Differentiation and phylogenetic relation of dermatophyte species by molecular analysis

(分子生物学的手法による皮膚糸状菌の菌種鑑別およびその系統関係)

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CONTENTS

	Pages
Genelal introduction ----------	2
Capter I ----------	6
Differentiation of *Arthroderma* species by random amplification of polymorphic DNA (RAPD) and Southern hybridization	
Capter II ----------	21
Identification of clinical Isolates of *Microsporum canis* and *M. gypseum* by random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses	
Capter III ----------	36
Differentiation of *Microsporum* species by random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses	
Capter IV ----------	50
Phylogenetic analysis of 8 dermatophyte species using chitin synthase 1 gene sequences	
Capter V ----------	61
Molecular analysis of chitin synthase 1 (*CHS1*) gene sequences of *Trichophyton mentagrophytes* complex and *T. rubrum*	
Conclusion ----------	76
Acknowledgment ----------	82
References ----------	83
Tables ----------	89
Legends for Figures ----------	95
Figures ----------	103

General Introduction

Dermatophytes, classified into 3 genera, *Epidermophyton*, *Microsporum* and *Trichophyton*, are causative agents of human and animal dermatophytoses. These dermatophytes include more than 40 species and nearly half of these species have been shown to produce sexual states which are classified in the genus *Arthroderma* [1, 24, 25, 27]. The dermatophytes have been identified by morphological and biochemical analyses as well as through mating experiments. However, it is frequently difficult to differentiate these species morphologically and biochemically. Some isolates of the dermatophytes, which are confirmed to have a sexual state, often lack any mating activity. Therefore, reliable and simple determinative methods are necessary in the field of dermatophyte study.

Moreover, phylogenetic study of dermatophytes is important in order to understand the evolution of dermatophytes and their definitive classification. The phylogenetic study on dermatophytes, however, has not been properly investigated by conventional characteristics. Therefore, the relationship between dermatophyte species are of

taxonomic interest in medical mycology, and conventional methods are required in order to clarify the phylogenetic relationship of each dermatophyte.

As a new approach, molecular analysis has recently been introduced into the field of medical mycology. Random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses have been utilized for the classification and identification of several fungal species [6, 17, 22, 28]. Analysis of chitin synthase 1 (*CHS1*) gene has also been carried out on *Candida albicans*, *Aspergillus nidulans*, *Histoplasma capsulatum* and *Sporothrix schenckii* [3-5]. Phylogenetic analyses of these fungi using the sequences of the*CHS1* gene indicated their genetic relationship confirming the previous results by the conventional classification [3]. However, the molecular characteristics and phylogenetic analysis of dermatophytes species have not been well investigated.

In the present study, I tried to differentiate dermatophytes species by RAPD and Southern hybridization analyses, in chapter I, II and III. After that, in chapter IV and V, the

phylogenetic relationship of dermatophytes was investigated by *CHS1* gene analysis.

Chapter I

Differentiation of *Arthroderma* species by random amplification of polymorphic DNA (RAPD) and Southern hybridization

Abstract

To develop the molecular differentiation analysis of dermatophytes, I carried out RAPD and Southern hybridization analyses using genomic DNAs of 6 *Arthroderma* species that were *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae* and *A. racemosum*. The RAPD analysis gave different band patterns specific to each of the 6 *Arthroderma* fungi. However, minor differences of the banding patterns were observed between the strains of (+) and (-) mating types of *A. gypseum*, *A. fulvum* and *A. incurvatum*. Southern blot analysis using a probe (1S) obtained from *A. grubyi* DNA gave specific bands only in the DNA samples of *A. grubyi* and *A. incurvatum*. On the other hand, Southern blot analysis using a probe (C3) obtained from A. otae DNA gave specific bands in all the 6 *Arthroderma* species examined and the size of the bands were specific to each species. These findings indicated that RAPD and Southern hybridization analyses were useful to differentiate these *Arthroderma* species.

Introduction

Molecular analysis, as a new approach has been applied for a variety of fungi including dermatophytes [11, 12, 18]. Molecular characteristics of some dermatophyte species were shown in aligned sequences of 600-bp fragments of 18S and 25S ribosomal RNA genes and restriction fragment length polymorphisms (RFLP) of the mitochondrial DNAs [9, 11,16].

On the other hand, Random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses have been utilized for classification and identification of several fungal species but not of dermatophytes [6, 17, 22, 28]. Therefore, RAPD and Southern hybridization analyses of dermatophytes were of interest in medical mycology.

In the present study, genomic DNAs of 6 *Arthroderma* species, which are teleomorph of *Microsporum* species, were investigated for molecular differentiation, since they are typical species in the genus *Microsporum* on the molophogical view point [10, 15, 21, 23] and are definitely classified by

teleomorph production.

Materials and methods

Strains

Twelve standard strains of *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae* and *A. racemosum* as shown in Table 1 were used for the experiment. Two strains of (+) and (-) mating types of each species were included. These strains are type strains described in the original papers [10, 15, 21, 23] and have been maintained by culturing on 1/10 Sabouraud dextrose agar in the Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Isolation of genomic DNA

Dermatophytes were grown in Sabouraud liquid medium at 27oC for 10 days. The mycelium samples were collected by centrifugation at 3,000 rpm for 5 min and then homogenized in liquid nitrogen. The samples were lysed with 1mg of zymolyase-100T (Takara , Kyoto, Japan) per ml in a lysis

buffer containing 0.1mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 10mM Tris hydrochloride (pH 8.0) and 0.3% 2-mercaptoethanol at 37°C for 16hrs. High molecular weight DNAs were obtained from these mycelium samples by phenol and chloroform extraction. These DNA samples dissolved in TE buffer (10mM Tris-HCl, pH8.0 and 1mM EDTA) were used for further analysis.

Random amplification of polymorphic DNA (RAPD) by polymerase chain reaction (PCR)

The genomic DNA samples (200ng) of dermatophytes were amplified by PCR in a reaction mixture (20ml) containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% Gelatin, 200 mM each deoxynucleoside triphosphate, 1.0 unit of Taq polymerase (Takara) and 0.5mg of the primer. The sequence of the random primer (FM1) was 5'-AGCCGCCTCCATGGCCCCAGG-3'. The PCR amplification was carried out for 40 cycles consisting of

template denaturation (1 min, at 94 °C), primer annealing (2 min, at 40 °C) and polymerization (1 min, at 72 °C). The PCR products were electrophoresed through 3% agarose gel and then stained with ethidium bromide.

Southern hybridization analysis

For Southern hybridization analyses, 1S probe was extracted from the approximately 130-bp band among the PCR-amplified bands from *A. grubyi* DNA, whereas C3 probe was extracted from the approximately 480-bp band among the PCR-amplified bands from *A. otae* DNA. These 1S and C3 probe DNAs were respectively gel-purified and cloned into pCRII vector (Invitrogen, San Diego, CA). These plasmid DNAs were purified with QIAGEN plasmid kit (QIAGEN, Studio City, CA) and sequenced by the dideoxy chain termination method using an Auto Read Sequencing kit (Pharmacia, Uppsala, Sweden).

Genomic DNA samples extracted from *Arthroderma* species were digested with a restriction endnuclease, *Eco*RI or

*Hind*III. The DNA digests were electrophoresed through 0.8% agarose gel and transferred to a hybridization membrane, GeneScreen Plus (NEN Research Products, Boston, MA). The DNA samples on the membrane were hybridized with a ^{32}P-labeled probe (1S or C3 probe) in a hybridization solution containing 1% SDS, 10% dextran sulfate, 1M NaCl, 100mg of salmon testis DNA per ml and 50 mM Tris-HCl (pH7.5) at 65oC for 16hrs. After hybridization, the filters were washed with a solution containing 2xSSC (1xSSC is 0.15M NaCl plus 0.015M sodium citrate) and 0.1% SDS at 55oC for 30 min and then autoradiographed.

Results

The RAPD patterns by PCR amplification were obtained as shown in Fig. 1. The DNA bands obtained by random amplification of the 6 *Arthroderma* species with the FM1 primer gave different band patterns. The RAPD patterns of *Arthroderma* species, *A. grubyi*, *A. gypseum*, *A. otae* and *A. iracemosum* were similar between the (+) and (-) mating types. The PCR products amplified from the (+) and (-) strains of *A. grubyi* gave 8 bands of 850, 500, 470, 450, 400, 330, 230 and 130bp. The RAPD patterns of *A. gypseum* were 5 bands of 750, 570, 550, 220 and 140bp in the (+) strain and 6 bands of 750, 570, 550, 300, 220 and 140bp in the (-) strain. The RAPD patterns of *A. incurvatum* were 5 bands of 800, 350, 310, 220 and 200bp, and the smallest band was slightly different in size between the (+) and (-) mating types. The RAPD pattern of *A. otae* was 6 bands of 480, 450, 350, 200, 129 and 75bp. The RAPD pattern of *A. racemosum* was 7 bands of 450, 400, 340, 300, 215, 130 and 120bp. The PCR amplification band

patterns were not identical between the (+) and (-) strains of *A. fulvum*: the (+) strain showed 6 bands of 1,000, 900, 550, 220, 140 and 75bp and the (-) strain showed 6 bands of 1,000, 550, 350, 220, 140 and 75bp (Fig.1).

The approximately 130-bp fragment (1S) among the bands amplified from *A. grubyi* and the approximately 480-bp fragment (C3) among the bands amplified from *A. otae* were respectively cloned into the plasmid vector and sequenced for the use of probes in Southern hybridization analysis. The 1S fragment consisted of 129 nucleotides containing an open reading frame that had a potential to code polypeptide of 94 amino acids (Fig. 4a). The C3 probe consisted of 476 nucleotides containing a single open reading frame encoding a putative polypeptide of 94 amino acids (Fig 4b).

These 1S and C3 fragments were used as probes for Southern hybridization analyses of the 6 *Arthroderma* species. Southern hybridization analysis using the 1S probe derived from *A. grubyi* gave distinct 9.0-kb EcoRI and 26-kb HindIII bands in *A. grubyi* and several faint bands in *A. incurvatum*, but not in

the other 4 *Arthroderma* species (Figs. 2a,b).

On the other hand, Southern hybridization analysis using the C3 probe gave distinct positive bands in all the samples of 6 *Arthroderma* species examined in this study and size of the bands were different among the 6 species : 1.8kb in *A. fulvum*, 16kb in *A. grubyi*, 2.2kb in *A. gypseum*, 4.2kb in *A. incurvatum*, 1.0kb in *A. otae* and 20kb in *A. racemosum* in the *Eco*RI digests; 16.5kb in *A. fulvum*, 17kb in *A. grubyi*, 18kb in *A. gypseum*, 10.5kb in *A. incurvatum*, 6.9kb in *A. otae* and 20.3kb in *A. racemosum* in the *Hin*dIII digests (Figs. 3a,b).

Discussion

It has been shown that RAPD analysis is very useful to differentiate several fungus species such as *Neurospora crassa*, *Aspergillus* sp and *Metarhizium* sp [2, 17, 28] and requires only a small amount of total genomic DNA samples. The RAPD analysis can useful for interspecies differentiation, because sequences differences among species are more evident than those among strains of the same species [2, 17, 28]. I preliminary examined genomic DNAs of some *Arthroderma* species by amplification using five 10-mer, one 21-mer and two 25-mer primers (data not shown). The results indicated that only the 21-mer primer (FM1) of arbitrary nucleotide sequence could amplify the different band patterns among the *Arthroderma* species examined. By the RAPD analysis with 21-mer primer, we could identify PCR-amplified patterns specific to each of the 6 *Arthroderma* species. The RAPD patterns of the (+) and (-) mating types were slightly different in 3 species of *A. gypseum*, *A. fulvum* and *A. incurvatum*.

These minor differences in these 3 species might be due to the genomic differences of the mating type or intraspecies variations. Megnegneau *et al.* detected intraspecies variations of *A. niger* with RAPD analysis and suggested that *A. niger* could be categorized into 4 distinct groups [22]. These minor differences of RAPD patterns in *A. gypseum*, *A. fulvum* and *A. incurvatum* should be investigated further.

Southern blot analysis was carried out with genomic DNAs of 6 Arthroderma species using 1S and C3 probes. Differences in the genomic fragment size of each species in Southern blot hybridization analyses were considered to be determined by restriction endnuclease patterns of the species. The differences of the restriction endnuclease patterns among species were more distinct than those of strains belonging to one species. It might be identical with the analyses of RFLP of mitochondrial and ribosomal DNAs [11, 22]. Therefore, Southern hybridization analysis can be considered as an useful method to differentiate species. Hybridization analysis using the 1S probe showed species-specific bands in *A. grubyi* and *A.*

incurvatum. This result suggested that the 1S probe could be used for identification of *A. grubyi* and *A. incurvatum* among 6 *Arthroderma* species. Although, the 1S fragment contained a coding sequence of 42 amino acids, it did not show any significant homology with known registered sequences in the database. Molecular cloning of a gene containing the sequence of 1S fragment might disclose the nature of some species-specific gene.

Southern hybridization analysis using the C3 probe revealed fragment sizes specific to each species of the *Arthroderma* fungi examined, indicating the presence of common sequence and different restriction endonuclease sites in the genomic DNAs of these *Arthroderma* species. Crowhurst *et al.* also detected the difference between *Mycosphaerella* sp. and *Fusarium* sp. with this technique [6]. The Southern hybridization analysis using the C3 probe was shown to be highly effective for differentiation of these *Arthroderma* species. The sequence of C3 fragment did not show any significant homology with those of known genes, however, the

sequence was commonly found in all of the 6 *Arthroderma* species, indicating that the fragment might be a part of some functional gene in *Arthroderma*.

The dermatophytes including these *Arthroderma* species have been generally identified by morphological and biochemical examination as well as mating experiments [1, 10, 15, 23-25, 27]. The RAPD and Southern hybridization analyses of *Arthroderma* species developed in this study will be useful molecular diagnostic approaches for differentiation of these *Arthroderma* species.

Chapter II

Identification of clinical isolates of *Microsporum canis* and *M. gypseum* by random amplification of polymorphic DNA (RAPD) and Southern hybridization Analyses

Abstract

Clinical isolates of *Microsporum canis* and *M. gypseum* from humans, dogs and cats were examined by random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses. The RAPD band patterns of 6 clinical isolates of *M. canis* were identical to those of standard strains of *Arthroderma otae*. Of 9 clinical isolates of *M. gypseum*, 7 isolates and 2 isolates showed RAPD patterns identical to those of standard strains of *A. gypseum* and *A. incurvatum*, respectively. Southern blot analysis using a probe (C3) obtained from *A. otae* DNA revealed that 6 clinical isolates of *M. canis* showed specific bands identical to those detected in the standard strains of *A. otae*. Of 9 clinical isolates of *M. gypseum*, 7 isolates and 2 isolates showed bands hybridized by the C3 probe identical to those detected in *A. gypseum* and *A. incurvatum*, respectively. Furthermore, the results from mating experiments on these 9 clinical isolates of *M. gypseum* completely agreed with the results from RAPD and Southern

hybridization analyses. These findings clearly indicate that RAPD and the Southern hybridization analyses are very useful for identification of clinical isolates of *M. canis* and *M. gypseum*.

Introduction

In the previous study, I carried out RAPD and Southern hybridization analyses using genomic DNAs of standard strains of 6 *Arthroderma* species (*A. fulvum, A. grubyi, A. gypseum, A. incurvatum, A. oate* and *A. racemosum*) and indicated the usefulness of these molecular analyses to differentiate these *Arthroderma* species [13].

Microsporum canis, the conidial state of *A. otae* and *M. gypseum,* the conidial states of *A. fulvum, A. gypseum* and *A. incurvatum* [10, 23, 27] have frequently been isolated from animal and human cases of dermatophytoses. These species are sometimes difficult to differentiate morphologically, when these isolates are unable to produce micro- and macroconidia and are also decreased in sexual activity of teleomorph production.

In the chapter II, I tried to confirmed RAPD and Southern hybridization analyses to be helpful in identification of clinical isolates of *M. canis* and *M. gypseum*, and to compare the results derived from the molecular analyses with those from mating

experiments on the clinical isolates of *M. gypseum*.

Materials and Methods

Strains

Clinical isolates and standard strains used in this study are shown in Table 2. All of the 6 clinical isolates of *M. canis* were obtained from dogs and cats with dermatophytosis. The 9 clinical isolates of *M. gypseum* comprised 5 isolates from humans, 3 isolates from dogs and 1 isolate from a rabbit (Table 1). Two strains of (+) and (-) mating types of each species of *A. otae, A. gypseum, A. incurvatum* and *A. fulvum* have been maintained by culturing on 1/10 Sabouraud dextrose agar in the Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Isolation of genomic DNA

Dermatophytes were cultured in Sabouraud liquid medium at 27°C for 10 days. The mycelial samples were collected by centrifugation at 3,000 rpm for 5 min and then homogenized in

liquid nitrogen. The samples were lysed with 1mg of zymolyase-100T (Takara , Kyoto, Japan) per ml in a lysis buffer containing 0.1mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 10mM Tris hydrochloride (pH 8.0) and 0.3% 2-mercaptoethanol at 37°C for 16hrs. High molecular weight DNAs were obtained from these mycelial samples by phenol and chloroform extraction. These DNA samples dissolved in TE buffer (10mM Tris-HCl, pH8.0 and 1mM EDTA) were used for further analysis.

RAPD analysis

The genomic DNA samples (200ng) of dermatophytes were amplified by polymerase chain reaction (PCR) in a reaction mixture (20ml) containing 10mM Tris -HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 200 mM each deoxynucleoside triphosphates, 1.0 unit of *Taq* polymerase (Takara) and 0.5mg of the primer. A random primer (FM1: 5'-AGCCGCCTCCATGGCCCCAGG-3'), which was used in

our previous study using standard of *Arthroderma* species, was used for the PCR amplification [13]. The PCR amplification was carried out for 40 cycles consisting of template denaturation (1 min, at 94 °C), primer annealing (2 min, at 40 °C) and polymerization (1 min, at 72 °C). The PCR products were electrophoresed through 3% agarose gel and then stained with ethidium bromide.

Southern hybridization analysis

The Southern hybridization analyses employed C3 probe, which was extracted from an approximately 480-bp band among the bands amplified from *A. otae* DNA with the FM1 primer in our previous study [13]. The genomic DNA samples extracted from fungi were digested with a restriction endonuclease, *Eco*RI or *Hin*dIII. The DNA digests were electrophoresed through 0.8% agarose gel and transferred to a hybridization membrane, GeneScreen Plus (NEN Research Products, Boston, MA). The DNA samples on the membrane were hybridized with a ^{32}P-labeled probe in a hybridization

solution containing 1% SDS, 10% dextran sulfate, 1M NaCl, 100mg of salmon testis DNA per ml and 50 mM Tris-HCl (pH7.5) at 65°C for 16hrs. After hybridization, the filters were washed with a solution containing 2XSSC (1XSSC is 0.15M NaCl plus 0.015M sodium citrate) and 0.1% SDS at 55°C for 30 min and then autoradiographed.

Mating experiments

Clinical isolates of *M. gypseum* were respectively crossed with (+) and (-) standard strains of *A. gypseum* and *A. incurvatum* on soil and hair medium at 24°C for more than 6 weeks [10].

Results

The RAPD patterns by PCR from all of the 6 clinical isolates of *M. canis* showed 6 bands of 480, 450, 350, 200, 130 and 75bp, which were the same as those amplified from standard strains of *A otae* (Fig. 5). Of the 9 clinical isolates of *M. gypseum*, 7 isolates (VUT-96007, VUT-96008, VUT-96009, VUT-96010, VUT-96011, VUT-96012 and VUT-96013) showed 5 or 6 RAPD bands of 750, 570, 550, (300), 220 and 140bp, which were identical to those seen in the standard strains of *A. gypseum.* Mean while RAPD patterns of 2 other clinical isolates of *M. gypseum* (VUT-96014 and VUT-96015) were shown as 5 bands of 800, 350, 310, 220 and 210bp, being identical to those amplified from *A. incurvatum.* RAPD band pattern identical to that of *A. fulvum* was not detected in any of the 9 clinical isolates of *M. gypseum* examined (Fig. 6).

Southern hybridization analysis using C3 probe derived from *A. otae* DNA gave distinct bands in all the samples of *Microsporum* species examined in this study (Fig. 7, 8). The

Southern hybridization analysis of the genomic DNAs of the six *M. canis* clinical isolates revealed 1.0-kb *Eco*RI and 6.9-kb *Hin*dIII fragments, which were identical to the bands detected in the standard *A. otae* strains (Fig 7). Of the nine *M. gypseum* clinical isolates, seven isolates (VUT-96007, VUT-96008, VUT-96009, VUT-96010, VUT-96011, VUT-96012 and VUT-96013) showed 2.2-kb *Eco*RI and 18-kb *Hin*dIII fragments hybridized with the C3 probe, being identical to the bands in standard *A. gypseum* strains. Other two *M. gypseum* isolates (VUT-96014 and VUT-96015) showed 4.2-kb *Eco*RI and 10.5-kb *Hin*dIII bands hybridized with the C3 probe, which were identical to the bands shown in standard *A. incurvatum* strains (Fig 8).

The results of mating experiments on the clinical isolates of nine *M. gypseum* are shown in Table 3. From the mating experiments, seven of nine *M. gypseum* isolates were identified to be *A. gypseum,* consisting of 5 (+) and (-) mating type isolates. On the other hand, two of the nine *M. gypseum* isolates were identified to be *A.incurvatum* (-)

These results from mating experiments completely agreed with the results by RAPD and Southern hybridization analyses.

Discussion

The RAPD and Southern hybridization analyses have been shown to be useful for identification of fungi including *Aspergillus sp.* and *Penicillium sp.* [17, 28]. These results suggest that RAPD and Southern hybridization analyses have advantages over previous identification systems such as restriction fragment length polymorphism (RFLP) and electrophoretic karyotypes analyses. In addition, RAPD analysis requires only a small amount of DNA to obtain the results. RAPD and Southern hybridization analyses are very simple and provide clear results for identification of fungal species [6, 17]. Furthermore, RAPD is also applicable to investigate intraspecies variations. In my previous report, I demonstrated that standard strains of 6 *Arthroderma* species that were *A. fulvum, A. grubyi, A. gypseum, A. incurvatum, A. otae* and *A. racemosum* could be differentiated by RAPD and Southern hybridization analyses. The RAPD analysis gave different band patterns specific to each of the 6 *Arthroderma* species. Moreover, Southern blot analysis using the C3 probe

which I prepared from *A. otae* gave specific bands in all the 6 *Arthroderma* species and the size of the bands was specific to each species. Based on these findings from our previous study, I applied RAPD and Southern blotting analyses to the identification of clinical isolates of *M. canis* and *M. gypseum* from humans and animals [13]. The present study revealed that clinical isolates of *M. canis* showed RAPD and Southern hybridization patterns identical to those of standard strains of *A. otae*. Furthermore, isolates of *M. gypseum* showed RAPD and Southern hybridization patterns identical to those of standard strains of *A. gypseum* or *A. incurvatum*. Furthermore, I confirmed the results of molecular analyses by mating experiments on the clinical isolates of *M. gypseum*. Results of the mating experiments completely agreed with the results by RAPD and Southern hybridization analyses.

Identification of *Microsporum* species has been carried out by morphological and biochemical characterization as well as mating experiments [10, 23]. However, it is very difficult to differentiate *A. gypseum* from *A. incurvatum* from their

morphological features. Furthermore, *M. gypseum* isolates often lack mating activity. On the other hand, the RAPD and Southern hybridization analyses can be used to identify the isolates of *M. canis* and *M. gypseum*, even when these isolates are pleomorphic. The RAPD and Southern hybridization analyses of *Arthroderma* species developed in this study will be useful as the molecular diagnostic approaches for these *Arthroderma* species.

Chapter III

Differentiation of *Microsporum* species by random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses

Abstract

Molecular characterization of 18 species belonging to genus *Microsporum* isolated from human and animal dermatophytosis was carried out by random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses. All of these 18 *Microsporum* species showed different band patterns specific to each species in RAPD analysis.

Southern hybridization analysis using C3 probe derived from on RAPD band from *Arthroderma otae*, teleomorph of *M. canis*, revealed distinct bands in 14 of the 18 *Microsporum* species but not in the other 4 species. The sizes of the hybridized bands of the 14 species were specific to each species except 3 species (*A. otae*, *M. equinum* and *M. ferrugineum*) which showed the band of the same size. These findings indicated that the RAPD and the Southern hybridization analyses could be effective to differentiate the species of *Microsporum*.

Introduction

Molecular characteristics were analysed in chapter I and II for differentiation of 6 *Arthroderma* specie by RAPD and the Southern hybridization analyses [13]. These findings indicated that the RAPD and the Southern hybridization analyses could be effective to differentiate these *Arthroderma* species which are teleomorph of *Microsporum* species. Genus *Microsporum* includes about 18 species and out of them, *M. audouinii* (including strains of *M. duboidii*), *M. equinum*, *M. ferrugineum*, *M. gallinae*, *M. langeloninii* and *M. ripariae* have not been shown to produce sexal states. These species are frequently difficult to differentiate morphologically, when these isolates are decreased in biochemical activities and in production of micro- and macroconidia. Therefore, molecular classification was necessary to identify *Microsporum* species definitely.

In the present study, I tried to differentiate additional 12 *Microsporum* species as well as the 6 species previously

examined by RAPD and Southern hybridization analyses.

Materials and Methods

Strains

Strains examined are shown in Table 4. Twenty strains of 10 *Arthroderma* species, teleomorphs of *Microsporum* species, are all standard strains of (+) and (−) mating types of the species [27].

Ten strains of 8 *Microsporum* species (*M. audouinii*, *M. boullardii*, *M. duboidii*, *M. equinum*, *M. ferrugineum*, *M. langeloninii*, *M. ripariae* and *M. gallinae*) are isolates from humans and animals with dermatophytosis. They have been maintained by culturing on 1/10 Sabouraud dextrose agar in the Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo

Isolation of genomic DNA

Dermatophytes were cultured in 50ml of Sabouraud liquid medium at 27°C for 10 days. The mycelial cells of

dermatophytes were collected by centrifugation at 3,000 rpm for 5 min. The mycelial cells were frozen in liquid nitrogen and then homogenized, and they were lysed by a lysis buffer containing 1mg of zymolyase-100T (Takara , Kyoto, Japan) per ml, 0.1mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 10mM Tris hydrochloride (pH 8.0) and 0.3% 2-mercaptoethanol at 37°C for 16hrs. High molecular weight DNAs were extracted from the mycelial cells by phenol and chloroform. DNA samples dissolved in TE buffer (10mM Tris-HCl, pH8.0 and 1mM EDTA) were used for further analysis.

Polymerase chain reaction (PCR) amplification

PCR amplification was performed in a reaction mixture (40µl) containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin (GenHunter, Massachusetts, USA), 200mM of each deoxynucleoside triphosphates, 1.0 unit of *Taq* polymerase (Takara), 0.5µg of primer and 200ng of total genomic DNA. A single primer (FM1: 5'-

AGCCGCCTCCATGGCCCCAGG-3') of arbitrary sequence [13] was used for PCR amplification. The PCR amplification was carried out for 40 cycles of template denaturation (1 min, at 94 °C), primer annealing (2 min, at 40 °C) and polymerization (1 min, at 72 °C). The PCR products were separated on 3% agarose gel.

Southern hybridization analysis

Genomic DNA samples from various *Microsporum* species were digested with a restriction endnuclease, *Eco*RI or *Hin*dIII. The DNA samples were electrophoresed on 1.2% agarose gel and transferred to hybridization membrane (Biodyne Transfer Membrane, Pall, NY, USA). The DNA samples on the membrane were hybridized with a ^{32}P-labeled C3 probe derived from on RAPD band of *A.otae* [13] in a hybridization solution containing 1% SDS, 10% dextran sulfate, 1M NaCl, 100μg of salmon testis DNA per ml and 50 mM Tris-HCl, pH7.5 at 65°C for 16hrs. After hybridization, the filters were washed with 2XSSC (1XSSC is 0.15M NaCl plus 0.015M sodium citrate)

added with 0.1% SDS at 55°C for 30 min and autoradiographed.

Results

In my previous study [13], 6 *Arthroderma* species (*A. fulvum, A. grubyi, A. gypseum, A. incurvatum, A. otae* and *A. racemosum*) showed different bands specific to each species in RAPD analysis. In the present study, I examined the RAPD patterns of additional 12 species of *Arthroderma* and *Microsporum* (*A. borellii, A. cajetani, A. obtusum, A. persicolor, M. audouinii, M. boullardii, M. diboidii, M. equinum, M. ferrugineum, M. langeloninii, M. ripariae* and *M. gallinae*) with FM1 primer in parallel with the 6 species previously examined. DNAs from these 18 species of *Microsporum* and *Arthroderma* showed different RAPD band patterns specific to each species (Fig. 9).

In my previous study [13], 6 *Arthroderma* species (*A. fulvum, A. grubyi, A. gypseum, A. incurvatum, A. otae* and *A. racemosum*) gave bands of sizes specific to each species in Southern hybridization analyses with C3 probe. In Southern hybridization analysis with C3 probe, the *Eco*RI digests of the

14 of 18 species of *Arthroderma* and *Microsporum* including the 6 species previously examine gave bands of 1.8kb in *A. fulvum*, 16kb in *A. grubyi*, 2.2kb in *A. gypseum*, 4.2kb in *A. incurvatum*, 1.0kb in *A. otae*, 22kb in *A. obtusum*, 20kb in *A. racemosum*, 1.0kb in *M. audouninii*, 13kb in *M. duboidii*, 1.0kb in *M. equinum*, 1.0kb in *M. ferrugineum*, 21kb in *M. gallinae*, 1.0kb in *M. langeloninii* and 23kb in *M. ripariae* (Fig. 10).

The same Southern hybridization analysis using their *Hin*dIII digests gave specific bands in 14 of 18 species examined as shown in the analysis using their *Eco*RI digests 16.5kb in *A. fulvum*, 17kb in *A. grubyi*, 18kb in *A. gypseum*, 10.5kb in *A. incurvatum*, 6.9kb in *A. otae*, 25kb in *A. obtusum*, 20.3kb in *A. racemosum*, 8.1kb in *M. audouninii*, 15kb in *M. duboidii*, 6.9kb in *M. equinum*, 6.9kb in *M. ferrugineum*, 23kb in *M. gallinae*, 5.2kb in *M. langeloninii* and 6.0kb in *M. ripariae* (Fig. 11). However, *A. borelli*, *A. cajetani*, *A. persicolor* and *M. boullardii* did not show any specific band in the Southern hybridization analysis using the C3 probe. Eleven species, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. obtusum*,

A. racemosum, *M. audouninii*, *M. duboidii*, *M. gallinae*, *M. langeloninii* and *M. ripariae*, showed bands of sizes specific to each species, however, 3 species, *M. equinum* and *M. ferrugineum* and *A. otae* gave bands of the same sizes in both of the *Eco*RI and *Hin*dIII digests.

Discussion

I previously found that the genomic DNA from the 6 *Arthroderma* (*Microsporum*) species showed RAPD band patterns specific to each species [13]. In this study, RAPD analysis using the same primer revealed band patterns specific to each of the 18 species of dermatophytes examined. The RAPD analysis is considered to be useful for interspecies differentiation of *Microsporum* species as shown in other species of fungi [2, 17, 28].

Kawasaki *et al.* reported that restriction fragment length polymorphisms (RFLP) of mitochondrial DNA from *M. audouinii*, *M. equinum*, *M. ferrugineum*, *M. langeloninii*, *M. ripariae* and *M. canis* (*A. otae*) showed the same pattern [11], suggesting that these species might be treated as a variant of *M. canis* [11]. However, the present study indicated that *M. audouinii*, *M. langeloninii*, *M. ripariae* and *M. canis* could be differentiated by RAPD and Southern hybridization analysis. Although the 3 species of *M. canis* (*A. otae*), *M. equinum* and

M. ferrugineum showed different RAPD band patterns, they showed the fragments of the same size in Southern hybridization analysis. Furthermore, the phylogenetic tree of 25S ribosomal RNA indicated that *M. canis* and *M. ferrugineum* were in the close genetic relationship [16]. Therefore, the 3 species of *M. canis M. equinum* and *M. ferrugineum* are considered to for a cluster with close relationship among the various *Microsporum* species.

A. borelli, *A. cajetani*, *A. persicolor* and *M. boullardii* did not show any specific fragment hybridized with the C3 probe derived from *A. otae* in Southern hybridization analysis. A phylogenetic tree from the RFLP analysis on their mitochondrial DNA also indicated that these 4 species were distant to *A. otae* [11]. From these findings, it is conceivable that *A. borelli*, *A. cajetani*, *A. persicolor* and *M. boullardii* are genetically distinct from *A. otae* in the multiple *Microsporum* species examined in this study.

Kawasaki *et al.* indicated that RFLP of mitochondrial DNA from *A. fulvum* and *M. boullardii* showed the same

pattern, and suggested that these 2 species should be classified in one species [11]. However, in the present study, RAPD band patterns of *A. fulvum* and *M. boullardii* were different, and the Southern hybridization using the C3 probe gave a specific band in *A. fulvum* but did not in *M. boullardii*, indicating that *M. boullardii* and *A. fulvum* can be differentiated by these molecular analysis systems.

The RAPD and Southern hybridization analyses developed in this study will be useful in the molecular diagnostic approach to differentiate these *Microsporum* (*Arthroderma*) species.

Chapter IV

Phylogenetic analysis of 8 dermatophyte species using chitin synthase 1 gene sequences

Abstract

Nucleotide sequences of chitin synthase 1 (*CHS1*) gene of 8 species of dermatophytes, *Arthroderma benhamiae*, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae*, *A. simii* and *A. vanbreuseghemii* were obtained and analyzed for their phylogenetic relationship. A 600-bp genomic DNA fragment of the *CHS1* gene was amplified from these dermatophytes by polymerase chain reaction (PCR) and sequenced. The *CHS1* nucleotide sequences of these 8 dermatophyte species showed more than 85% similarity between the species. The phylogenetic analysis of their sequences revealed 3 clusters, first cluster consisting of *A. benhamiae*, *A. simii* and *A. vanbreuseghemii*, and second cluster consisting of *A. fulvum*, *A. gypseum* and *A. incurvatum*, and third cluster consisting of *A. grubyi* and *A. otae*. The phylogenetic analysis of *CHS1* gene in this study will provide useful information for classification and understanding the evolution of these dermatophyte species.

Introduction

In previous study, 18 *Microsporum* species examined were clearly differentiated by RAPD and the Southern hybridization analyses. However, these molecular analyses could not reveal the phylogenetic relationship of dermatophytes. The phylogenetic study of dermatophytes is important to understand the evolution of dermatophytes and their definitive classification. Though phylogenetic analyses of some dermatophyte species have been carried out using the sequences of 18S and 25S ribosomal RNAs [9, 16]. However, they have not well revealed the phylogeny of dermatophytes.

On the other hand, chitin synthase 1 (*CHS1*) gene has been cloned in *Candida albicans*, *Aspergillus nidulans*, *Histoplasma capsulatum* and *Sporothrix schenckii* [3-5]. Comparison of their sequences revealed a highly conserved region which can be used to prepare primers for polymerase chain reaction (PCR) amplification of this gene [3, 4]. Chitin, ß1-4-linked polymer of N-acetylglucosamine, is a fibrous cellulose-like

polysaccharide that constitutes the cell wall of many species of fungi including dermatophytes. Phylogenetic analyses of these fungi using the sequences of *CHS1* gene indicated their genetic relationship confirming the results by the conventional classification [3, 4].

Therefore, in the present study, to establish their phylogenetic relationship of dermatophytes, I analyzed genetic phylogeny of the *CHS1* gene sequences from 8 species of dermatophytes, *Arthroderma benhamiae*, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae*, *A. simii* and *A. vanbreuseghemii* which were main causative agents of human and small animal dermatophytoses.

Materials and methods

Strains

The standard strains of *A. benhamiae*, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae*, *A. simii* and *A. vanbreuseghemii* (Table 5) were used for the experiment. All of them are + mating type strains of each species and have been maintained by culturing on 1/10 Sabouraud dextrose agar in the Department of Veterinary Internal Medicine, Graduate School of Agriculturual and Life Sciences, the University of Tokyo.

Isolation of genomic DNA

The standard strains of dermatophytes were grown in Sabouraud liquid medium at 27°C for 10 days. The mycelium samples were collected by centrifugation at 3,000 rpm for 5 min and then homogenized in liquid nitrogen. These samples were lysed with 1mg of zymolyase-100T (Takara , Kyoto, Japan) per ml in a lysis buffer containing 0.1mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 10mM Tris hydrochloride (pH

8.0) and 0.3% 2-mercaptoethanol at 37°C for 16hrs. High molecular weight DNAs were obtained from these mycelium samples by phenol and chloroform extraction. These DNA samples dissolved in TE buffer (10mM Tris-HCl pH8.0 and 1mM EDTA) were used for analysis.

PCR amplification

The genomic DNA samples (200ng) of dermatophytes were amplified by PCR in a reaction mixture (20µl) containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 200 mM each deoxynucleoside triphosphate, 1.0 unit of *Taq* polymerase (Takara) and 0.5µg of the primer. The sequences of the degenerate primers for *CHS1* gene were based on the sequences reported by Chen-Wu et al (4) : primer 1, 5'-CTG AAG CTT ACT(ACG) ATG TAT(C) AAT(C) GAG(A) GAT(C)-3'; primer 2, 5'-GTT CTC GAG (C)TTT (A)GTA (C)TTC (A)GAA (A)GTT (T)CTG-3'.

The PCR amplification was carried out for 30 cycles consisting of template denaturation (1 min, at 94 °C), primer

annealing (2 min, at 50°C) and polymerization (3 min, at 72 °C). The PCR products were electrophoresed through 2% agarose gel and then stained with ethidium bromide.

Cloning of PCR products

The PCR product from each species was respectively gel-purified and cloned into pCRII vector (Invitrogen, San Diego, CA). These plasmid DNAs were extracted with QIAGEN plasmid kit (QIAGEN, Studio City, CA) and sequenced by the dideoxy chain termination method using an Auto Read Sequencing kit (Pharmacia, Uppsala, Sweden).

Phylogenetic analysis

To examine the phylogenetic relationships, the nucleotide sequences were analyzed by Clustal W multiple sequence Alignment programs [26] and a phylogenic tree was constructed by the TREEVIEW displaying phylogenies program [20]. Bootstrap analysis was performed on 1000 random samples taken from multiple alignment as described by Felsenstein [8]

and analyzed by the Clustal W programs.

Results

Amplification of dermatophyte DNAs with degenerate *CHS1* primers yielded a expected fragment of 600-bp. The *CHS1* nucleotide sequences of the 8 species of dermatophytes showed a high degree of sequence similarity among them : more than 85% in nucleotide level and more than 85% in amino acid level. The phylogenetic analysis of the nucleotide sequences of the 8 dermatophytes species showed three clusters, first cluster consisting of *A. benhamiae*, *A. simii* and *A. vanbreuseghemii*, second cluster consisting of *A. fulvum*, *A. gypseum* and *A. incurvatum*, and third cluster consisting of *A. grubyi* and *A. otae* (Fig. 12). The sequences reported in this paper have been deposited in the GenBank data base [accession nos. AB003558, *Arthroderma benhamiae*; AB003559, *A. fulvum*; AB003560, *A. gypseum*; AB003561, *A. grubyi*; AB003562, *A. incurvatum*; AB003563, *A. otae*; AB003564, *A. simii*; AB003565, *A. vanbreuseghemii*].

Discussion

The *CHS1* gene of these dermatophytes showed 75 - 85% amino acid sequence similarity with those of non-dermatophyte fungi, *Wangeilla dermatitidis, Penicillium chrysogenum, Coccidioides immitis* and *Emericella nidulans* [3, 4]. The phylogenetic tree based on the *CHS1* sequences was obtained in this study to indicate the presence of 3 distinct groups in the *Arthroderma* species. The first group is consisted of *A. benhamiae*, *A. simii* and *A. vanbreuseghemii*. These 3 species are teleomorphs of *Trichophyton mentagrophytes* [1, 24, 25]. The second group comprises *A. fulvum*, *A. gypseum* and *A. incurvatum*, corresponding to teleomorphs of *Microsporum gypseum* [23, 7]. *A. grubyi* (*M. vanbreuseghemii*) and *A. otae* (*M. canis*) were categorized into the third group. The results obtained in this study seem to be very similar to a previous phylogenetic study based on the mitochondrial DNA [12] on the point that there are 3 distinct groups in the *Arthroderma* species.

On the other hand, phylogenetic analyses of dermatophytes by using the sequences of 18S and 25S ribosomal RNAs peviously reported [9, 16] showed the phylogenetic trees that indicated groupings similar to those based on the results obtained from the *CHS1* gene sequences. The sequence analysis of 25S ribosomal RNA also indicated the phylogenetic tree that *M. canis*, *M. gypseum* and *T. mentagrophytes* were grouped into different clusters.

Molecular biological analyses will add useful informations to the conventional taxonomy of fungi which has been based on their morphological and physiological characteristics. The sequence analysis of *CHS1* gene in this study should be useful for classification and understanding the evolution of dermatophytes.

Chapter V

Molecular analysis of chitin synthase 1 (*CHS1*) gene sequences of *Trichophyton mentagrophytes* complex and *T. rubrum*

Abstract

Nucleotide sequences of chitin synthase 1 (*CHS1*) gene of 10 dermatophytes, *Arthroderma benhamiae*, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae*, *A. simii*, *A. vanbreuseghemii*, *Trichophyton mentagrophytes* var. *interdigitale* (*T. interdigitale*) and *T. rubrum* were analyzed for their phylogenetic relationship. About 620-bp genomic DNA fragments of the *CHS1* gene were amplified from these dermatophytes by polymerase chain reaction (PCR) and sequenced. The *CHS1* nucleotide sequences of these 10 dermatophytes showed more than 85% similarity between the species. The phylogenetic analysis of the nucleotide sequences of the 10 dermatophytes species showed 3 clusters, first cluster consisting of *A. benhamiae*, *A. simii*, *A. vanbreuseghemii*, *T. rubrum* and *T. interdigitale*, second cluster consisting of *A. fulvum*, *A. gypseum* and *A. incurvatum*, and third cluster consisting of *A. grubyi* and *A. otae*. The first cluster of 5 *Trichophyton* species revealed that *A. benhamiae*, *A. simii*, *A.*

vanbreuseghemii and *T. rubrum* were genetically distinct from each other but *T. interdigitale* was genetically very close to *A. vanbreuseghemii*.

On the other hand, specific restriction endnuclease site of *Hin*fI was shown in the *CHS1* gene fragment of *T. rubrum* but not in those of *A. benhamiae*, *A. simii*, *A. vanbreuseghemii* and *T. interdigitale*.

The molecular analysis of *CHS1* gene in this study will provide useful information for the classification of these *Trichophyton* species and understanding their evolution.

Introduction

Trichophyton mentagrophytes and *T. rubrum* are most frequently isolated from human and animal dermatophytoses and they have been identified by morphological and biochemical analyses as well as mating experiments. However, it is frequently difficult to differentiate these isolates of these *Trichophyton* species morphologically, since they could occasionally produce no macro- and microconidia, and lack mating activity. *T. mentagrophytes* is reported to be the conidial states of at least 3 teleomorphic species (*A. benhamiae*, *A. simii* and *A. vanbreuseghemii*). This complex is supposed to include the other species in which the sexual state is unknown. *T. rubrum* has not been confirmed its teleomorph production. Therefore, the differentiation of *T. mentagrophytes* complex and *T. rubrum* is of taxonomic interest. In my previous study, I sequenced and analyzed chitin synthase 1 (*CHS1*) genes of 8 dermatophytes species including *A. benhamiae*, *A. simii* and *A. vanbreuseghemii* for

understanding their phylogenetic relationship [14].

In the present study, to investigate the phylogenetic relationship of these *Trichophyton* species which are most frequently isolated in human and animal cases of dermatophytoses. I analyzed the nucleotide sequences of *CHS1* gene from 3 clinical isolates of *T. mentagrophytes* and 9 isolates *T. rubrum* comparing with those of the standard strains of 8 dermatophytes.

Furthermore, I analyzed the *CHS1* genes from *T. mentagrophytes* complex and *T. rubrum* by the RFLP analysis.

Materials and methods

Strains

The standard strains of *A. benhamiae*, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae*, *A. simii* and *A. vanbreuseghemii* were used for this study (Table 6). Clinical isolates of *T. interdigitale* and *T. rubrum* were derived from humans and dogs with dermatophytosis (Table 6). These standard strains and clinical isolates have been maintained by culturing on 1/10 Sabouraud dextrose agar at the Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Isolation of genomic DNA

The standard strains and clinical isolates of dermatophytes were grown in Sabouraud liquid medium at 27°C for 10 days. The mycelial samples were collected by centrifugation at 3,000 rpm for 5 min and then homogenized in liquid nitrogen. The samples were lysed with 1mg of zymolyase-100T (Takara,

Kyoto, Japan) per ml in a lysis buffer containing 0.1mM EDTA, 1% sodium dodecyl sulfate (SDS), 10mM Tris hydrochloride (pH 8.0) and 0.3% 2-mercaptoethanol at 37°C for 16hrs. High molecular weight DNAs were obtained from these mycelium samples by phenol and chloroform extraction. These DNA samples dissolved in TE buffer (10mM Tris-hydrochloride, pH8.0 and 1mM EDTA) were used for polymerase chain reaction (PCR) amplification.

Polymerase chaine reaction (PCR) amplification

The genomic DNA samples (200ng) of dermatophytes were amplified by PCR in a reaction mixture (20µl) containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 200 mM each deoxynucleoside triphosphate, 1.0 unit of *Taq* polymerase (Takara) and 0.5µg of a pair of primer. The sequences of the degenerate primers for *CHS1* gene were based on the sequences of *CHS1* gene of *Candida albicans* (4) :

primer1,5'-CTGAAGCTTACT(ACG)ATGTAT(C)AAT

(C)GAG(A)GAT(C)-3' ; primer 2, 5'-GTTCTCGAG(C)TT

T(A)GTA(C)TTC(A)GAA(A)GTT(T)CTG-3'. The PCR amplification was carried out for 30 cycles consisting of template denaturation (1 min, at 94 °C), primer annealing (2 min, at 50°C) and polymerization (3 min, at 72 °C). The PCR products were electrophoresed through 2% agarose gel and then stained with ethidium bromide.

Cloning and sequencing of the PCR products

The PCR products from each species were respectively gel-purified and cloned into pCRII vector (Invitrogen, San Diego, CA). These plasmid DNAs were extracted with QIAGEN plasmid kit (QIAGEN, Studio City, CA) and sequenced by the dideoxy chain termination method using an Auto Read Sequencing kit (Pharmacia, Uppsala, Sweden).

Phylogenetic analysis

To examine the phylogenetic relationship, the nucleotide sequences were analyzed by Clustal W multiple sequence alignment programs [26] and a phylogenic tree was constructed

by TREEVIEW displaying phylogenies program [20]. Bootstrap analysis was performed on 1000 random samples taken from multiple alignment as described by Felsenstein [8] and bootstrap analysis was carried out by the Clustal W programs.

Restriction analysis of the PCR products

PCR-amplified fragments of *CHS1* gene of *Arthroderma* and *Trichophyton* species were digested with a restriction endnuclease *Hin*fI. The DNA digests were electrophoresed through 2% agarose gel and then stained with ethidium bromide.

Results

Amplification of dermatophyte DNAs with degenerate *CHS* primers yielded fragments of about 620bp, consistent with the sizes of *CHS1* gene fragments from fungal species previously reported [3-5]. Nucleotide sequence analysis of the *CHS1* gene fragments from the strains of five *Trichophyton* species indicated the sequence similarities more than 85% among the five species of dermatophytes (Fig. 13). Especially high degree of nucleotide sequence similarity more than 99% was noted between the *CHS1* gene fragments of *A. vanberuseghemii* and *T. interdigitale*. Intra-species sequence variation among nine isolates of *T. rubrum* and that among three 3 isolates of *T. interdigitale* were extremely limited (less than 1%). The phylogenetic analysis of the nucleotide sequences of the 10 dermatophytes species showed 3 clusters, first cluster consisting of *A. benhamiae*, *A. simii*, *A. vanbreuseghemii*, *T. rubrum* and *T. interdigitale*, second cluster consisting of *A. fulvum*, *A. gypseum* and *A. incurvatum*, and third cluster consisting of *A.*

grubyi and *A. otae.* (Fig. 14). The first cluster of five *Trichophyton* species revealed that *A. benhamiae, A. simii, A. vanbreuseghemii* and *T. rubrum* were genetically distinct from each other but *T. interdigitale* was genetically very close to *A. vanbreuseghemii* (Fig. 14). The sequences reported in this paper have been deposited in the GenBank data base [accession nos.- *Trichophyton interdigitale,* AB005794; *T. rubrum,* AB005793].

A specific restriction endnuclease site of *Hin*fI was detected in the sequence of *CHS1* gene fragment of *T. rubrum* (nt 369bp-373bp) but not in those of *A. benhamiae, A. simii, A. vanbreuseghemii* and *T. interdigitale.* The 620-bp bands of *CHS1* gene fragment amplified from standard strains of *A. benhamiae, A. simii* and *A. vanbreuseghemii,* and three clinical isolates of *T. interdigitale* were not cleaved by *Hin*fI digests, although these were additional bands of about 500bp corresponding to *CHS2* or *CHS3* gene (Fig. 15). In contrast, *Hin*fI digestion of the *CHS1* gene fragment amplified from nine clinical isolates of *T. rubrum* yielded two bands of 370bp and

250bp in addition with bands of 620bp derived from *CHS1*-related gene (Fig. 14). From these results, the PCR restriction analysis of *CHS1* gene showed a clear differentiation in the band patterns between *T. rubrum* and other four *Trichophyton* species.

Discussion

The phylogenetic analysis of the nucleotide sequences of the 10 dermatophytes species showed 3 clusters, first cluster consisting of *A. benhamiae*, *A. simii*, *A. vanbreuseghemii*, *T. rubrum* and *T. interdigitale*. These 5 species are genus of *Trichophyton* species. The second and third clusters consisting of *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum* and *A. otae*, corresponding to teleomorphs of *Microsporum*.

On the other hand, the first cluster of five *Trichophyton* species revealed that *A. benhamiae*, *A. simii*, *A. vanbreuseghemii* and *T. rubrum* were genetically distinct from each other but *T. interdigitale* which was genetically very close to *A. vanbreuseghemii*. The results obtained in this study seemed to be similar to that in a previous phylogenetic study on mitochondrial DNA at the point that *A. vanbreuseghemii* and *T. interdigitale* are genetically very close [18, 19]. RFLP analysis of mitochondrial DNA also indicated that the RFLP patterns of *T. interdigitale* and *A. vanbreuseghemii* were identical [18, 19].

Moreover, it has been shown that *T. interdigitale* is morphologically very similar to *A. vanbreuseghemii* [25]. Therefore, *T. interdigitale* can be considered to be very close or identical to *A. vanbreuseghemii* from various genetic analyses.

On the other hand, from the phylogenetic tree based on the *CHS1* gene sequences, *T. rubrum* was distantly related to the other 4 *Trichophyton* species. Mitochondrial DNA analysis also showed that *T. rubrum* strains formed a cluster distant from the other 4 species [19]. The sequence analysis of 25S ribosomal RNA showed a phylogenetic tree that *T. rubrum* and *T. mentagrophytes* were grouped into two different clusters [16]. Therefore, all of the genetic analyses including that obtained in this study indicated the genetic identity of *T. rubrum* among the *Trichophyton* species.

The specific restriction endnuclease site of *Hin*fI was detected in the *CHS1* gene fragment from *T. rubrum* but not in those from *A. benhamiae*, *A. simii A. vanbreuseghemii* and *T. interdigitale*. Sequence analysis of *CHS1* gene requires several

experimental products including the sequencing apparatus, but the PCR-restriction analysis is very easy to carry out in a laboratory. Therefore, this PCR-restriction analysis of *CHS1* gene could be applicable to differentiation of clinical isolates of *T. rubrum* from *T. mentagrophytes* complex.

Molecular biological analyses are considered to provide useful informations to the conventional taxonomy of fungi which has been based on their morphological and biochemical characteristics. The sequence analysis of *CHS1* gene in this study should be helpful for classification and understanding the evolution of *Trichophyton* species.

Conclusion

In recent years, molecular analyses have been considered a reliable and simple method to differentiate the fungal species [6, 17, 22, 28]. However, molecular analyses of dermatophytes species have not been well investigated. Therefore, I tried to differentiate dermatophyte species by random amplification of polymorphic DNA (RAPD) and Southern hybridization analyses.

In chapter I, genomic DNAs of 6 *Arthroderma* species (*A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae* and *A. racemosum*) which are teleomorphs of *Microsporum* species, were investigated for molecular differentiation by RAPD and Southern hybridization analyses. The RAPD analysis gave different band patterns specific to each of the 6 *Arthroderma* fungi. However, minor differences in the band patterns were observed between the strains of (+) and (-) mating types of *A. gypseum*, *A. fulvum* and *A. incurvatum*. Southern blot analysis using a 1S probe (130-bp) obtained from *A. grubyi* DNA gave specific bands only in the DNA samples of *A. grubyi* and *A. incurvatum*. On the other hand, Southern blot analysis using

a C3 probe (480-bp) obtained from *A. otae* DNA gave specific bands in all the 6 *Arthroderma* species examined and the size of the bands were specific to each species.

In chapter II, I carried out an identification of the clinical isolates of *M. canis* and *M. gypseum* by RAPD and Southern hybridization analyses, according to the results obtained in chapter I. The results revealed that clinical isolates of *M. canis* showed RAPD and Southern hybridization patterns identical to those of the standard strains of *A. otae*. The isolates of *M. gypseum* also showed RAPD and Southern hybridization patterns identical to those of standard strains of *A. gypseum* or *A. incurvatum*. Moreover, the results of the mating experiments on the clinical isolates of *M. gypseum* completely agreed with the these results by RAPD and Southern hybridization analyses, confirming that molecular analysis is applicable to the identification of *M. canis* and *M. gypseum*.

In chapter III, I tried to differentiate an additional 12 *Microsporum* species in addition to the 6 species examined by the RAPD and Southern hybridization analyses established in

chapter I. All of these 18 *Microsporum* species showed different band patterns specific to each species in RAPD analysis. Southern hybridization analysis using a C3 probe revealed distinct bands in 14 of the 18 *Microsporum* species but not in the other 4 species. The sizes of the hybridized bands of the 14 species were specific to each species except for 3 species (*A. otae*, *M. equinum* and *M. ferrugineum*) which showed a band of the same size. The results obtained in chapters I, II and III clearly indicated that RAPD and the Southern hybridization analyses were very useful in the identification of *Microsporum* species.

On the other hand, phylogenetic study of dermatophytes is important in understanding the evolution of dermatophytes. The phylogenetic study on dermatophytes, however, has not been well investigated by conventional characteristics. Therefore, the relationship between dermatophyte species are of taxonomic interest in medical mycology. The phylogenetic analysis of chitin synthase 1 (*CHS1*) gene has been carried out in some pathogenic fungi. The *CHS1* genes of some

dermatophytes were investigated in order to understand their phylogenetic relationship. In chapter IV, I obtained the nucleotide sequences of the *CHS1* gene from 8 species of dermatophytes, *A. benhamiae*, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae*, *A. simii* and *A. vanbreuseghemii*, and investigated their phylogenetic relationship based on the sequences of the *CHS1* gene. The *CHS1* genes of these dermatophytes showed a 75 - 85% amino acid sequence similarity with those of non-dermatophyte fungi. The phylogenetic analysis of their sequences revealed three clusters, the first cluster consisting of *A. benhamiae*, *A. simii* and *A. vanbreuseghemii*, the second cluster consisting of *A. fulvum*, *A. gypseum* and *A. incurvatum*, and the third cluster consisting of *A. grubyi* and *A. otae*.

In chapter V, nucleotide sequences of the *CHS1* gene of 10 dermatophytes, *Arthroderma benhamiae*, *A. fulvum*, *A. grubyi*, *A. gypseum*, *A. incurvatum*, *A. otae*, *A. simii*, *A. vanbreuseghemii*, *Trichophyton mentagrophytes* var. *interdigitale* (*T. interdigitale*) and *T. rubrum* were analyzed for

their phylogenetic relationship. The phylogenetic analysis of their sequences revealed that *A. benhamiae*, *A. simii*, *A. vanbreuseghemii* and *T. rubrum* were genetically distinct from each other, but *T. interdigitale* was genetically very close to *A. vanbreuseghemii*.

Further, the specific restriction endnuclease site of *Hin*fI was shown in the *CHS1* gene fragment of *T. rubrum* but not in those of *A. benhamiae*, *A. simii*, *A. vanbreuseghemii* and *T. interdigitale*. The PCR-restriction analysis of the *CHS1* gene could be applicable in differentiating the clinical isolates of *T. rubrum* from the isolates of *T. mentagrophytes* complex.

This study revealed that RAPD and Southern hybridization analyses could be helpful for the classification and differentiation of dermatophytes. Moreover, the molecular analysis of the *CHS1* gene will provide useful information on the phylogenetic relationship of dermatophytes and their classification.

Acknowledgments

I wish to express my deepest gratitude to Dr. Atsuhiko Hasegawa, Dr. Hajime Tsujimoto and Dr Toshihiro Watari (Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo) for their great supports and advice during this study. I thank Dr Yuka Nakamura, Dr. Shinichi Watanabe and Dr. Hisashi Takahashi (Department of Dermatology, Teikyo University School of Medicine) for helpful suggestions and providing some samples. I also thank to Miss Haruka Matsushiro for advised technical assistance. Finally, I am grateful to all members of Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, for their continuos encouragement and discussion.

References

1. **Ajello, L. and Cheng, Shu-Lan.** 1967. The perfect state of *Trichophyton mentagrophytes. Sabouraudia.* **4:** 230-234.

2. **Bidochka, J. M., Mcdonald, A. M., Leger, J. St. R. & Roberts, W. D.** 1994. Differentiation of species and strains of entomopathogenic fungi by random amplification of polymorphic DNA. *Curr. Genet.* **25:** 107-113.

3. **Bowen, A. R., Chen-Wu, J.L., Momany, M., Young, R., Szanizlo, P. J. and Robbins, P. W.** 1992. Classification of fungal chitin synthases. *Proc. Natl. Acad. Sci. USA.* **89:** 519-523.

4. **Chen-Wu, J.L., Zwicker, J., Bowen, A. R. and Robbins, P. W.** 1992. Expression of chitin synthase gene during yeast and hyphal growth phases of *Candida albicans. Mol. Microbiol.* **6:**497-502.

5. **Chua, S. S., Momany, M., Mendoza, L and Szanizlo, P. J.** 1994. Identification of three chitin synthase genes in the dimorphic fungal pathogen *Sporothrix schenckii. Curr.*

Microbiol. **29:** 151-156.

6. **Crowhurst, N. R., Hawthorne, T. B., Rikkerink, H. A. E. and Templeton, D. M.** 1991. Differentiation of *Fusarium solani* f. sp. cucurbitae races 1 and 2 by random amplification of polynorphic DNA. *Curr. Genet.* **20:** 391-396.

7. **Demange, C., Contet-Audonneau, N., Kombila, M., Miegeville, M., Berthonneau, M., Vroey, De. Ch. and Percebos, G.** 1992. *Microsporum gypseum* complex in man and animals. *J. Med. Vet. Mycol.* **30:** 301-308.

8. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution.* **39:** 783-791.

9. **Harmsen, D., Schwinn, A., Weig, M., Bröcker, E.-B. and Heesemann, J.** 1995. Phylogeny and dating of some pathogenic keratinophilic fungi using small subunit ribosomal RNA. *J . Med. Vet . Mycol.* **33:** 299-303.

10. **Hasegawa, A. & Usui, K.** 1975. *Nannizzia otae* sp. nov, the perfect state of *Microsporum canis* Bodin. *Japan. J. Med.*

Mycol. **16:**148-153

11. **Kawasaki, M., Aoki, M. and Ishizaki, H.** 1995. Phylogenetic relationships of some *Microsporum* and *Arthorderma* species inferred mitochondrial DNA analysis *Mycopathologia.* **130:** 11-21.

12. **Kawasaki, M., Aoki, M., Ishizaki, H., Nishimura, K. and Miyaji, M.** 1996. Phylogeny of *Epidermophyton floccosum* and other dermatophytes. *Mycopathologia.* **134:** 121-128

13. **Kano, R., Matsushiro, H., Watari, T., Tsujimoto, H and Hasegawa, A.** 1997. Differentiation of *Arthroderma* species by random amplification polymorphic DNA (RAPD) and Southern hybridization analyses. *Mycoses.* **40:** in press.

14. **Kano, R., Nakamura, Y., Watari, T., Watanabe, S., Takahashi, H., Tsujimoto H. and Hasegawa, A.** 1997. Phylogenetic Analysis of 8 Dermatophyte Species Using Chitin Synthase 1 Gene Sequences. *Mycoses.* **40:** in press.

15. **Georg, K. L., Ajello, L., Friedman, L. and**

Brinkman, A. S. 1962. A new species of *Microsporum* pathogenic to man and animals. *Sabouraudia.* **1:** 189-196

16. **Leclerc, C. M., Philippe, H. and Guého, E.** 1994. Phylogeny of dermatophytes and dimorphic fungi based on large subunit ribosomal RNA sequence comparisons. *J. Med. Vet. Mycol.* **32:** 331-341.

17. **Megnegneau, B., Debets, F. and Hoekstra, F. R.** 1993. Genetic variability and relatedness in the complex group of black Aspergilli based on random amplification of polymorphic DNA. *Curr. Genet.* **23:** 323-329.

18. **Mochizuki,T. K., Takada, K., Watanabe, S., Kawasaki, M. and Ishizaki H.** 1990. Taxonomy of *Trichophyton nterdigitale* (*Trichophyton mentagrophytes* var. *interdigitale*) by restriction enzyme analysis of mitochondrial DNA. *J. Med. Vet. Mycol.* **28:** 191-196.

19. **Nishio, K., Kawasaki, M. and Ishizaki, M.** 1992. Phylogeny of the genera *Trichophyton* using mitochondrial DNA analysis. *Mycopathologia.* **117:** 127-132.

20. **Page, R. D. M.** 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12:** 357-358.

21. **Rush-Munro, M. F., Smith, M. B. and Borelli, D.** 1970. The perfect state of *Microsporum racemosum*. *Mycologia.* **62:** 856-859

22. **Someren, A. K. M., Samson, A. R. and Visser, J.** 1991. The use of RFLP analysis in classification of the black Aspergilli: reinterpretation of *Aspergillus niger* aggregate. *Curr. Genet.* **19:** 21-26.

23. **Stockdale, M. P**. 1963. The *Microsporum gypseum* complex (*Nannizzia incurvata* stockd., *N. gypsea* (NANN.) comb. nov., *N. fulva* sp. nov.). *Sabouraudia.* **3:** 114-126

24. **Stockdale, M. Phyllis., Mackenzie, W. R. D., Austwick, K. C. P.** 1967. *Arthroderma simii* sp. nov., the perfect state of *richophyton simii* (Pinoy) comb. nov. *Sabouraudia.* **4:** 112-123.

25. **Takashio, M.** 1977. The *Trichophyton mentagrophytes*

complex, p. 271-276 In: Iwata, K. (Ed.) Recent Advances in medical and veterinary mycology. University of Tokyo Press, Tokyo.

26. **Thompson, J.D., Higgins, D.G. and Gibson, T.J.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nuc. Acid. Res.* **22:** 4673-4680.

27. **Weitzman, I., McGinnis, M. R., Padhye, A. A. and Ajello, L.** 1986. The genus *Arthroderma* and its later synonym *Nannizzia*. *mycotaxon.* **15:** 505-518.

28. **Williams, G. K. J., Kubelik, R. A., Livak, J. K., Rafalski, J. A. and Tigngey, V. S.** 1990. DNA polymorphisms amplified by arbitray primers are useful as genetic markers. *Nuc. Acid. Res.* **18:** 6531-6535.

Table 1. Species and strains used in this study

Species (mating type)	Strain
A. fulvum (+)	VUT-4006 (CMI 86179)
A. fulvum (-)	VUT-4007 (CMI 86179)
A grubyi (+)	VUT-78043 (UAMH 1465=CDC X470)
A. grubyi (-)	VUT-78044 (UAMH 1466=CDC X471)
A. gypseum (+)	VUT-4004 (CMI 86526)
A. gypseum (-)	VUT-4005 (CMI 86176)
A. incurvataum (+)	VUT-4002 (CMI 86523)
A. incurvataum (-)	VUT-4003 (CMI 86518)
A. otae (+)	VUT-77054 (IAM 12728=ATCC 44334)
A. otae (-)	VUT-77055 (IAM 12729=ATCC 44335)
A. rasemosum (+)	VUT-76031 (CDC X902)
A. rasemosum (-)	VUT-76032 (CDC X903)

Table 2. Standard strains and clinical isolates used this study

Species (mating type)	Strain	
Standard strains		
A. otae (+)	VUT*-77054	(ATCC 44334)
A. otae (-)	VUT-77055	(ATCC 44335)
A. gypseum (+)	VUT-4004	(CMI 86526)
A. gypseum (-)	VUT-4005	(CMI 86176)
A. fulvum (+)	VUT-4006	(CMI 86179)
A. fulvumm (-)	VUT-4007	(CMI 86180)
A. incurvatum(+)	VUT-4002	(CMI 86523)
A. incurvatum (-)	VUT-4003	(CMI 86518)
Clinical isolates		
M. canis	VUT-96001	[Cat]**
M. canis	VUT-96002	[Cat]
M. canis	VUT-96003	[Dog]
M. canis	VUT-96004	[Dog]
M. canis	VUT-96005	[Dog]
M. canis	VUT-96006	[Dog]
M. gypseum	VUT-96007	[Dog]
M. gypseum	VUT-96008	[Human]
M. gypseum	VUT-96009	[Dog]
M. gypseum	VUT-96010	[Human]
M. gypseum	VUT-96011	[Human]
M. gypseum	VUT-96012	[Rabbit]
M. gypseum	VUT-96013	[Dog]
M. gypseum	VUT-96014	[Human]
M. gypseum	VUT-96015	[Human]

* VUT : School of Veterinary Medicine, the University of Tokyo
* * [ ]: isolated from dermatophytosis

Table 3. Mating experiments of the clinical isolates of *M. gypseum* with the standard strains of *A. incurvatum* and *A. gypseum*

	A. incurvatum		*A. gypseum*	
	(+)VUT-4002	(-)VUT-4003	(+)VUT-4004	(-)VUT-4005
Clinical isolates of *M. gypseum*				
VUT-96007	−	+	−	
VUT-96008	−	−	+	
VUT-96009	−	−	+	
VUT-96010	−	+	−	
VUT-96011	−	−	+	
VUT-96012	−	−	+	
VUT-96013	−	−	+	
VUT-96014	+	−	−	
VUT-96015	+	−	−	

−, ascospores are present; +, ascospores are absent.

Table 4. Species and strains used in this study

Species (mating type)	Strain	
A. borellii (+)	VUT-78041	(UAMH 3571=CDC Y.81)
A. borellii (-)	VUT-78042	(UAMH 3572=CDC Y.82)
A. cajetani (+)	VUT-9017	(ATCC 14387=CDC B434)
A. cajetani (-)	VUT-9018	(ATCC 14388=CDC B435)
A. fulvum (+)	VUT-4006	(CMI 86179)
A. fulvum (-)	VUT-4007	(CMI 86179)
A. grubyi (+)	VUT-78043	(UAMH 1465=CDC X470)
A. grubyi (-)	VUT-78044	(UAMH 1466=CDC X471)
A. gypseum (+)	VUT-4004	(CMI 86526)
A. gypseum (-)	VUT-4005	(CMI 86176)
A. incurvataum (+)	VUT-4002	(CMI 86523)
A. incurvataum (-)	VUT-4003	(CMI 86518)
A. obtusum (+)	VUT-71001	(CDC X719=ATCC 24446)
A. obtusum (-)	VUT-71002	(CDC X718=ATCC 24435)
A. otae (+)	VUT-77054	(IAM 12728=ATCC 44334)
A. otae (-)	VUT-77055	(IAM 12729=ATCC 44335)
A. persicolor (+)	VUT-77160	(CMI 117073)
A. persicolor (-)	VUT-77161	(CMI 117064)
A. rasemosum (+)	VUT-76031	(CDC X902)
A. rasemosum (-)	VUT-76032	(CDC X903)
M. audouinii	VUT-75065	Clinical isolate
M. boullardii	VUT-75036	Clinical isolate
M. duboidii	VUT-9007	Clinical isolate
M. equinum	VUT-97012	Clinical isolate
M. equinum	VUT-75015	Clinical isolate
M. ferrugineum	VUT-97013	Clinical isolate
M. ferrugineum	VUT-78065	Clinical isolate
M. gallinae	VUT-76002	Clinical isolate
M. langeloninii	VUT-75038	Clinical isolate
M. ripariae	VUT-75020	Clinical isolate

Table 5. Species and strains in this study

Species (Mating type)	Strain	
A. benhamiae (+)	VUT-77011	(IAM 12704=RV 26678)
A. fulvum (+)	VUT-4006	(CMI 86179)
A. grubyi (+)	VUT-78043	(UAMH 1465=CDC X470)
A. gypseum (+)	VUT-4004	(CMI 86526)
A. incurvataum (+)	VUT-4002	(CMI 86523)
A. otae (+)	VUT-77054	(IAM 12728=ATCC 44334)
A. simii (+)	VUT-77009	(CMI 101693=CBS 448.65)
A. vanbreuseghemii (+)	VUT-77007	(CBS 646.73=RV27960)

Table 6. Species and strains used in this study

Species (Mating type)	Strain	
A. benhamiae (+)	VUT-77011	(IAM 12704=RV 26678)
A. benhamiae (-)	VUT-77012	(IAM 12705=RV 26680)
A. fulvum (+)	VUT-4006	(CMI 86179)
A. grubyi (+)	VUT-78043	(UAMH 1465=CDC X470)
A. gypseum (+)	VUT-4004	(CMI 86526)
A. incurvataum (+)	VUT-4002	(CMI 86523)
A. otae (+)	VUT-77054	(IAM 12728=ATCC 44334)
A. simii (+)	VUT-77009	(CMI 101693=CBS 448.65)
A. simii (-)	VUT-77010	(CMI 101695=CBS 447.65)
A. vanbreuseghemii (+)	VUT-77007	(CBS 646.73=RV27960)
A. vanbreuseghemii (-)	VUT-77008	(CBS 642.73=RV27961)
T. interdigitale	VUT-97004 *	[Human]
T. interdigitale	VUT-97007	[Human]
T. interdigitale	VUT-97009	[Human]
T. rubrum	VUT-97014	[Human]
T. rubrum	VUT-97015	[Human]
T. rubrum	VUT-97016	[Human]
T. rubrum	VUT-97017	[Human]
T. rubrum	VUT-97018	[Human]
T. rubrum	VUT-97019	[Human]
T. rubrum	VUT-97020	[Dog]
T. rubrum	VUT-97021	[Dog]
T. rubrum	VUT-97022	[Dog]

* [ ]: isolated from dermatophytosis

Legends for Figures

Fig. 1. Random amplification of polymorphic DNA amplified by PCR from *Arthroderma* species. All genomic DNA samples were amplified with the 21-mer primer (FM1). Lanes: 1, *A. otae* (+) VUT-77054; 2, *A. oate* (-) VUT-77055; 3, *A. grubyi* (+) VUT-78043; 4, *A. grubyi* (-) 78045; 5, *A. gypseum* (+) 4004; 6, *A. gypseum* (-) 4005; 7, *A. fulvum* (+) 4006; 8, *A. fulvum* (-) 4007; 9, *A. incurvatum* (+) 4002; 10, *A. incurvatum* (-) 4003; 11, *A. racemosum* (+) 76031; 12, *A. racemosum* (-) 76032. * used as probes for Southern hybridization analysis. RAPD bands which showed difference between the (+) and (-) strains of the same species.

Fig. 2a,b. Southern hybridization analysis of genomic DNA of *Arthroderma* species with 1S probe (a, *Eco*RI ; b, *Hin*dIII). Lanes: 1, *A. otae* (+) VUT-77054; 2, *A. oate* (-) VUT-77055; 3, *A. grubyi* (+) VUT-78043; 4, *A. grubyi* (-) 78045; 5, *A. gypseum* (+) 4004; 6, *A. gypseum* (-) 4005; 7, *A. fulvum* (+) 4006; 8, *A. fulvum* (-) 4007; 9, *A. incurvatum* (+) 4002; 10, *A.*

incurvatum (-) 4003; 11, *A. racemosum* (+) 76031; 12, *A. racemosum* (-) 76032.

Fi. 3a,b. Southern hybridization analysis of genomic DNA of *Arthoroderma* species with C3 probe (a, *Eco*RI ; b, *Hin*dIII). Lanes: 1, *A. otae* (+) VUT-77054; 2, *A. oate* (-) VUT-77055; 3, *A. grubyi* (+) VUT-78043; 4, *A. grubyi* (-) 78045; 5, *A. gypseum* (+) 4004; 6, *A. gypseum* (-) 4005; 7, *A. fulvum* (+) 4006; 8, *A. fulvum* (-) 4007; 9, *A. incurvatum* (+) 4002; 10, *A. incurvatum* (-) 4003; 11, *A. racemosum* (+) 76031; 12, *A. racemosum* (-) 76032.

Fig. 4a,b. Nucleotide and deduced amino acid sequences of 1S probe fragment (a) and C3 probe fragment (b). The nucleotide sequences are numbered from the first nucleotide of the DNA fragment.

Fig 5. RAPD patterns of the standard strains of *A. otae* and clinical isolates of *M. canis*. Lane 1: a standard strain of *A. otae* (+); Lane 2: a standard strain of *A. otae* (-); Lanes 3 to 8: clinical isolates of *M. canis*. The genomic DNA samples were amplified with a 21-mer primer

(FM1).

Fig 6. RAPD patterns of the standard strains of *A. gypseum*, *A. incurvatum*, *A. fulvum* and clinical isolates of *M. gypseum*. Lane 1: a standard strain of A. *gypseum* (+) ; Lane 2: a standard strain of *A. gypseum* (-); Lane 10: a standard strain of A. *incurvatum* (+); Lane 11: a standard strain of *A. incurvatum* (-); Lane 14: a standard strain of *A. fulvum* (+) ; Lane 15: a standard strain of *A. fulvum* (-); Lanes 3 to 9, 12 and 13: clinical isolates of *M. gypseum*. The genomic DNA samples were amplified with a 21-mer primer (FM1).

Fig 7a,b. Southern hybridization analysis on genomic DNAs standard strains of *A. otae* and clinical isolates of *M. canis* with C3 probe (a, *Eco*RI; b, *Hin*dIII). Lane 1: a standard strain of *A. otae* (+); Lane 2: a standard strain of *A. otae* (-); Lanes 3 to 8: clinical isolates of *M. canis*.

Fig 8a,b. Southern hybridization analysis on genomic DNAs of *A. gypseum*, *A. incurvatum*, *A. fulvum* and clinical isolates of *M. gypseum* with C3 probe (a, *Eco*RI ; b,

*Hind*III). Lane 1: a standard strain of *A. gypseum* (+) ; Lane 2: a standard strain of *A. gypseum* (-); Lane 10: a standard strain of *A. incurvatum* (+); Lane 11: a standard strain of *A. incurvatum* (-); Lane 14: a standard strain of *A. fulvum* (+) ; Lane 15: a standard strain of *A. fulvum* (-); Lanes 3 to 9, 12 and 13: clinical isolates of *M. gypseum*.

Fig. 9. Band patterns of random amplification of polymorphic DNA of various *Arthroderma* and *Microsporum* species. All of the genomic DNA samples were amplified with a 21-mer primer (FM1) of arbitrary sequence. Lanes: 1, *A. borellii* (+); 2, *A. borellii* (-); 3, *A. cajetani* (+); 4, *A. cajetani* (-); 5, *A. fulvum* (+); 6, *A. fulvum* (-); 7, *A. grubyi* (+); 8, *A. grubyi* (-); 9, *A. gypseum* (+); 10, *A. gypseum* (-); 11, *A. incurvatum* (+); 12, *A. incurvatum* (-); 13, *A. obtusum* (+); 14, *A. obtusum* (-); 15, *A. otae* (+); 16, *A. oate* (-); 17, *A. persicolor* (+); 18, *A. persicolor* (-); 19, *A. racemosum* (+); 20, *A. racemosum* (-); 21, *M. audouinii*; 22, *M. boullardii*; 23, *M. duboidii*; 24, *M. equinum*; 25, *M. equinum*; 26, *M. ferrugineum*; 27, *M. ferrugineum*; 28, *M. langeloninii*; 29, *M.*

gallinae; 30, *M. ripariae*.

Fig. 10. Southern hybridization analysis of *Eco*RI digested DNA samples of *Arthroderma* and *Microsporum* species with C3 probe. Lanes: 1, *A. borellii* (+); 2, *A. borellii* (-); 3, A. cajetani (+); 4, *A. cajetani* (-); 5, *A. fulvum* (+); 6, *A. fulvum* (-); 7, *A. grubyi* (+); 8, *A. grubyi* (-); 9, *A. gypseum* (+); 10, *A. gypseum* (-); 11, *A. incurvatum* (+); 12, *A. incurvatum* (-); 13, *A. obtusum* (+); 14, *A. obtusum* (-); 15, *A. otae* (+); 16, *A. oate* (-); 17, *A. persicolor* (+); 18, *A. persicolor* (-); 19, *A. racemosum* (+); 20, *A. racemosum* (-); 21, *M. audouinii*; 22, *M. boullardii;* 23, *M. duboidii*; 24, *M. equinum;* 25, *M. equinum*; 26, *M. ferrugineum*; 27, *M. ferrugineum*; 28, *M. gallinae*; 29, *M. langeloninii*; 30, *M. ripariae*.

Fig. 11. Southern hybridization analysis of *Hin*dIII digested DNA samples of *Arthroderma* and *Microsporum* species with C3 probe. Lanes: 1, *M. audouinii*; 2, *M. boullardii*; 3, *M. duboidii*; 4, *M. equinum*; 5, *M. equinum*; 6, *M. ferrugineum*; 7, *M. ferrugineum*; 8, *M. gallinae*; 9, *M. langeloninii*; 10, *M. ripariae*; 21,

M. audouinii; 22, *M. boullardii*; 23, *M. duboidii*; 24, *M. equinum*; 25, *M. equinum*; 26, *M. ferrugineum*; 27, *M. ferrugineum*; 28, *M. gallinae*; 29, *M. langeloninii*; 30, *M. ripariae*.

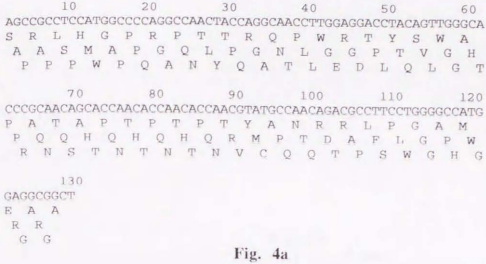
Fig. 12 A tree showing phylogenic relationships among *CHS1* gene fragments of 8 species. The DNA sequences were compared by Clustal W multiple sequence alignment programs and a phylogenic tree was constructed by the TREEVIEW displaying phylogenies program. Bootstrap analysis was performed on 1000 random samples and analyzed by the Clustal W programs. Numbers at branches were determined by the bootstrap analysis indicating the times in 1000 repeat subsamples in monophyletic grouping.

Fig. 13 Nucleotide sequences of *CHS1* gene of 5 dermatophytes, *A. benhamiae*, *A. simii*, *A. vanbreuseghemii*, *T. interdigitale* and *T. rubrum*. About 620-bp genomic DNA fragments of the *CHS1* gene were amplified from these dermatophytes by PCR and sequenced.

Fig. 14 A tree showing phylogenetic relationships among *CHS1* gene fragments of 10 dermatophytes. The DNA sequences were compared by Clustal W multiple sequence alignment programs and a phylogenetic tree was constructed by the TREEVIEW displaying phylogenies program. Bootstrap analysis was performed on 1000 random samples and analyzed by the Clustal W programs. Numbers at branches were determined by the bootstrap analysis indicating the times in 1000 repeat sub samples in monophylogenic grouping.

Fig. 15 PCR restriction analysis of *CHS1* gene fragments from *Arthroderma* and *Trichophyton* species. The *CHS1* gene fragments amplified from *Arthroderma* and *Trichophyton* species were digested with *Hin*fI and electrophoresed through 2% agarose gel and stained with ethidium bromide. Lanes: 1, *A. benhamiae* VUT-77011 ; 2, *A. benhamiae* VUT-77012; 3, *A. simii* VUT-77009; 4, *A. simii* VUT-77010; 5, *A. vanbreuseghemii* VUT-77007; 6, *A. vanbreuseghemii* VUT-77008 7, *T. interdigitale*

VUT-97004; 8, *T. interdigitale* VUT-97007; 9, *T. interdigitale* VUT-97009; 10, *T. rubrum* VUT-97014; 11, *T. rubrum* VUT-97016; 12, *T. rubrum* VUT-97017; 13, *T. rubrum* VUT-97018; 14, *T. rubrum* VUT-97019; 15, *T. rubrum* VUT-97020 ; 16, *T. rubrum* VUT-97021 ; 15, *T. rubrum* VUT-97022. Left arrow head indicate the *CHS* fragments of 620bp which was not cleaved by *Hin*fI digestion. Right arrows indicate two *CHS1* gene fragments of approximately 400bp and 200bp which were digested with *Hin*fI.


10 20 30 40 50 60
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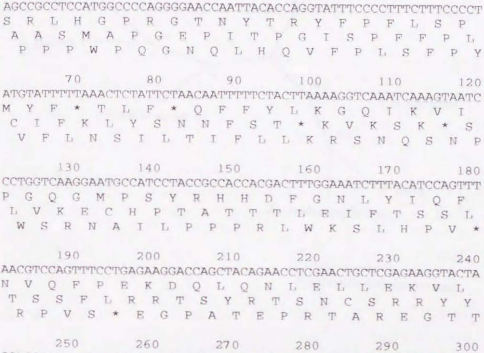
S R L H G P R P T T R Q P W R T Y S W A
A A S M A P G Q L P G N L G G P T V G H
P P P W P Q A N Y Q A T L E D L Q L G T
70 80 90 100 110 120
CCCGCAACAGCACCAACACCAACACCAACGTATGCCAACAGACGCCTTCCTGGGGCCATG
P A T A P T P T P T Y A N R R L P G A M
P Q Q H Q H Q H Q R M P T D A F L G P W
R N S T N T N T N V C Q Q T P S W G H G
130
GAGGCGGCT
E A A
R R
G G

Fig. 4a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A A S M A P G E P I T P G I S P F P L
P P P W P Q G N Q L H Q V F P L S F P Y
70 80 90 100 110 120
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M Y F * T L F * Q F F Y L K G Q I K V I
C I F K L Y S N N F S T * K V K S K * S
V F L N S I L T I F L L K R S N Q S N P
130 140 150 160 170 180
CCTGGTCAAGGAATGCCATCCTACCGCCACCACGACTTTGGAAATCTTTACATCCAGTTT
P G Q G M P S Y R H H D F G N L Y I Q F
L V K E C H P T A T T T L E I F T S S L
W S R N A I L P P P R L W K S L H P V *
190 200 210 220 230 240
AACGTCCAGTTTCCTGAGAAGGACCAGCTACAGAACCTCGAACTGCTCGAGAAGGTACTA
N V Q F P E K D Q L Q N L E L L E K V L
T S S F L R R T S Y R T S N C S R R Y Y
R P V S * E G P A T E P R T A R E G T T
250 260 270 280 290 300
CCACCACGTTTGACCCAGGAGGCTCCACCACCAGACAGCATGGTAGAGGACTTTGCCTTG
P P R L T Q E A P P P D S M V E D F A L
H H V * P R R L H H Q T A W * R T L P W
T T F D P G G S T T R Q H G R G L C L G
310 320 330 340 350 360
GAGAATGTTGACAGCAACGGAGGCCAAGCTAGAGCCCAAGGAGCTGCCAGAGGTGATGAT
E N V D S N G G Q A R A Q G A A R G D D
R M L T A T E A K L E P K E L P E V M M
E C * Q Q R R P S * S P R S C Q R * * *
370 380 390 400 410 420
GATGAAGAAGATGGCATACCGCCCGGCGCTGAGAGAATGCAGTGCGCATCTCAGTAAGCG
D E E D G I P P G A E R M Q C A S Q * A
M K K M A Y R P A L R E C S A H L S K R
* R R W H T A R R * E N A V R I S V S G
430 440 450 460 470 480
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V R N I * R K P G A W G M E X A Q I
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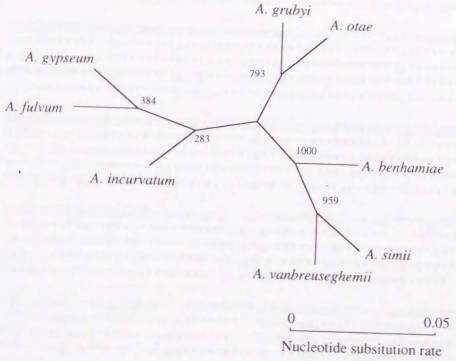
Fig. 4b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A. gypseum
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A. fulvum
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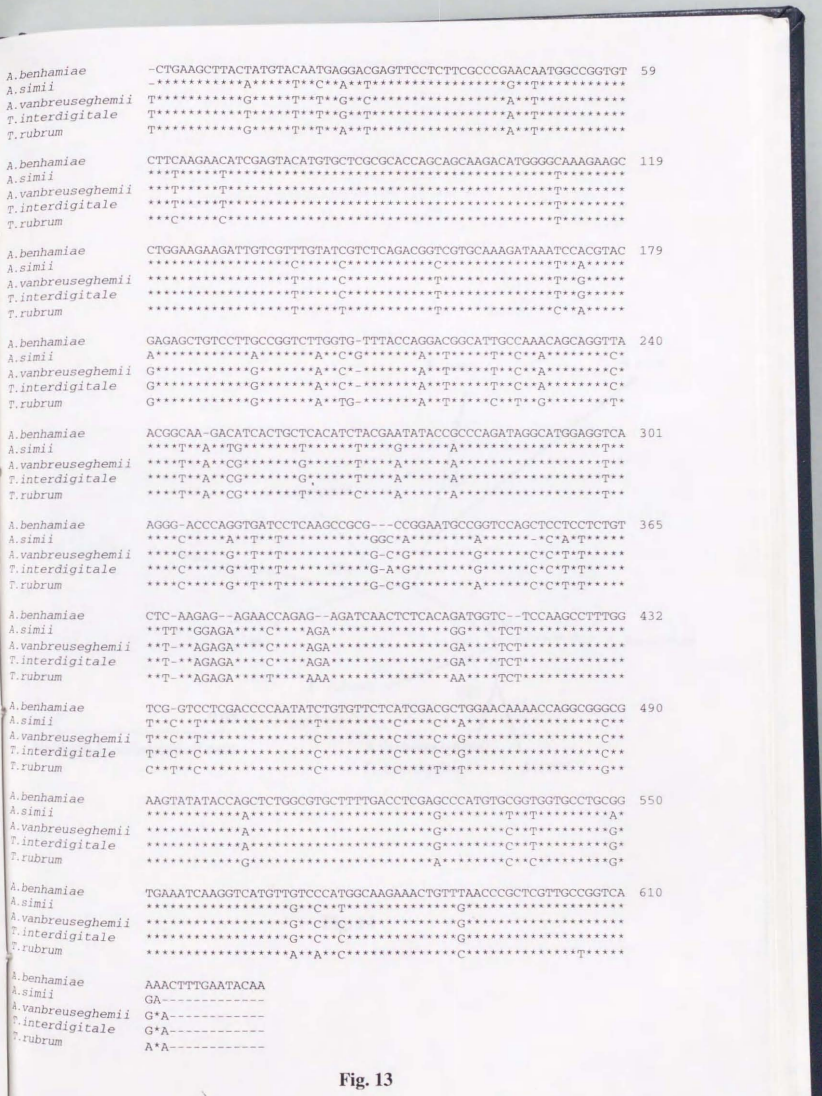
A. incurvatum
959
A. simii
A. vanbreuseghemii
0
0.05
Nucleotide subsitution rate

Fig. 12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A.vanbreuseghemii
T.interdigitale
T.rubrum
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550
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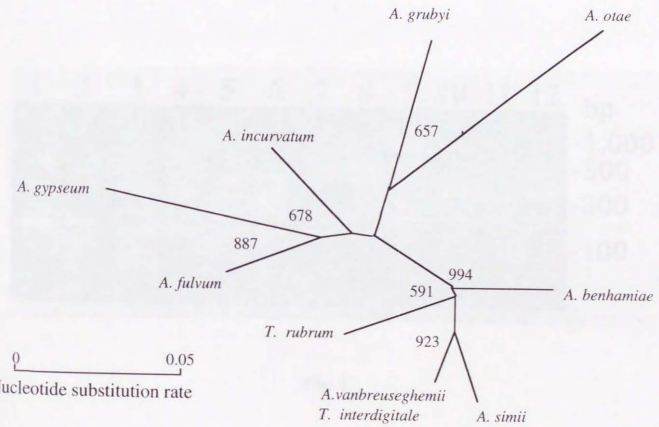
Fig. 13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A. otae
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A. incurvatum
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A. fulvum
A. benhamiae
T. rubrum
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0
0.05
Nucleotide substitution rate
A.vanbreuseghemii
T. interdigitale
A. simii

Fig. 14

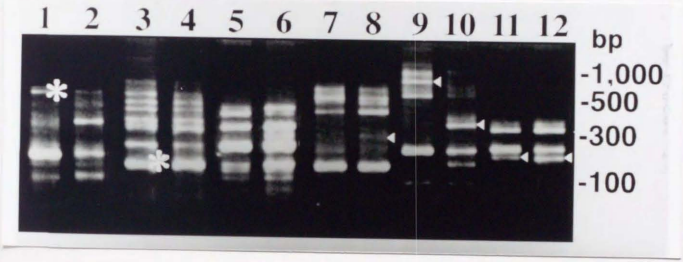


Fig. 1

Fig. 2a

Fig. 2b

Fig. 3a

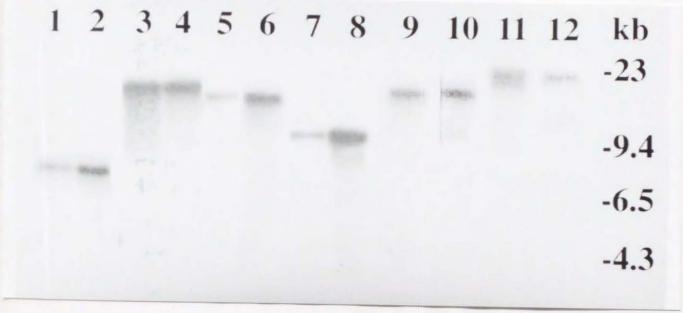


Fig. 3b

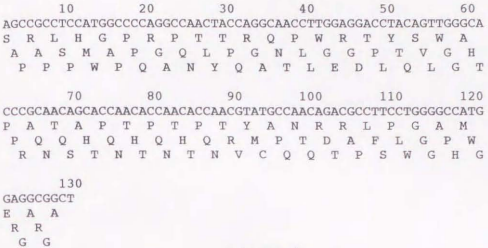


Figure 4a

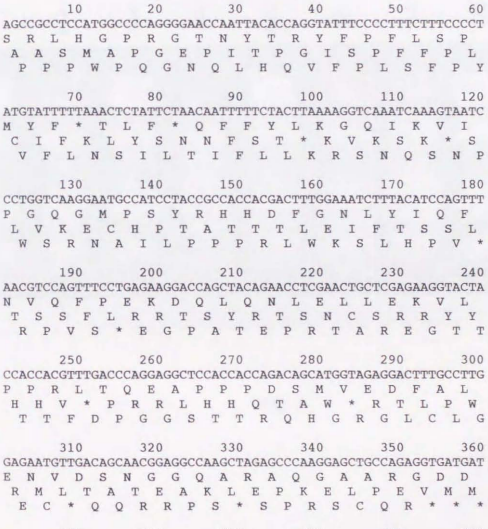


Figure 4b

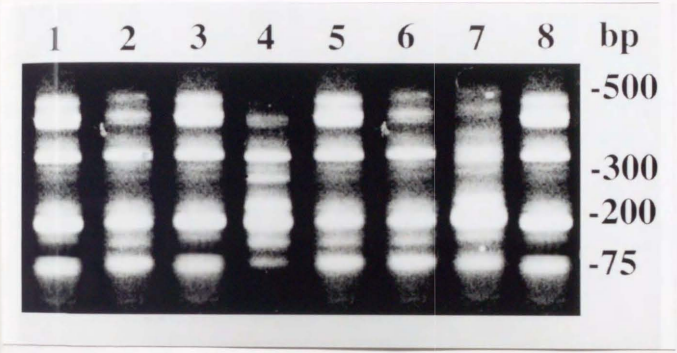


Fig. 5

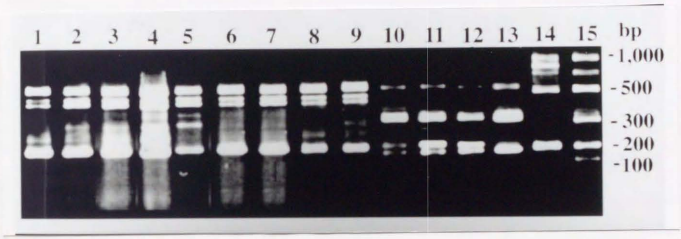


Fig. 6

Fig. 7a

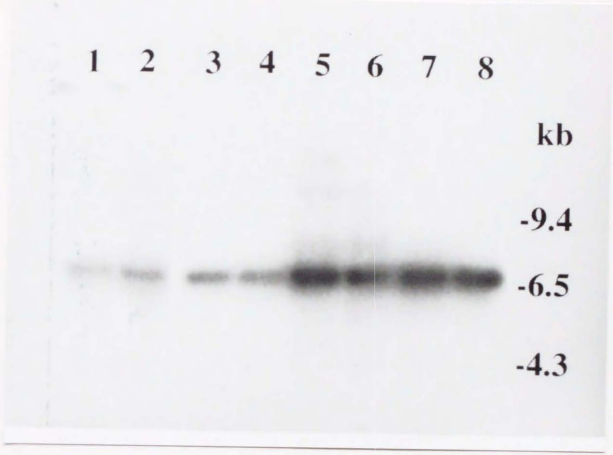


Fig. 7b

Fig. 8a

Fig. 8b

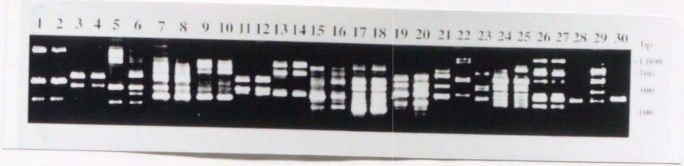
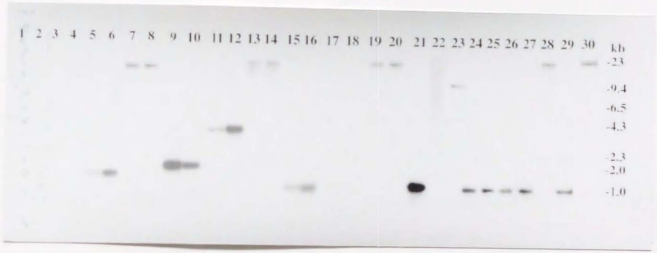


Fig. 9



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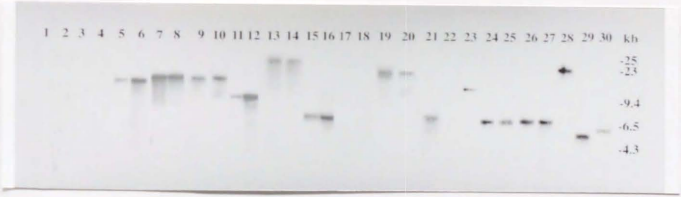
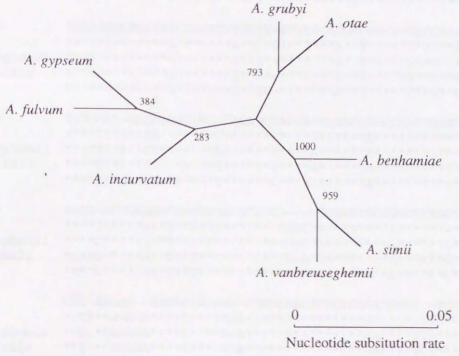


Fig. 11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283
1000
A. benhamiae
A. incurvatum
959
A. simii
A. vanbreuseghemii
0
0.05
Nucleotide subsitution rate

Fig. 12

```
A.benhamiae          -CTGAAGCTTACTATGTACAATGAGGACGAGTTCCTCTTCGCCCGAACAATGGCCGGTGT  59
A.simii              -**********A*****T**C**A**T*****************G**T**********
A.vanbreuseghemii    T**********G*****T**T**G**C*****************A**T**********
T.interdigitale      T**********T*****T**T**G**T*****************A**T**********
T.rubrum             T**********G*****T**T**A**T*****************A**T**********

A.benhamiae          CTTCAAGAACATCGAGTACATGTGCTCGCGCACCAGCAGCAAGACATGGGGCAAAGAAGC 119
A.simii              ***T*****T*************************************************T********
A.vanbreuseghemii    ***T*****T*************************************************T********
T.interdigitale      ***T*****T*************************************************T********
T.rubrum             ***C*****C*************************************************T********

A.benhamiae          CTGGAAGAAGATTGTCGTTTGTATCGTCTCAGACGGTCGTGCAAAGATAAATCCACGTAC 179
A.simii              ******************C*****C***********C*************T**A*****
A.vanbreuseghemii    ******************T*****C***********T*************T**G*****
T.interdigitale      ******************T*****C***********T*************T**G*****
T.rubrum             ******************T*****T***********T*************C**A*****

A.benhamiae          GAGAGCTGTCCTTGCCGGTCTTGGTG-TTTACCAGGACGGCATTGCCAAACAGCAGGTTA 240
A.simii              A***********A*******A**C*G*******A**T*****T**C**A********C*
A.vanbreuseghemii    G***********G*******A**C*-*******A**T*****T**C**A********C*
T.interdigitale      G***********G*******A**C*-*******A**T*****T**C**A********C*
T.rubrum             G***********G*******A**TG-*******A**T*****C**T**G********T*

A.benhamiae          ACGGCAA-GACATCACTGCTCACATCTACGAATATACCGCCCAGATAGGCATGGAGGTCA 301
A.simii              ****T**A**TG******T******T****G******A*****************T**
A.vanbreuseghemii    ****T**A**CG*******G******T****A******A*****************T**
T.interdigitale      ****T**A**CG*******G******T****A******A*****************T**
T.rubrum             ****T**A**CG*******T******C****A******A*****************T**

A.benhamiae          AGGG-ACCCAGGTGATCCTCAAGCCGCG---CCGGAATGCCGGTCCAGCTCCTCCTCTGT 365
A.simii              ****C*****A**T**T**********GGC*A********A******-*C*A*T*****
A.vanbreuseghemii    ****C*****G*T**T**********G-C*G*******G******C*C*T*T*****
T.interdigitale      ****C*****G*T**T**********G-A*G*******G******C*C*T*T*****
T.rubrum             ****C*****G*T**T**********G-C*G*******A******C*C*T*T*****

A.benhamiae          CTC-AAGAG--AGAACCAGAG--AGATCAACTCTCACAGATGGTC--TCCAAGCCTTTGG 432
A.simii              **TT**GGAGA****C****AGA**************GG****TCT**********
A.vanbreuseghemii    **T-**AGAGA****C****AGA**************GA****TCT**********
T.interdigitale      **T-**AGAGA****C****AGA**************GA****TCT**********
T.rubrum             **T-**AGAGA****T****AAA**************AA****TCT**********

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A.simii              T**C*T**********T*********C****C**A**********C**
A.vanbreuseghemii    T**C*T**********C*********C****C**G**********C**
T.interdigitale      T**C*C**********C*********C****C**G**********C**
T.rubrum             C**T**C**********C*********C****T**T**********G**

A.benhamiae          AAGTATATACCAGCTCTGGCGTGCTTTTGACCTCGAGCCCATGTGCGGTGGTGCCTGCGG 550
A.simii              ***********A**********************G*******T**T********A*
A.vanbreuseghemii    **********A**********************G*******C**T********G*
T.interdigitale      **********A**********************G*******C**T********G*
T.rubrum             **********G**********************A*******C**C********G*

A.benhamiae          TGAAATCAAGGTCATGTTGTCCCATGGCAAGAAACTGTTTAACCCGCTCGTTGCCGGTCA 610
A.simii              ******************G**C**T**********G*******************
A.vanbreuseghemii    ******************G**C**C**********G*******************
T.interdigitale      ******************G**C**C**********G*******************
T.rubrum             ******************A**A**C**********C*********T*****

A.benhamiae          AAACTTTGAATACAA
A.simii              GA-------------
A.vanbreuseghemii    G*A-------------
T.interdigitale      G*A-------------
T.rubrum             A*A-------------
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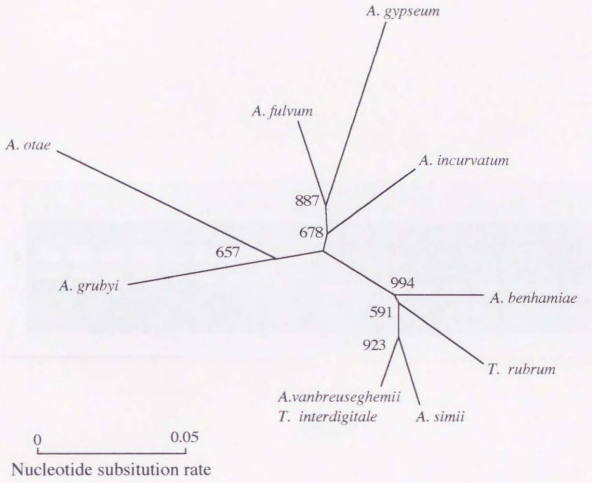
Fig. 13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T. rubrum
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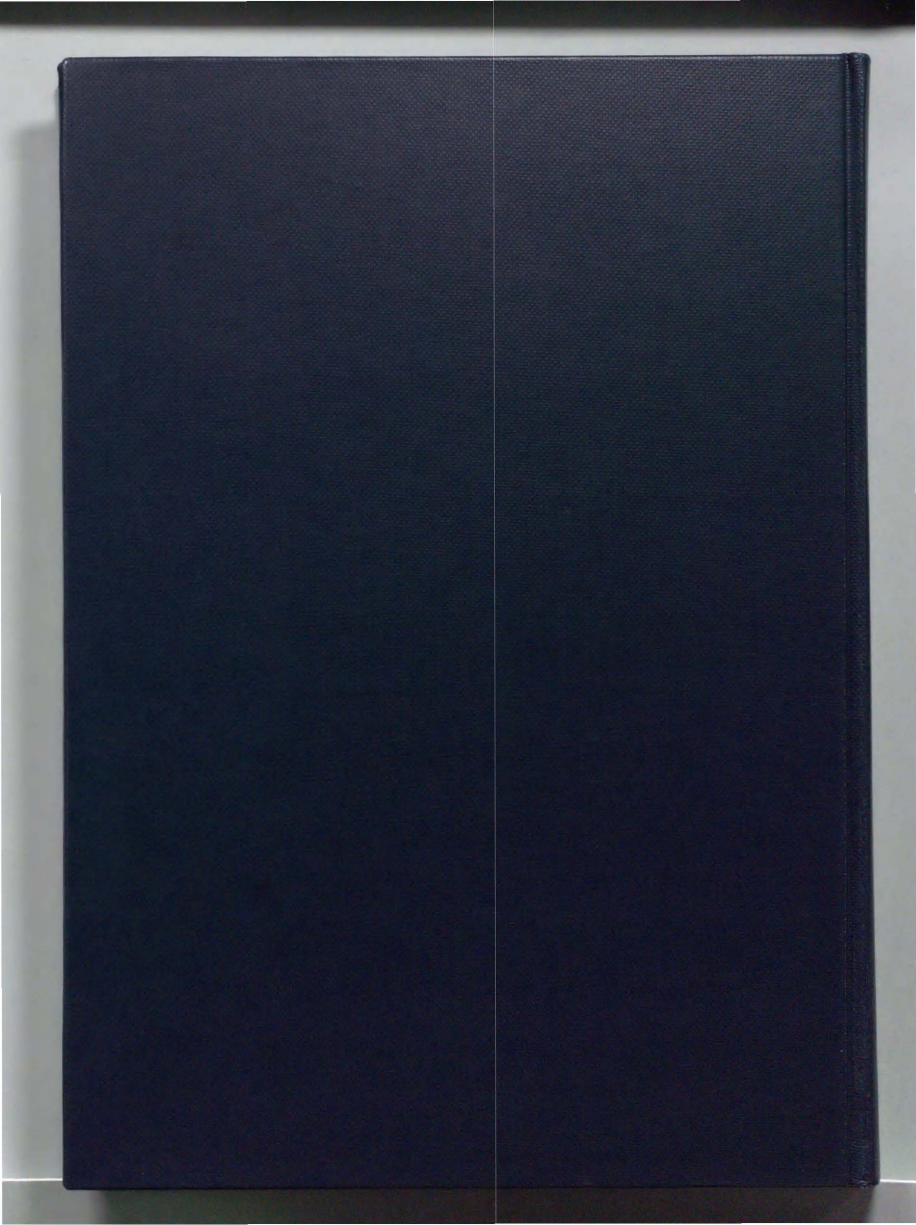
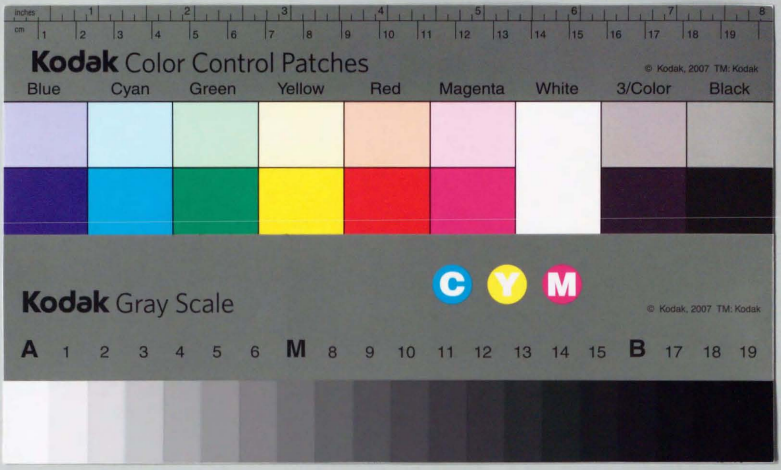
T. interdigitale
A. simii
0
0.05
Nucleotide subsitution rate

Fig. 14

Fig. 15

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cm
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Green
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White
3/Color
Black
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C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19