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博士学位論文

Species identification and strain typing of dermatophytes by single-strand conformation polymorphism (SSCP) analysis of the ribosomal DNA and polymerase chain reaction analysis of subrepeat elements in the intergenic spacer region of *Trichophyton rubrum*

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【要　　旨】

The PCR-single strand conformation polymorphism (PCR-SSCP) analysis is a rapid and convenient technique for the detection of mutations and allelic variants. We applied this technique for the identification of various dermatophyte species. We also examined genetic polymorphisms in intra-species of 2 major dermatophytes, *T. rubrum and T. mentgrophytes var. interdigitale*. The PCR products including internal transcribed spacer (ITS) 1 region of ribosomal DNA (rDNA) of *Trichophyton rubrum, T. mentagrophytes var. interdigitale, T. verrucosum, Microsporum canis, M. gypseum, Epidermophyton floccosum* were analyzed. Specific SSCP patterns for each species were obtained. This result indicated that the system is useful for species identification. SSCP analysis of the amplified products from the posterior part of the D1/D2 (5′ end domain of the 26S rDNA) regions manifested the presence of 2 distinctive types of *T. mentagrophytes* var. *interdigitale*. The difference proved to be one nucleotide substitution, C → T. There was no clear correlation between the genotype patterns and morphological phenotypes of this fungus. On the other hand, no intra-species polymorphism was detected in *T. rubrum* by PCR-SSCP analysis using the region including ITS1, 5.8S rDNA, ITS2 and D1/D2, totally spanning about 1.2 kbp. Subsequent PCR analysis of non-transcribed intergenic spacer (IGS) region of *T. rubrum* showed the occurrence of numerous intra-species polymorphisms. The system should be useful for epidemiological surveillance of dermatophyte infection and give us new insights into pathogenicity of dermatophyte species.

Key words　Dermatophytes, Identification, SSCP, PCR, Polymorphism

INTRODUCTION

Dermatophytes are a group of closely related keratinophilic fungi capable of invading keratinized tissue (skin, hair and nails), causing dermatophytoses (commonly known as tinea or ringworm) in humans and animals. Taxonomically, dermatophytes consist of three genera: *Epidermophyton, Microsporum* and *Trichophyton*. Except for *Epidermophyton* with one recognized species (*E. floccosum*), *Microsporum* and *Trichophyton* have various species, many of which are pathogenic for humans[1]. Although more than 40 dermatophyte species have been

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accepted as pathogenic [2] , less than 10 dermatophyte species are frequently isolated from humans. Accurate identification of these species is important clinically and epidemiologically, since the prognosis and therapy may vary depending on each species.

The conventional laboratory identification methods for these dermatophytes are based on the examination of colony appearance and microscopic morphology. These methods are occasionally time-consuming, as *in vitro* culture may require 2-4 weeks. Furthermore, due to atypical phenotypic isolates with unusual conidia formation or pigment production, the identification of each dermatophyte species is occasionally difficult, even by the use of nutritional requirement and mating tests. For example, *T. rubrum* without red pigment production can be easily confused with *T. mentagrophytes* var. *interdigitale.*

On the other hand, identification methods based on the genotype analysis are considered to be more accurate and reproducible. Recently various molecular analyses based on PCR techniques, such as random amplified polymorphic DNA (RAPD) analysis[3] [4] , PCR fingerprinting method [5] , arbitrarily primed PCR technique[6] [7] and direct sequencing of the internal transcribed spacer (ITS) region [8] [9] have been developed for the identification of dermatophyte species.

In eukaryotes, the genes for 18S (16S-like), 5.8S, and 26S (23S-like) rDNA are usually arranged as tandem repeats separated by a non-transcribed intergenic spacer (IGS). Two internal transcribed spacer (ITS) regions (ITS1 and ITS2) are located between the fungal genomic 18S and the 26S rDNA. The D1/D2 region is another divergent domain located within the 26S rDNA. As reported previously [8-14] , these regions have been shown to be useful for phylogenetic analysis and species identification of various fungi. The IGS contains variable numbers of repetitive sequence motifs which are responsible for rDNA repeat length polymorphisms [15] [16] .

PCR-SSCP analysis is an electrophoretic technique that has been developed for the detection of mutations, mainly in human genetics[17] [18] . Under non-denaturing conditions, single-stranded DNAs will fold into secondary structures (conformations) according to their nucleotide sequences and their physicochemical environment such as running temperature and pH of the gels. Because of distinctive electrophoretic mobility, different conformations can be separated by non-denaturing polyacrylamide gel electrophoresis. Recently, PCR-SSCP analysis has been applied for various clinically isolated pathogenic fungi[19-22] .

In this study, we amplified the ITS1 region of rDNA, and performed SSCP analysis to identify various clinically available dermatophyte species, such as *T. rubrum, T. mentagrophytes* var. *interdigitale, T. verrucosum, M. canis, M. gypseum, E. floccosum*. We further examined intra-species polymorphisms of 2 major pathogenic dermatophytes for human, *T. rubrum* and *T. mentgrophytes* var. *interdigitale* by using the PCR-SSCP technique. As no intra-species polymorphism was detected by SSCP analysis of *T. rubrum*, we amplified subrepeat elements in the IGS region for strain typing. Our results indicate that combination of these molecular techniques provide us useful information for species identification and intra-species polymorphisms of pathogenic dermatophyte fungi.

MATERIALS AND METHODS

Fungi used. Six dermatophyte species, *T. rubrum* (IFM 51254), *T. mentagrophytes* var. *interdigitale* (IFM 51256), *T. verrucosum* (IFM 51259), *M. canis* (IFM 51260), *M. gypseum* (IFM 47489) and *E. floccosum* (IFM 51261) were obtained from the Research Center for Pathogenic Fungi & Microbial Toxicoses, Chiba University (IFM culture collection). In addition, 87 strains of dermatophyte isolated from patients, *T. rubrum* (n=55) and *T. mentagrophytes* var. *interdigitale* (n=32) were examined. The strains of clinical isolates were identified by conventional methods, on the basis of their colony appearance and microscopic characteristics.

DNA isolation. A modification of the procedure described previously was used in extracting DNA from fungi [23] [24] . Briefly, all organisms were cultured on PDA (potato dextrose agar: Difco, USA) at 27 ℃ for 21 days. A small mount of fungi grown on PDA was suspended in 300μl of extraction buffer consisting of 200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA and 0.5% sodium

dodecyl sulfate. Then it was crushed with conical grinder and incubated at 100℃ for 15 min and then placed on ice for 10 min. The supernatants collected after centrifugation at 13,000 rpm for 5 min was extracted once with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and subsequently extracted once with equal volume of chloroform/isoamyl alcohol (24:1). The extract combined was mixed with 50 μl of 3.0 M sodium acetate and precipitated with 500 μl of isopropanol at room temperature for 15 min. And then the DNA was washed with 500 μl of cold 70% ethanol, dried, and suspended in 100 μl of sterile distilled water. Extracted DNA was diluted 1:100 in distilled water in preparation for use as a template in PCR.

PCR amplification for SSCP analysis. The following primers were used for the amplification of ITSs regions and D1/D2 domain. ITS5: 5′-GGAAGTAAAAGTCGTAACAAGG-3′, ITS2: 5 v -GCTGCGTTCTTCATCGATGC-3′, ITS3: 5′-GCATCGATGAAGAACGCAGC-3′, and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ [14] for ITSs, and NL1: 5′-GCATATCAATAAGCGGAGGAAAAG-3′, NL2A: 5′-CTTGTTCGCTATCGGTCTC-3′, NL-3A: 5′-GAGACCGATAGCGAACAAG-3′ and NL4G: 5′-GGTCCGTGTTTCAAGACG-3′ [12) 25] for D1/D2, respectively. PCR was performed in a 0.5 ml microcentrifuge tube, with a total volume of 50 μl of the reaction mixture containing 25.75 μl of distilled water, 5.0 μl of each two primers (2.0 pmol/μl), 0.25 μl of Ex Taq polymerase (1.25 U) (Takara, Shiga, Japan), 4.0 μl of dNTP mixture (2.5 mM for each) (Takara), 5.0 μl of 10 × Ex Taq buffer (Takara) and 5.0 μl of genomic DNA samples. The amplification reaction were performed by a TM 3000 PCR thermal cycler (Takara) with the following cycling parameters; ITS regions: 1 cycle of 94 ℃ for 4 min, 35 cycles of 94℃ for 2 min, 55℃ for 2 min, 72℃ for 2 min, and 1 cycle of 72℃ for 10 min, D1/D2 regions: 1 cycle of 95 ℃ for 5 min, 36 cycles of 94 ℃ for 1 min, 52 ℃ for 2 min, 72℃ for 2 min, and 1 cycle of 72℃ for 5 min.

SSCP analysis. The gel was consisted of 2.5 ml MED gel solution (BioWhittaker Molecular Applications), 600 μl of 10 × Tris-borate-EDTA (TBE), and 6.9 ml of distilled water. The gel solution was degassed under vacuum pump until no bubbles were visible. After the acrylamide solution was degassed, 40 μl of ammonium persulfate and 6 μl of N,N,N,N-tetramethylenediamide (TEMED) were added and mixed to induce gel polymerization prior to the gels being poured. The minislab gel system (gel size, 90 × 80 × 0.75 mm: ATTO Co., Tokyo, Japan) was used for electrophoresis. Gels were solidified for 3 h at room temperature. The solidified gels were set into the buffer chamber containing 0.6 × TBE buffer. Two μl of PCR products were mixed with 18 μl of loading dye (95% formamide, 0.5% bromophenol blue). Samples were heated at 95℃ for 2 min and then cooled on ice till loaded onto gels. All electrophoresis were performed under the appropriate constant temperatures described at Results and Figures. Gels were pre-run at 250 V for 30 min. Then 6 μl of samples were loaded onto the gel and then electrophoresed at 75 V for 30 min to samples set into the gels, then electrophoresed at 250 V. Running times are depending on each running temperatures, which were also indicated at Result and Figures. On terminating electrophoresis, gels were stained with ethidium bromide and then photographed.

Sequence analysis. The amplified PCR products were purified by using SUPREC-02 DNA purification cartridges (Takara) according to the manufacturer's instructions. Sequencing reactions were performed with the DNA Sequencing Kit Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems Inc.) according to the manufacturer's instructions. Electrophoresis and automated analysis of sequence were performed with a model ABI prism 377 DNA sequencer. Alignment was performed using CLUSTAL W multiple sequence alignment programs.

PCR analysis of the IGS region of rDNA for T. rubrum. Two primers were designed from the sequence of T. rubrum in the GenBank/EMBL/DDBJ databases with the accession number AF222887. The primers for IGS region were IGS3F: 5′-TATCATCGCACCGACATCATAC-3′and IGS62R: 5′-TATGGGAGAGCTAGAGCCAGAT-3′. PCR was performed in a 0.2 ml microcentrifuge tube, with a total volume of 25 μl of the reaction mixture containing 0.25 μl of distilled water, 2.5 μl of each two primers (2.0 pmol/μl), 0.25 μl of LA Taq polymerase (1.25 U) (Takara, Shiga, Japan), 4.0 μl of dNTP mixture (2.5 mM for each) (Takara),

12.5 μl of GCI buffer (Takara) and 3.0 μl of genomic DNA samples. The amplification reaction was performed with the following cycling parameters: 1 cycle of 95 ℃ for 5 min, 28 cycles of 95℃ for 1.5 min, 62℃ for 1 min, 72℃ for 1.5 min, and 1cycle of 72℃ for 10 min.

RESULTS

Species identification of dermatophytes. The universal primer pair, ITS5 and ITS2, drawn in Fig. 1 produced the amplified products with approximately 360 bp containing the ITS1 region for all dermatophyte species tested. The products were used for SSCP analysis. The PCR-SSCP patterns of six species of medically important dermatophytes at 35℃ for 60 min are shown in Fig. 2. *M. canis, M. gypseum, T. verrucosum, E. floccosum* and *T. rubrum* showed characteristic two distinctive bands with different electrophoretic mobility, while *T. mentagrophytes* var. *interdigitale* showed one band. A phenotypically atypical isolate of *T. rubrum*, IFM 51255, which produced brownish pigment and granular colony surface gave identical pattern as that of the typical strain of this species. These data demonstrated that SSCP analysis could be used as a sensitive and easily-performed method for the species identification of these dermatophytes.

Intra-species polymorphism analysis for dermatophytes. For this study, 32 strains of *T. mentagrophytes* var. *interdigitale* were analyzed by using PCR-SSCP technique. The following four PCR fragments were amplified from different regions on rDNA. They are the fragments, Tm-I, Tm-II, Tm-III, and Tm-IV, which were amplified with the primer pairs of ITS5 and ITS2, ITS3 and ITS4, NL1 and NL2A, NL3A and NL4G, and included the regions of ITS1, ITS2, D1 and D2, respectively (Fig. 1). Fig. 3 shows the SSCP patterns for the four amplified fragments: A for Tm-I, B for Tm-II, C for Tm-III and D for Tm-IV. As can be seen in Figure 3 (A), (B) and (C), all of 32 strains gave identical SSCP pattern for the respective amplified fragment. This evidence revealed that the base sequence of the three regions are well conserved and then no intra-species polymorphism was found in *T. mentagrophytes* var. *interdigitale*. On the other hand, SSCP analysis of Tm-IV gave two different band patterns and separated this fungal species into two sub- groups, (a) and (b), as shown in Fig. 3 (D). To investigate the reason of this different pattern, base sequence of the amplified fragments, Tm-IV, obtained from *T. mentagrophytes* var. *interdigitale* IFM 51257 and IFM 51258 which were selected as a representative strain form groups of the pattern (a) and (b) each, were compared as shown in Fig. 4. The difference between the two patterns was revealed to be one nucleotide substitution (C → T) at the position of 144.

Similarly, SSCP analysis of the four fragments, Tr-I, Tr-II, Tr-III and Tr-IV, amplified with the same primer pairs as described above was carried out against 55 strains of

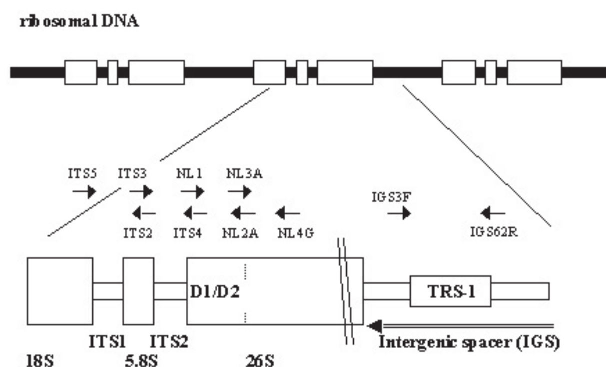

Fig 1. Scheme of the rDNA regions and the location of the primers used. The primer pairs of ITS2 and ITS3, ITS4 and NL1, and NL2A and NL3A are complementary sequences, respectively. The scheme of IGS region, which contains TRS-1 with repeat sequences is shown according to the published of *T. rubrum*[26] .

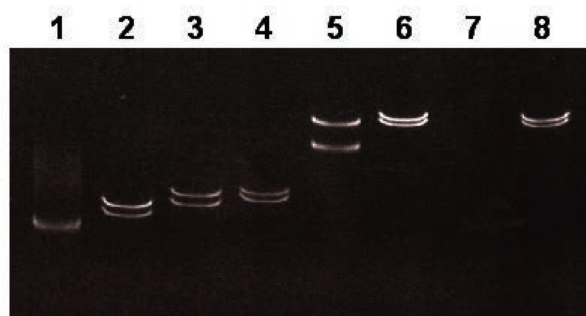

Fig 2. SSCP patterns of the fragments containing ITS1 region amplified with primers ITS5 and ITS2 from 6 species of dermatophytes. Electrophoresis was performed at 35 ℃ for 60 min. Lanes: 1, *T. mentagrophytes* var. *interdigitale*; 2, *M. canis;* 3, *M. gypseum*; 4, *T. verrucosum*; 5, *E. floccosum*; 6, *T. rubrum*. Lane 7 is blank and lane 8 is phenotypically atypical isolate of *T. rubrum* (IFM 51255).

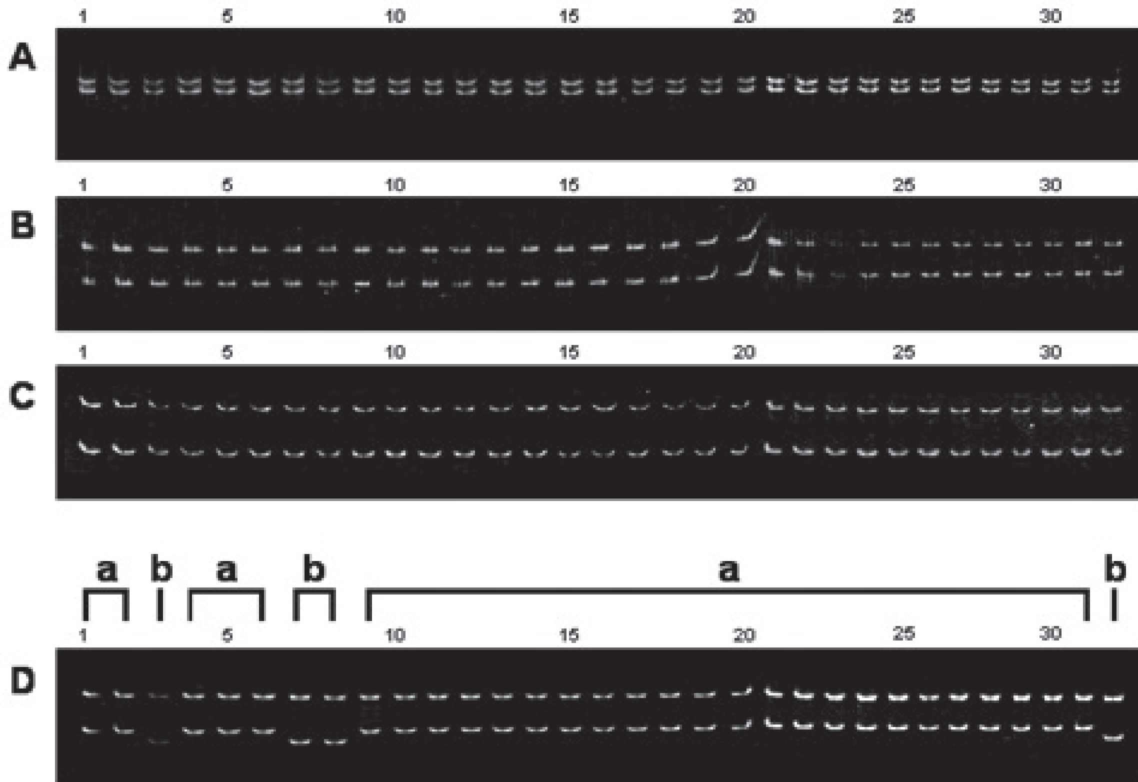

Fig. 3. SSCP patterns of the four amplified fragments on rDNA obtained from 32 strains of *T. mentagrophytes* var. *interdigitale*. A: Tm-I, electrophoresis condition at 35℃ for 60 min ; B: Tm-II, at 30℃ for 70 min ; C: Tm-III, at 10℃ for 180 min ; D: Tm-IV, at 35℃ for 40 min.

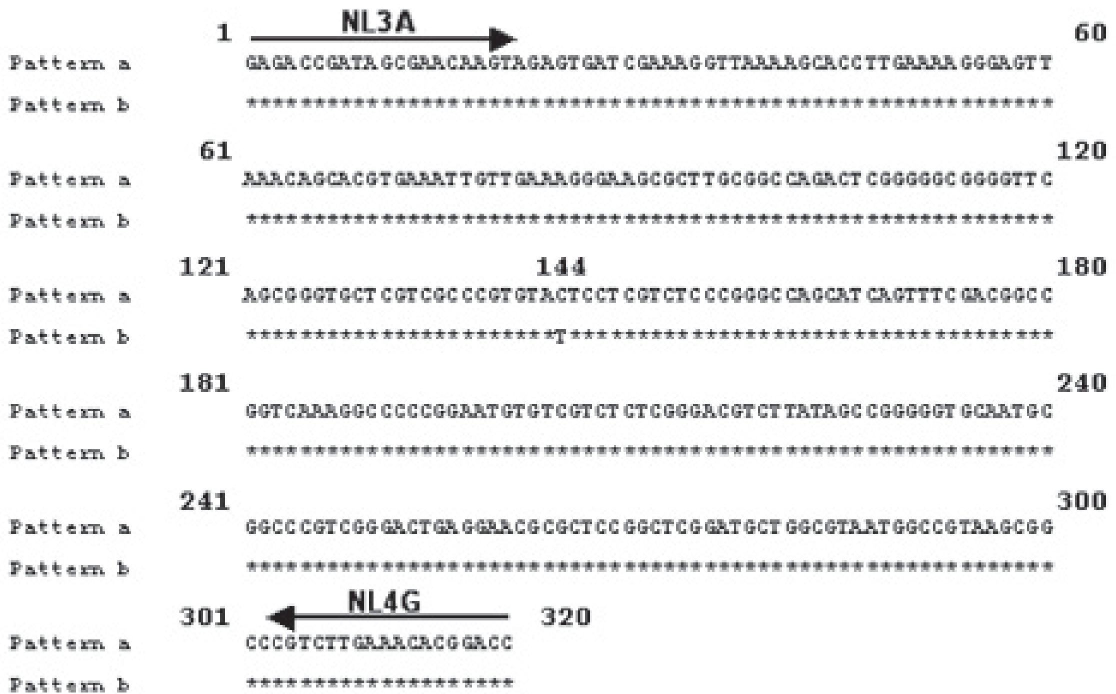

Fig. 4. Comparison of the base sequence of the fragments Tm-IV from *T. mentagrophytes* var. *interdigitale* IFM 51257 and IFM 51258 which were selected as a representative strain of pattern (a) and (b) each. Asterisks indicate the same sequences between (a) and (b). Arrows indicate the location of primers, NL3A and NL4G.

T. rubrum. Fig. 5 shows SSCP patterns for Tr-I (A), Tr-II (B), Tr-III (C) and Tr-IV (D). The SSCP analysis afforded the completely identical pattern in every four fragments for all of the tested strains. This result suggested that no occurrence of intra-species polymorphism in ITS and D1/D2 regions of this fungal species (totally spanning about 1.2 kbp).

PCR analysis of the IGS region for T. rubrum. As no intra-species polymorphism was found in ITS and D1/D2 regions for *T. rubrum* by SSCP analysis, additional PCR analysis was performed in the IGS region for *T. rubrum*. Primers IGS3F and IGS62R were used to amplify the IGS region (Fig. 1). The amplified PCR products contained the recently-described [26] subrepeat elements (TRS-1) of IGS region. The analysis of 15 strains of *T. rubrum* is shown in Fig. 6. The strains used in this analysis were the same examined for the SSCP analysis (Fig. 5: lanes 1 to 15). Any visible PCR products were not obtained from the strain of lane 13. *T. rubrum* strains isolated from 15 different samples represented distinctive 12 types; the two strains in lanes 2 and 3, 6 and 9, 7 and 11 showed every identical PCR types, while all strains in other lanes showed distinctively different bands in the size of base pairs amplified. As expected, two *T. rubrum* strains isolated from the same patient showed the identical PCR type (lane2: tinea pedis and lane 3: tinea unguium, respectively).

DISCUSSION

Trichophyton rubrum and *T. mentagrophytes* var. *interdigitale* are the two major pathogenic dermatophyte species for human. About 80% of dermatophyte infection is caused by the two species. Accurate identification for the dermatophyte species is clinically important, because in recent years there has been progress toward the development of treatment strategies that target for each dermatophyte species. Many antimycotic agents have different spectra of activity. For example, the new azole compounds have been shown to exhibit different minimal inhibitory concentrations to dermatophyte species, such as *T. rubrum* and *T. mentagrophytes* [27].

Recently various molecular analyses based on PCR techniques have been developed for the identification of

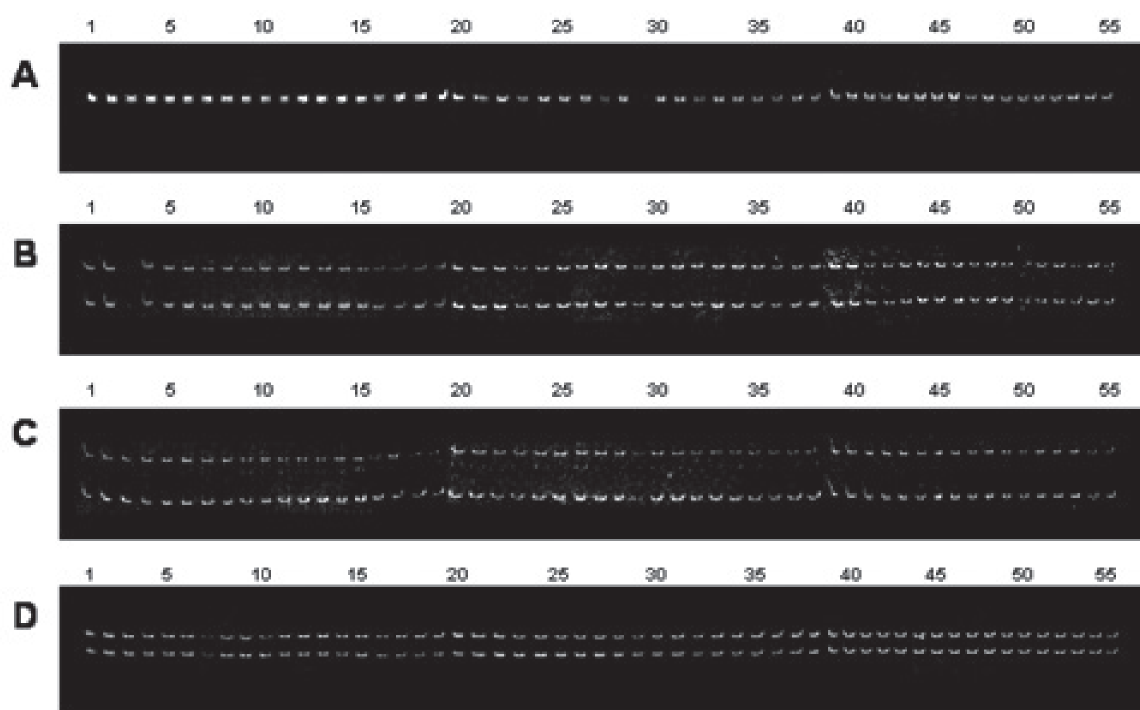

Fig. 5. SSCP patterns of the four amplified fragments on rDNA obtained from 55 strains of *T. rubrum*. A: Tr-I, electrophoresis condition at 35℃ for 60 min ; B: Tr-II, electrophoresis condition at 30℃ for 70 min ; C: Tr-III, at 10℃ for 180 min ; D: Tr-IV, at 40℃ for 50 min.

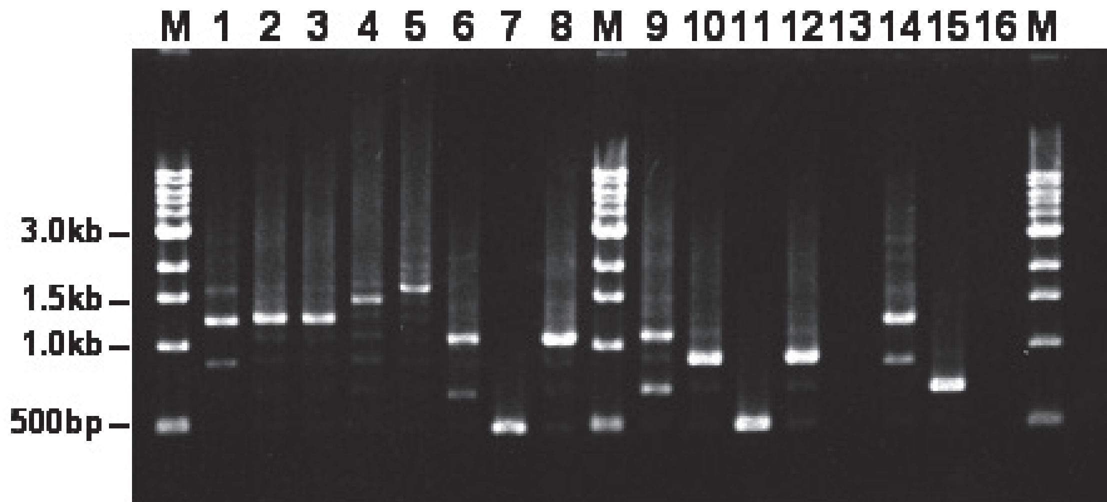

Fig. 6. Amplification of TRS-1 subrepeat element of IGS region. Lane 16: negative control ; M: molecular weight marker.

dermatophytes, such as random amplified polymorphic DNA (RAPD) analysis [3) 4)], PCR fingerprinting method [5)], arbitrarily primed PCR technique [6) 7)] and direct sequencing of the ITS region [8) 9)]. Compared to these techniques, PCR-SSCP analysis is more sensitive, easily-performed and highly reproducible. In this study, by using PCR-SSCP analysis containing the ITS1 region, we could adequately distinguish major dermatophytes, such as *T. rubrum*, *T. mentagrophytes* var. *interdigitale, T. verrucosum, M. canis, M. gypseum* and *E. floccosum*. No intra-species polymorphisms in the ITS regions in dermatophytes against *T. rubrum, T. mentagrophytes* var. *interdigitale,* and *M. canis* (data not shown) made the PCR-SSCP analysis successful for species identification.

Recently, PCR-SSCP analysis has been applied for species identification of various pathogenic fungi other than dermatophytes[19-22)]. The SSCP patterns of PCR fragments depend on the experimental conditions, particularly on the composition of the electrophoretic gels and running temperature. Glycerol added to change pH of the gels diminished the SSCP signals. Running temperature affected both the time of electrophoresis and SSCP patterns. As we could not obtain reproducible SSCP patterns under the electrophoresis at room temperature, SSCP analysis was performed under the defined temperature system. Since the time required for electrophoresis decreased at higher running temperature, we selected the highest temperature, which could detect the specific band patterns. Conditions of electrophoresis including the running temperature should be optimized for the specific sequence of the PCR fragments derived from each fungal species. Contrary to the previous study [22)], lower temperature system was unsuccessful to detect the difference in a shorter time.

Strain typing based on morphological phenotypes of dermatophyte species is occasionally difficult because of a lack of reproducibility. The morphological differences in intra-species are often not stable on subculture or may simply be artifacts due to different growth conditions[28)]. Strain typing methods based on fungal genotype is expected to be more useful for dermatophyte species. Although recent molecular analyses are adequate for the species identification, they are not sensitive enough for strain typing for dermatophyte. PCR-SSCP method can detect single nucleotide polymorphisms among hundreds of nucleotide sequences. We examined intra-species polymorphisms for two major dermatophyte species, *Trichophyton rubrum* and *T. mentgrophytes* var. *interdigitale* by using this technique. SSCP analysis of the amplified products, Tm-IV, from the posterior part of the D1/D2 regions disclosed 2 distinctive patterns of *T. mentagrophytes* var. *interdigitale.* The difference between the two patterns found to be one nucleotide, C → T substitution. On the other hand, no intra-

species polymorphism was detected for *T. rubrum* by using the products from ITS region including 5.8S rDNA and D1/D2 regions, totally spanning about 1.2 kb nucleotides. Preliminary SSCP analysis for multiple strains of *M. canis* (n=13) disclosed no intra-species polymorphism in the ITS and D1/D2 regions. (data not shown). These results support the previous study [29], that ITS regions are conserved in intra-species for *T. rubrum*. Furthermore, the ITS regions of *T. mentagrophytes* var. *interdigitale* and *M. canis* are conservative in intra-species. The D1/D2 regions are also conservative in *T. rubrum* and *M.canis*. Although we detected intra-species polymorphisms for *T. mentagrophytes* var. *interdigitale* in the D2 region, the difference was only one nucleotide substitution. These results suggest that the ITS-D1/D2 regions are conservative in intra-species for these fungi.

As resistance to antifungal drugs continues to expand, elucidation of mechanisms of resistance becomes increasingly important screening for single nucleotide mutations of structural and regulatory genes by SSCP patterns may be a useful tool in understanding mechanisms of antifungal drug resistance. Such mutations have been identified by SSCP analysis in other pathogens. For example, single point mutations in RNA polymerase have been identified by this technique in rifampicin-resistant *Mycobacterium tuberculosis* [30] and mutations in 14-α-demethylase (target enzyme encoding gene of azoles) have been detected in fluconazole-resistant *Candida albicans* [31]. Recently, mutations of dihydropteroate synthase (DHPS) gene have been detected in sulfa-resistant *Pneumocystis carinii* [32].

Recent molecular analyses of strain typing for *T. rubrum* have been mostly unsuccessful[4) 28) 33)]. So far analyses of subrepeat elements in the nontranscribed spacer of *T. rubrum* are successful applications [26) 29) 34)]. As there was no intra-species polymorphism in the ITS and D1/D2 regions, we examined PCR analysis of IGS subrepeat elements for clinically isolates of *T. rubrum* for strain typing. In this study, we detected 12 distinctive PCR types for 15 clinical-isolated strains of T. rubrum. In the previous study [26], strain typing of T. rubrum isolated from European countries, such as United Kingdom, showed that 75% of all isolates belonged to one of four patterns and 40% of strains belonged to a single pattern. However, the Japanese isolates demonstrated various PCR types. Our results also represented that the clinical isolates of *T. rubrum* in Japan are more variable in genetic types of subrepeat elements in the IGS.

In the previously reported paper [26], the pattern of PCR analysis of subrepeat elements of IGS region showed ladder-like, multiple weak signal bands, besides one, two or multiple strong signal bands. The significance of these multiple, ladder-like bands pattern remains to be determined.

As discussed in the previous studies[26], we could not mention clear relationships between the genotypes of dermatophyte species (e.g. SSCP types of *T. mentagrophytes* var. *interdigitale* and PCR types of *T. rubrum*) and morphological phenotypes or clinical types (e.g., tinea pedis or tinea corporis or tinea unguium). In the future, easily-performed, reproducible strain typing methods based on molecular techniques, which reflect morphological phenotype or pathogenicity, should be applied for dermatophyte infection.

In conclusion, SSCP analysis of the PCR products including ITS1 region of fungal rDNA is useful for species identification for dermatophytes. Application of this analysis for T. mentagrophytes var. interdigitale, enabled us to detect the polymorphism due to one nucleotide substitution. In addition, we detected variable PCR types for the strains of T. rubrum by PCR analysis of IGS subrepeat element. Combining these techniques should make it possible to detect accurate species identification and strain typing of dermatophyte species.

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