A2	N2
3516	5	B	Japan	Tsukuba 4	IV	II	H2	A2	N2
3518	5	B	Japan	Tsukuba 6	IV	II	H2	A2	N2
3519	5	B	Japan	Tsukuba 7	IV	II	H2	A2	N2
3520	5	B	Japan	Tsukuba 8	IV	II	H2	A2	N2
3691	5	B	Japan	TIMM 1015	IV	II	H2	A2	N2
3701	5	B	Japan	TIMM 1032	IV	II	H2	A2	N2
3707	5	B	Japan	TIMM 1041	IV	II	H2	A2	N2
3708	5	B	Japan	TIMM 1044	IV	II	H2	A2	N2
4020	5	B	Spain	144	IV	II	H2	A2	N2
4023	5	B	Spain	1048	IV	II	H2	A2	N2
4027	5	B	Spain	1500	IV	II	H2	A2	N2
2735	6	B	Japan	SM1202	IV	II	H2	A2	N2
2745	6	B	Japan	SM1212	IV	II	H2	A2	N2
2750	6	B	Japan	K1010	IV	II	H2	A2	N2
2759	6	B	Japan	K1022	IV	II	H2	A2	N2
2784	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
2870	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
2916	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3362	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3376	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3515	6	B	Japan	Tsukuba 3	IV	II	H2	A2	N2
3530	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3593	6	B	Japan	IFM 46287	IV	II	H2	A2	N2
3602	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3603	6	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3692	6	B	Japan	TIMM 1016	IV	II	H2	A2	N2
3693	6	B	Japan	TIMM 1018	IV	II	H2	A2	N2
3916	7	B	Australia	AMMR15. 33	IV	II	H2	A2	N2
3918	7	B	Australia	AMMR15. 35	IV	II	H2	A2	N2
2734	7	B	Japan	SM1201	IV	II	H2	A2	N2
2755	7	B	Japan	K1016	IV	II	H2	A2	N2
2756	7	B	Japan	K1018	IV	II	H2	A2	N2
2768	7	B	Japan	K1075	IV	II	H2	A2	N2
2769	7	B	Japan	K1077	IV	II	H2	A2	N2
2774	7	B	Japan	K1095	IV	II	H2	A2	N2
2775	7	B	Japan	K1096	IV	II	H2	A2	N2
2779	7	B	Japan	K1100	IV	II	H2	A2	N2
3360*	7, AB122043	B	Japan	Clinical isolate	IV	II	H2	A2	N2
3507	7	B	Japan	Clinical isolate	IV	II	H2	A2	N2
2741	8	B	Japan	SM1208	IV	II	H2	A2	N2
2760	9	B	Japan	K1023	IV	II	H2	A2	N2
2736	9	B	Japan	SM1203	IV	II	H2	A2	N2
2877	9	B	Japan	Clinical isolate	IV	II	H2	A2	N2
2763	10	B	Japan	K1027	IV	II	H2	A2	N2

(continued)

Table 1. *S. schenckii* clinical isolates and other species used in this study (3)

KMU no.	mtDNA type, Accession no.	Group	Country	Origin	rDNA Type	Group	ITS-RFLP pattern with		
							Hae II	*Apa* I	*Nla* III
2687*	11, AB122044	A	France	IP1020	I	I	H1	A1	N1
4011*	11, AB128008	A	Mexico	160	I	I	H1	A1	N1
3974	11	A	South Africa	MRC 7349	I	I	H1	A1	N1
4001	11	A	South Africa	MRC 7385	I	I	H1	A1	N1
3314	12	B	Japan	Clinical isolate	IV	II	H2	A2	N2
2762	13	B	Japan	K1025	IV	II	H2	A2	N2
3580	14a	A	Costa Rica	IFM 46270	I	I	H1	A1	N1
3581	14a	A	Costa Rica	IFM 46271	I	I	H1	A1	N1
3582	14a	A	Costa Rica	IFM 46274	I	I	H1	A1	N1
3583	14a	A	Costa Rica	IFM 46276	I	I	H1	A1	N1
3584	14a	A	Costa Rica	IFM 46277	I	I	H1	A1	N1
3585	14a	A	Costa Rica	IFM 46279	I	I	H1	A1	N1
3586	14a	A	Costa Rica	IFM 46280	I	I	H1	A1	N1
3588	14a	A	Costa Rica	IFM 46282	I	I	H1	A1	N1
3589*	14a, AB128010	A	Costa Rica	IFM 46283	I	I	H1	A1	N1
3590	14a	A	Costa Rica	IFM 46284	I	I	H1	A1	N1
3591	14a	A	Costa Rica	IFM 46285	I	I	H1	A1	N1
3592	14a	A	Costa Rica	IFM 46286	I	I	H1	A1	N1
3594	14a	A	Costa Rica	IFM 46288	I	I	H1	A1	N1
3596	14a	A	Costa Rica	IFM 46290	I	I	H1	A1	N1
3597	14a	A	Costa Rica	IFM 46291	I	I	H1	A1	N1
3598	14a	A	Costa Rica	IFM 46292	I	I	H1	A1	N1
3599	14a	A	Costa Rica	IFM 46294	I	I	H1	A1	N1
3600	14a	A	Costa Rica	IFM 46295	I	I	H1	A1	N1
3601	14a	A	Costa Rica	IFM 46297	I	I	H1	A1	N1
3501*	14b, AB128009	A	USA	TIMM 0962	I	I	H1	A1	N1
3486*	14c, AB122045	A	USA	CDC B5537	I	I	H1	A1	N1
3488	14c	A	USA	CDC B5534	I	I	H1	A1	N1
3504*	15, AB122046	A	USA	TIMM 0965	I	I	H1	A1	N1
3652	16	A	Argentina	Ss M	II	I	H1	A3	N1
3653*	16, AB122047	A	Argentina	102. 137	II	I	H1	A3	N1
3654	16	A	Argentina	102. 391	II	I	H1	A3	N1
3659	16	A	Argentina	102. 138	II	I	H1	A3	N1
3655*	17, AB122048	A	Argentina	1611/24	I	I	H1	A1	N1
3658	17	A	Argentina	1611/21	I	I	H1	A1	N1
3664	17	A	Argentina	10144/205	I	I	H1	A1	N1
3940*	17, AB128011	A	South Africa	MRC 5372	I	I	H1	A1	N1
3946	17	A	South Africa	MRC 5379	I	I	H1	A1	N1
3951	17	A	South Africa	MRC 6853	I	I	H1	A1	N1
3955	17	A	South Africa	MRC 6872	I	I	H1	A1	N1
3957	17	A	South Africa	MRC 6857	I	I	H1	A1	N1
3958	17	A	South Africa	MRC 6858	I	I	H1	A1	N1
3959	17	A	South Africa	MRC 6860	I	I	H1	A1	N1
3965	17	A	South Africa	MRC 6866	I	I	H1	A1	N1
3966	17	A	South Africa	MRC 6867	I	I	H1	A1	N1
3976	17	A	South Africa	MRC 6952	I	I	H1	A1	N1
3977	17	A	South Africa	MRC 6953	I	I	H1	A1	N1
3978	17	A	South Africa	MRC 6954	I	I	H1	A1	N1
3985	17	A	South Africa	MRC 6961	I	I	H1	A1	N1
4004	17	A	South Africa	MRC 7388	I	I	H1	A1	N1
4005	17	A	South Africa	MRC 7389	I	I	H1	A1	N1
3617*	18, AB122049	A	Venezuela	9380	I	I	H1	A1	N1
3627	19	A	Venezuela	268	I	I	H1	A1	N1
3628*	19, AB122050	A	Venezuela	9763	I	I	H1	A1	N1
3637	19	A	Venezuela	255	I	I	H1	A1	N1
4018	20	B	Spain	5	IV	II	H2	A2	N2
4019	20	B	Spain	133	IV	II	H2	A2	N2
4021	20	B	Spain	156	IV	II	H2	A2	N2
3621	20	B	Venezuela	7471	IV	II	H2	A2	N2
3625	20	B	Venezuela	8345	IV	II	H2	A2	N2
3634	20	B	Venezuela	8916	IV	II	H2	A2	N2
3635	20	B	Venezuela	9887	IV	II	H2	A2	N2
3640	20	B	Venezuela	8915	IV	II	H2	A2	N2
3912	21	B	Australia	AMMR15. 28	IV	II	H2	A2	N2
3913	21	B	Australia	AMMR15. 29	IV	II	H2	A2	N2
3492*	22, AB122051	A	USA	CDC B4668	III	I	H1	A3	N3
3998*	23, AB122052	A	South Africa	MRC 7382	I	I	H1	A1	N1
4303	24	B	China	CMU 3143	IV	II	H2	A2	N2
4316	24	B	China	CMU C3	IV	II	H2	A2	N2
2787*	AB128012	*S. schenckii* var. *luriei*		CDC B1040**					
4394		*S. curviconia*		CBS 959. 73**					
4393		*S. inflata*		CBS 239. 68**					
2819*	AB122053	*C. stenoceras*		CBS 237. 32**					

(continued)

* indicates strains used for sequence analysis. ** indicates the type strain of each species.
AMMRL: Australian National Reference Laboratory in Medical Mycology, Australia.
ATCC: American Type Culture Collection, USA. CBS: Centraalbureau voor Schimmelcultures, The Netherlands.
CDC: Centers for Disease Control and Prevention, USA. CMU: China Medical University, China.
Duke: Duke University, USA. IFM: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan.
IP: Institut Pasteur, France. K: Kyushu University, Japan. KMU: Kanazawa Medical University, Japan.
MRC: Medical Research Council, Programme on Mycotoxins and Experimental Carcinogenesis, South Africa.
SM: Shiga University of Medical Science, Japan. TIMM: Research Center for Medical Mycology, Teikyo University, Japan.

dH_2O. The thermal cycler was set at 94°C for 4 min, 35 cycles of 94°C for 1 min, 58°C for 2 min and 72°C for 1.5 min, and a final temperature of 4°C. DNA fragments of about 620 bp comprising ITS1, 5.8S rDNA and ITS2 regions were obtained.

Restriction enzymes

An approx. 600 bp stretch of the ITS region of rDNA from 1 strain each of groups A (KMU 2500) and B (KMU 2052) as categorized by mtDNA, was sequenced. The software GENETYX-MAC ver. 10.1 (Software Development Co., Ltd., Tokyo, Japan) was used to select restriction enzymes that can detect differences between their sequences. Three restriction enzymes, *Hae* II, *Apa* I, *Nla*III (TOYOBO, Osaka, Japan) capable of showing differences in bands of 50 bp or more were then used for the RFLP analysis of all the strains in this study.

Restriction enzyme analysis

Five microliters of the PCR products was digested with each of the three restriction enzymes at 37°C for 2 h, according to the manufacturer's instructions. The digested products were subjected to electrophoresis on 5% acrylic amide gel for 40 min, stained with ethidium bromide, and visualized under UV light. The procedure to obtain the bands took about 8 h.

Construction of phylogenetic tree

The base sequence of the ITS regions, including 5.8S rDNA, of 28 strains of *S. schenckii*, 1 strain each of *S. schenckii* var. *luriei* and *C. stenoceras* were determined (Table 1). Of the 28 *S. schenckii* strains, 23 comprised 12 types in Group A and 5 comprised 3 types in Group B as categorized by mtDNA. Using the primer pair ITS5 and ITS4, we amplified approx. 650 bp fragments, purified them by precipitation in ethanol, and used them as template for cycle sequencing. After PCR using the primers ITS5, ITS2, ITS3 and ITS4[11] with a BigDye Terminator Ready Reaction Kit ver. 2.0 (Applied Biosystems, Foster City, USA) we used an ABI Prism 310 Genetic Analyzer (Applied Biosystems) to read the base sequences.

We aligned the base sequences using GENETYX-MAC, then refined the alignments manually. Based on the alignments, we constructed the phylogenetic tree by the NJ method using GENETYX-MAC, taking into account the gaps (inserted or deleted bases).

Results

Digestion with Restriction Enzymes

With *Hae* II, the 204 strains each gave one of two RFLP patterns: H-1 with 300, 120 and 100 bp bands or H-2 with 300, 200 and 120 bp bands. (Fig. 1). These were in agreement with the expected lengths (KMU 2500: 301, 113, 108

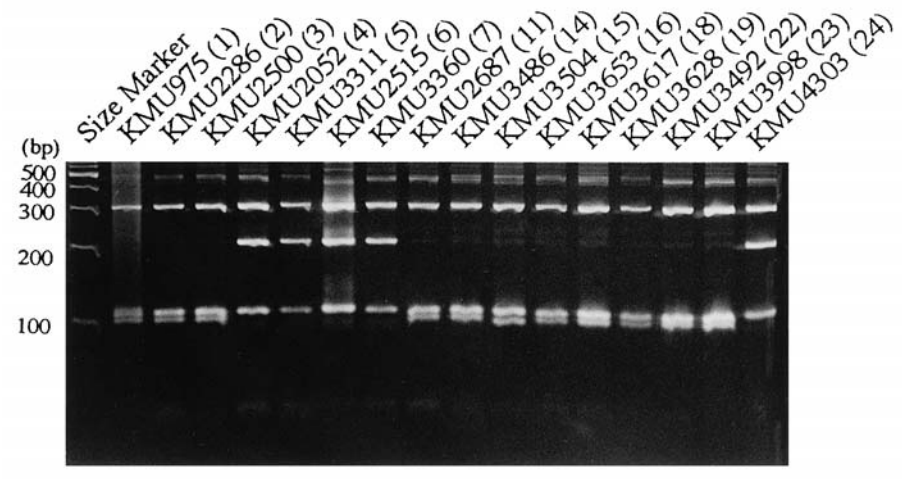

Fig. 1. ITS-RFLP patterns with *Hae* II.

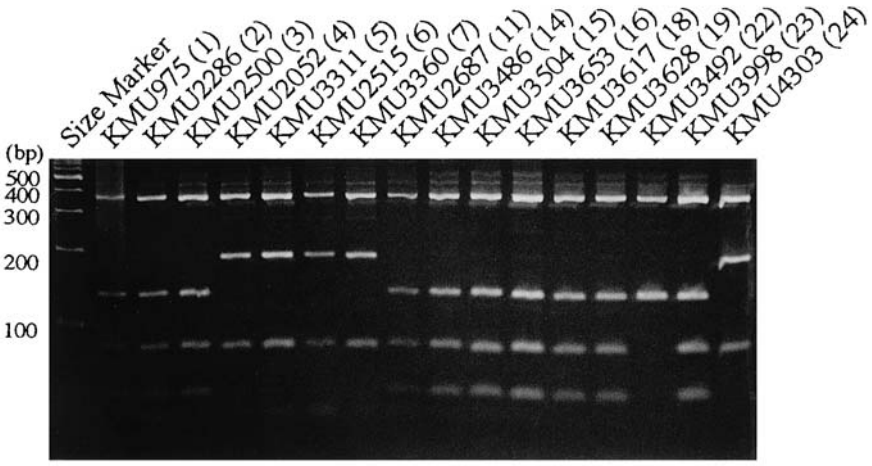

Fig. 2. ITS-RFLP patterns with *Nla* III.

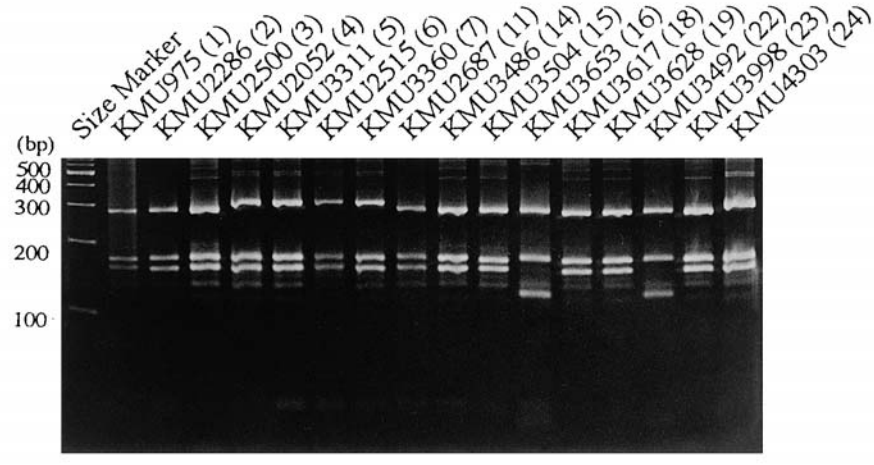

Fig. 3. ITS-RFLP patterns with *Apa* I .

Table 2. Lengths of restriction fragments of 8 ITS-RFLP patterns

	rDNA Type I (KMU 2500)	rDNA Type II (KMU 3653)	rDNA Type III (KMU 3492)	rDNA Type IV (KMU 2052)
Hae II	H1*	H1	H1	H2
	301	302	301	302
				206
	113	113	113	112
	108+101**	108+101	108+102	
Apa I	A1	A2	A2	A3
				298
	277	277	277	
	168	168	169	167
	157			155
		125	124	
		(33)	(33)	
	(21)***	(21)	(21)	
Nla III	N1	N1	N2	N3
	356	357	356	356
				185
	134	134	134+134	
	80	80		79
	53	53		

*: H1 - N3 indicate names of RFLP patterns obtained by digestion with restriction enzyme *Hae* III, *Apa* I or *Nla* III.

**: Two bands of similar sizes form a band.

***: Bands in parentheses ran off the gel in this study

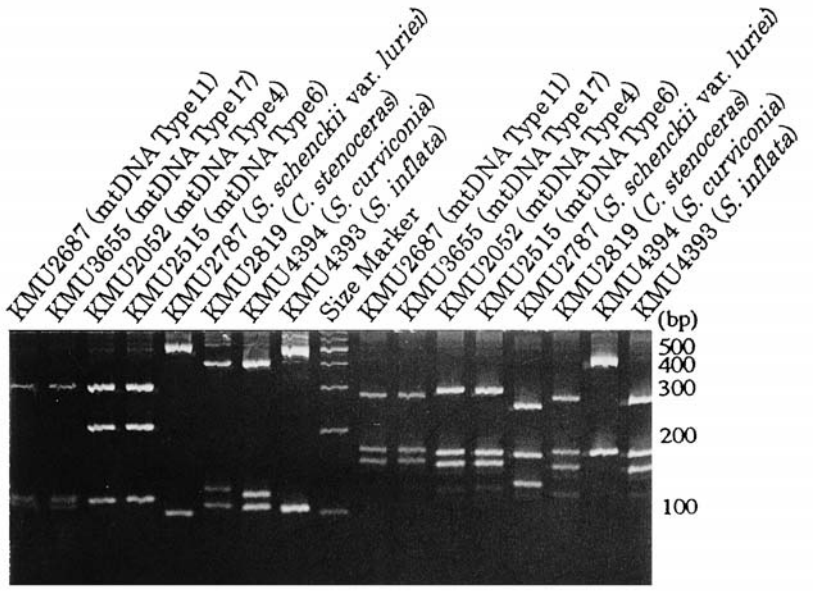

Fig. 4. ITS-RFLP patterns of 4 related species. The left half is RFLP patterns with *Hae* II and the right half is RFLP patterns with *Apa* I .

Table 3. The number of different bases between each mtDNA type

KMU no.	1	1	2a	2a	2b	3a	3a	3b	3c	3d	4	4	4	5	7	11	11	14a	14b	14c	15	16	17	17	18	19	22
975- I (1)																											
2285- I (1)	0																										
2286- I (2a)	4	4																									
3114- I (2a)	4	4	0																								
4014- I (2b)	4	4	2	2																							
2500- I (3a)	3	3	1	1	1																						
3944- I (3a)	3	3	1	1	1	0																					
3620- I (3b)	3	3	1	1	1	0	0																				
4040- I (3c)	3	3	1	1	1	0	0	0																			
4012- I (3d)	5	5	3	3	1	2	2	2	2																		
2052-IV (4)	19	19	18	18	19	18	18	18	18	20																	
3509-IV (4)	18	18	19	19	20	19	19	19	19	21	1																
3993-IV (4)	19	19	18	18	19	18	18	18	18	20	0	1															
3311-IV (5)	19	19	18	18	19	18	18	18	18	20	0	1	0														
3360-IV (7)	19	19	18	18	19	18	18	18	18	20	0	1	0	0													
2687- I (11)	3	3	3	3	3	2	2	2	2	4	18	19	18	18	18												
4011- I (11)	3	3	3	3	3	2	2	2	2	4	18	19	18	18	18	0											
3589- I (14a)	5	5	1	1	3	2	2	2	2	4	19	20	19	19	19	4	4										
3501- I (14b)	5	5	1	1	3	2	2	2	2	4	19	20	19	19	19	4	4	2									
3486- I (14c)	5	5	1	1	3	2	2	2	2	4	20	21	20	20	20	4	4	2	0								
3504- I (15)	8	8	4	4	6	5	5	5	5	7	19	20	19	19	19	7	7	3	1	1							
3653- II (16)	4	4	2	2	2	1	1	1	1	3	19	20	19	19	19	3	3	3	3	3	6						
3655- I (17)	3	3	1	1	3	2	2	2	2	4	20	19	20	20	20	4	4	2	2	2	5	4					
3940- I (17)	3	3	1	1	3	2	2	2	2	4	20	19	20	20	20	4	4	2	2	2	5	3	0				
3617- I (18)	6	6	4	4	4	3	3	3	3	5	20	21	20	20	20	3	3	5	5	5	8	4	5	5			
3628- I (19)	3	3	1	1	1	0	0	0	0	2	18	19	18	18	18	2	2	2	2	2	5	1	2	2	3		
3492-III (22)	7	7	5	5	5	4	4	4	4	6	21	22	21	21	21	6	6	6	5	5	8	4	6	6	7	4	
3998- I (23)	3	3	3	3	3	2	2	2	2	4	18	19	18	18	18	0	0	4	4	4	7	3	4	4	3	2	6

Roman numerals bound to KMU numbers indicate rDNA types. Arabic numerals in parentheses indicate mtDNA types and the alphabetical letters indicate mtDNA subtypes.

and 101 bp; and KMU 2052: 302, 206 and 112 bp). With *Nla* III, the 204 strains gave 3 RFLP patterns: N-1 with 360, 130, 80 and 50 bp bands, N-2 with 360 and 130 bp bands and N-3 with 360, 190 and 80 bp bands (Fig. 2). With *Apa* I, the strains gave 3 RFLP patterns: A-1 with 280, 170 and 160 bp bands, A-2 with 280, 170 and 130 bp bands, and A-3 with 300, 170 and 160 bp bands (Fig. 3). Table 2 shows the precise band sizes obtained from the base sequences.

Taking together the RFLP patterns from all

three restriction enzymes, the 204 strains comprised 4 types: rDNA type I (H-1, N-1, A-1), rDNA type II (H-1, N-1, A-2), rDNA type III (H-1, N-2, A-2) and rDNA type IV (H-2, N-3, A-3). The rDNA type I strains corresponded to mtDNA types 1 - 3, type 11, type 14, type 15, types 17 - 19 and type 23, the rDNA type II strains corresponded to mtDNA Type 16, and the rDNA type III strains corresponded to mtDNA Type 22, all of which belonged to Group A. On the other hand, the rDNA type IV strains all belonged to Group B, according to mtDNA analysis (Table 1).

With all three restriction enzymes, *S. schenckii*, *S. schenckii* var. *luriei*, *S. curviconia*, *S. inflata* and *C. stenoceras* each gave a different ITS-RFLP pattern (Fig. 4), making it possible to discriminate *S. schenckii* from the others.

Genetic Sequence

Sequences of about 620 bp comprising the end part of the SSUrRNA gene, ITS1, 5.8S rRNA gene, ITS2, and the beginning of the LSUrRNA gene, together with the information about the strains, were registered in GenBank. The accession numbers are listed in Table 1. The numbers of different (substituted, inserted or deleted) bases between each mtDNA type are listed in Table 3. The difference among rDNA type I strains was 0 - 8 bases or 1.3% maximum. The difference between the rDNA type I and the rDNA type II strains was 1 - 6 bases or 1.0% maximum, and the difference between the rDNA type I and the rDNA type III strains was 4 - 8 bases or 1.3% maximum. The sequences of the 620 bases of the 4 rDNA type IV strains were completely identical, except for a single base insertion in 1 strain. The number of different bases between rDNA type IV and rDNA types I, II and III was 18 - 21, and so the maximum difference between the *S. schenckii* strains was 3.2%. The maximum difference between *C. stenoceras* and *S. schenckii* was 10%.

Phylogenetic tree

In the phylogenetic tree based on the base sequences, the 23 strains belonging to rDNA type I, rDNA type II, and rDNA type III formed a clade on a large branch. Of these mtDNA type 11 was on the same branch as mtDNA type 23, and mtDNA type 3 was on the same branch as mtDNA type 19 (Fig. 5). The 5 rDNA type IV strains occupied another

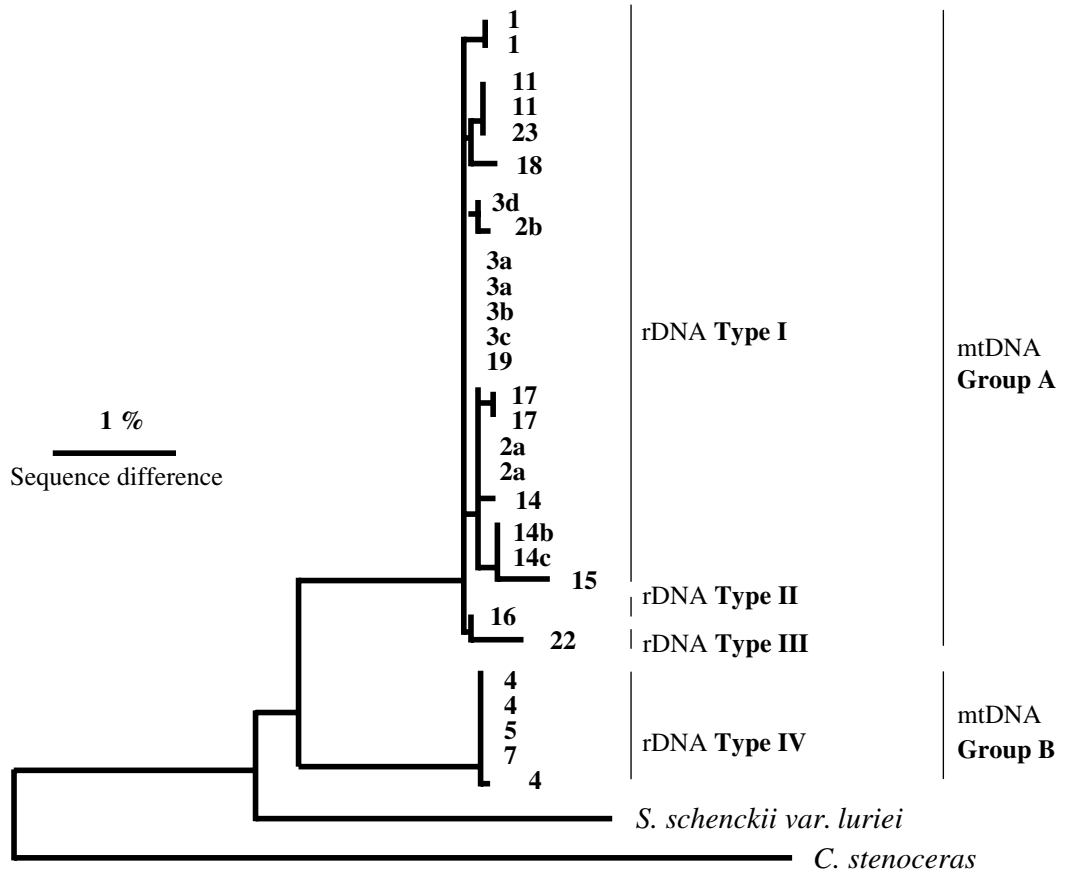

Fig. 5. A phylogenetic tree of *S. schenckii* types and subtypes, *S. schenckii* var. *luriei* and *C. stenoceras*. Arabic numerals indicate mtDNA types of *S. schenckii*. The alphabetical letters indicate mtDNA subtypes. Roman numerals indicate rDNA types.

large branch. From the tree, the rDNA types I - III strains can be considered as Group I, while the rDNA type IV strains can be considered as Group II, corresponding to mtDNA groups A and B, respectively. *S. schenckii* var. *luriei* and *S. schenckii*, were on different branches, somewhat distant from the branch occupied by *C. stenoceras*.

Discussion

In the present study, *S. schenckii* was categorized into 4 types according to ITS-RFLP patterns. These 4 types fell into groups I and II on a phylogenetic tree based on the base sequence of the ITS region, and corresponded to their mtDNA groups A and B. The typing of *S. schenckii* according to the ITS-RFLP analysis completely supported the typing according to the mtDNA-RFLP analysis.

Among the Group B strains, there was a difference of only one base in the sequence of their ITS region, while among the Group A strains as many as 8 bases were different. Therefore, the Group A strains are more diverse than the Group B strains. This supports the grouping according to mtDNA-RFLP analysis. Furthermore, from this analysis, the difference in the base sequences in mtDNA between the two groups is estimated to be 10% maximum[10], while the maximum difference in their ITS region is 3.5%. The smaller difference in the ITS region agrees with the finding that there are fewer rDNA types than there are mtDNA types. Moreover, the difference between the ITS regions of *S. schenckii* and *C. stenoceras* is 10%, which supports the claim[4] that they are different species even though they are morphologically, chemically, and immunologically related.

Both mtDNA-RFLP analysis and ITS-RFLP analysis showed that the ITS region varies by 9 % or more among strains of *F. pedrosoi*[17] in contrast with the case of *S. schenckii*[10]. Therefore, the size of the inter-specific variation in the ITS region greatly depends on the fungi. In practice, either mtDNA or rDNA should be analyzed according to the information required. The relation between the extent of the inter-specific variation of the ITS region and the phylogeneses or evolutions of the Eumycetes will be investigated in future.

On both the phylogenetic tree based on the base sequence of rDNA and on the tree based on mtDNA RFLP, *S. schenckii* was divided into 2 groups, and among the strains in Group I and Group A on the respective trees, mtDNA type 22 was the most distantly related. However, there were small differences between the rDNA and the mtDNA phylogenetic trees: on the mtDNA phylogenetic tree the subtypes each formed one clade, whereas on the rDNA phylogenetic tree, mtDNA subtypes 2a and 2b were on separate branches from each other, and the subtypes 3a, 3b and 3c were on the same branch while subtype 3d was on a different branch. From the rDNA base sequence, each mtDNA subtype should perhaps be considered as an independent type. On the mtDNA phylogenetic tree, mtDNA type 15, which belongs to rDNA type I, is not particularly distant. However, according to the rDNA base sequence mtDNA type 15 has more inserted and exchanged bases than does mtDNA type 22. The disagreement between the two trees may be caused by the fact that RFLP analysis does not necessarily detect all the differences in the sequences.

The 204 isolates of *S. schenckii* collected from many regions of the world were divided into 4 types according to ITS-RFLP analysis. Geographically, rDNA type I strains are predominant in Africa and South and North America, rDNA type II strains in South America, rDNA type III strains in North America and rDNA type IV strains in Australia and Asia. However, for a deeper understanding, we need to study more isolates, especially from regions not yet investigated.

To diagnose sporotrichosis conventionally, two weeks are required to culture *S. schenckii* obtained from specimens, and another week for morphological identification. In contrast, with ITS-RFLP analysis, only about 10 mg of cultured isolate is needed, and the identification and typing take only about 8 hours. Hence, this method is less labor-intensive, faster and clinically more useful than other molecular biological techniques including mtDNA-RFLP analysis.

The identifications of all clinical isolates of *S. schenckii* have been confirmed by mtDNA-RFLP without the problems faced when making identifications morphologically[6]. However, as they had all been "filtered" by infected humans, only pathogenic *S. schenckii* were isolated. For environmental isolates, however, *S. schenckii* cannot be discriminated morphologically, and it has become apparent from molecular biological techniques that many fungi have been called *S. schenckii*[18]. By ITS-RFLP analysis, the environmental isolates can be screened on a large scale, making this analysis useful in precise epidemiological surveys of *S. schenckii*.

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