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# Sequence Diversity of the Intergenic Spacer Region of the rRNA Gene of *Malassezia globosa* Colonizing the Skin of Patients with Atopic Dermatitis and Healthy Individuals

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**The lipophilic yeast *Malassezia globosa* is one of the major constituents of the mycoflora of the skin of patients with atopic dermatitis (AD). We compared the genotypes of *M. globosa* colonizing the skin surface of 32 AD patients and 20 healthy individuals for polymorphism of the intergenic spacer (IGS) 1 region of the rRNA gene. Sequence analysis demonstrated that *M. globosa* was divided into four major groups, which corresponded to the sources of the samples, on the phylogenetic tree. Of the four groups, two were from AD patients and one was from healthy subjects. The remaining group included samples from both AD patients and healthy subjects. In addition, the IGS 1 region of *M. globosa* contained short sequence repeats: $(CT)_n$, and $(GT)_n$. The number of sequence repeats also differed between the IGS 1 of *M. globosa* from AD patients and that from healthy subjects. These findings suggest that a specific genotype of *M. globosa* may play a significant role in AD, although *M. globosa* commonly colonizes both AD patients and healthy subjects.**

*Malassezia* species are lipophilic yeasts that are part of the normal human cutaneous commensal flora; they are isolated from sebaceous gland-rich areas of the skin, particularly on the chest, back, and head. They are also associated with several cutaneous diseases, including atopic dermatitis (AD), folliculitis, pityriasis versicolor, and seborrheic dermatitis (1, 7). In a taxonomic revision in 1996, the genus *Malassezia* was classified into seven different species: *M. furfur*, *M. globosa*, *M. restricta*, *M. obtusa*, *M. pachydermatis*, *M. slooffiae*, and *M. sympodialis* (9). Recently, we described an eighth species, *M. dermatis*, which was isolated from Japanese patients with AD (27). Since the taxonomic revision of the genus *Malassezia*, several studies have examined the distribution of the newly defined species of *Malassezia* on healthy human skin and lesions of skin diseases (2, 10, 21). However, culture media or sampling techniques often affect analyses of the *Malassezia* microflora. In a previous study, we used a nonculture method as an alternative to fungal culture to analyze the distribution of cutaneous *Malassezia* species (25). *M. globosa* and *M. restricta* were detected in approximately 90% of AD patients, and *M. furfur* and *M. sympodialis* were detected in approximately 40% of the subjects. In healthy subjects, *M. globosa*, *M. restricta*, and *M. sympodialis* were detected in approximately 40 to 60% of the subjects; *M. furfur* was found in only 4% of the subjects; and no other *Malassezia* species were detected. Therefore, these four species are common inhabitants of the skin of both AD patients and healthy individuals. In addition, while anti-*Malassezia* immunoglobulin E (IgE) antibody was detected in more than 90% of AD patients, no antibody was found in healthy subjects. Based on these results, *M. globosa* and *M. restricta* are thought to play a significant mycological role in AD. *M. globosa* is also part of the major microflora on the skin of healthy individuals. We used the intergenic spacer (IGS) region of the rRNA gene to investigate the genotypes of *M. globosa* colonizing the skin of AD patients and healthy subjects. The fungal rRNA gene consists of 5S, 5.8S, 18S (small subunit), and 26S (large subunit) subunits (Fig. 1). Two other regions are positioned between the subunits: the internal transcribed spacer (ITS) and the IGS. These two regions are further divided into two subregions. The 18S and 26S ribosomal DNAs (rDNAs) and ITS regions have been widely utilized in studies of molecular systematics and to identify microorganisms (17, 23). The IGS regions have higher rates of divergence than other subunits or regions. Some authors (5, 20, 24, 26) have demonstrated that the sequence of the IGS region shows remarkable intraspecies diversity.

In this study, we compared the levels of DNA sequence divergence among the IGS regions of *M. globosa*, which is the key candidate allergen in AD, obtained from the skin of AD patients and from healthy subjects.

## MATERIALS AND METHODS

**Sequencing the IGS region of *M. globosa* stock strains.** Two stock strains, CBS 7996 (type strain of *M. globosa*) and CBS 8745, were purchased from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). They were maintained on modified Leeming and Notman agar (LNA; 20 g of glucose, 50 g of malt extract, 1 g of polypeptone, 20 g of bile salts) (OXOID, Hampshire, United Kingdom), 1% Tween 40, 0.2% glycerol, and 50 μg of chloramphenicol per ml (Sankyo, Tokyo, Japan) at 32°C. Genomic DNA was extracted by the method of Makimura et al. (18). The IGS region containing 5S rDNA was amplified from each strain by using primers 26S-F and P1R, shown in Table 1. The reactions were performed in a final reaction mixture (50 μl) containing 10 pmol of each primer; 200 μM each dATP, dTTP, dGTP, and dCTP; 2.5 mM $MgCl_2$; 0.5 U of Takara Ex *Taq* polymerase (Takara, Shiga, Japan); and 10× reaction buffer (Takara). Amplification reactions were performed in a GeneAmp PCR system

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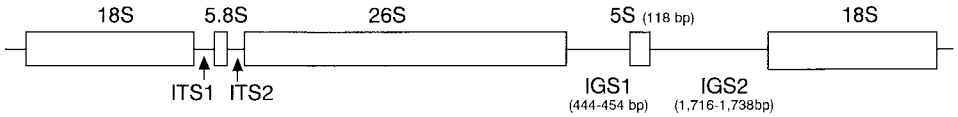


FIG. 1. Schematic representation of the rRNA gene in the type strain (CBS 7966) of *M. globosa*.

9700 (PE Applied Biosystems, Foster, Calif.) using the following cycling parameters: 94°C for 1 min; followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min; followed by a final extension at 72°C for 10 min. The PCR product was sequenced with an ABI PRISM cycle sequencing kit (PE Applied Biosystems) using the primers shown in Table 1 in accordance with the manufacturer's instructions.

**Subjects.** Thirty-six AD outpatients (24 males and 12 females; 20 to 64 years of age; mean age, 33.3 ± 10.5 years) at Tokyo Medical University Hospital and 30 healthy students (10 males and 20 females; 19 to 25 years of age; mean age, 20.9 ± 1.4 years) at Meiji Pharmaceutical University were involved in this study. AD was diagnosed according to the criteria of Hanifin and Rajka (11), and samples were collected from erythematous lesions on the face and neck. Routine skin care, including intermittent applications of mild steroid ointment or petrolatum, was administered before sampling. Written informed consent was obtained from each subject.

**Sequencing the IGS 1 region from patient samples.** *Malassezia* samples were collected by applying a 3- by 3-cm transparent OpSite dressing (Smith and Nephew Medical Ltd., Hull, United Kingdom), and the fungal DNA was extracted from the OpSite dressing as described previously (25). Briefly, the collected dressing was placed in 1 ml of lysing solution (100 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) and incubated at 100°C for 15 min. After deproteinization, DNA was precipitated with 2-propanol and Ethatimate (Nippon Gen, Toyama, Japan). The DNA pellet was resuspended in 30 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]). The DNA extracted (10 μl) from each sample was added to 40 μl of PCR master mixture, which consisted of 5 μl of 10× PCR buffer (Takara), 4 μl of 200 μM deoxynucleoside triphosphates, 10 pmol of each primer, and 0.5 U of Takara Ex *Taq* DNA polymerase (Takara). PCR was performed with an initial denaturation at 94°C for 1 min; followed by 30 cycles of 30 s at 94°C, 1 min at 54°C, and 30 s at 72°C; followed by a final extension at 72°C for 10 min with primers gb-F (GCTTTCGAGTGCATACCACAC) and gb-R (GGAAATAGGATGAGAGA AAC). The PCR products were cloned with a TA cloning kit (Invitrogen Corp., Carlsbad, Calif.), and three positive clones were sequenced with an ABI PRISM cycle sequencing kit (PE Applied Biosystems) and Sequence Rx Enhancer solution A (GIBCO BRL, Life Technologies, Rockville, Md.) in accordance with the manufacturers' instructions.

**Molecular phylogenetic analysis.** The sequences of the IGS 1 region were aligned using Clustal W (28). For neighbor-joining analysis (22), the distances between sequences were calculated with Kimura's two-parameter model (13). A bootstrap analysis was conducted with 100 replicates (8).

**Formation of chimeric molecules.** To confirm whether chimeric molecules formed under the PCR conditions used in this study, mixed genomic DNA from the eight known *Malassezia* species was used for PCR coamplification of the IGS region. Then, the IGS amplified from the mixed genomes was cloned, 30 clones were selected at random, and their sequences were determined.

**Nucleotide sequence accession number.** The nucleotide sequences determined in this study have been deposited with the DNA Data Bank of Japan (DDBJ) under accession no. AB099877, AB099878 (CBS 7966), AB099879, and AB099880 (CBS 8745).

TABLE 1. Primers used to amplify and sequence the IGS region

| Primer | Sequence (5′ to 3′) | Corresponding position in IGS sequence of strain CBS 7966 |
|---|---|---|
| Amplification | | |
| 26S-F | ATCCTTTGCAGACGAC TTGA | 3′ end of 26S rDNA |
| P1R | ACTGGCAGGATCAAC CAGAT | 5′ end of 18S rDNA |
| Sequencing | | |
| Forward | | |
| 26S-F | ATCCTTTGCAGACGAC TTGA | 3′ end of 26S rDNA |
| gb-F2 | CCGATCTGCGAAGTTA AGCA | 483–502 |
| gb-F3 | GATCATAGCCTCATCA TGTGCA | 997–1018 |
| gb-F4 | GAATACGTGACAATTT GTGTGG | 1380–1401 |
| gb-F5 | GTCGCACTGGAGAAA GATGT | 1765–1784 |
| Reverse | | |
| P1R | ACTGGCAGGATCAAC CAGAT | 5′ end of 18S rDNA |
| gb-R2 | ACATCTTTCTCCAGTG CGAC | 1784–1765 |
| gb-R3 | CCACACAAATTGTCAC GTATTC | 1401–1380 |
| gb-R4 | TGCACATGATGAGGC TATGATC | 1018–997 |
| gb-R5 | TGCTTAACTTCGCAGA TCGG | 502–483 |

## RESULTS

**IGS 1 sequences of *M. globosa*. (i) Stock strains.** Complete sequences of the IGS region, including 5S rDNA, were determined for two CBS stock strains of *M. globosa*. Their sequences ranged from 2288 to 2300 bp long. The *M. globosa* IGS 1 regions were 444 to 454 bp long, while the IGS 2 regions were 1,716 to 1,738 bp long. The IGS 1 and 2 regions of this microorganism showed 12.6, and 6.1% dissimilarity, respectively. Therefore, IGS 1 is more suitable than IGS 2 for differentiating closely related strains. *M. globosa* IGS 1 had four short sequence repeats (SSRs) of $(CT)_n$, $(CT)_n$, $(CT)_n$, and $(GT)_n$ at positions 29 to 49, 278 to 291, 380 to 485, and 242 to 267 in the IGS sequence of strain CBS 7996 (type strain of *M. globosa*), respectively. Alignments of IGS 1 of two strains of *M. globosa* are shown in Fig. 2. Because *M. globosa* had three $(CT)_n$s in its IGS 1 region, these are referred to as $(CT1)_n$, $(CT2)_n$, and $(CT3)_n$ in this article.

**(ii) Samples from the subjects.** *M. globosa* DNA was found in 32 of 36 AD patients and 20 of 30 healthy subjects. Under the PCR conditions described above, 420- to 467-bp fragments were amplified and analyzed.

**(iii) SSRs.** The number of sequence repeats of $(GT)_n$ and $(CT)_n$ in the IGS 1 region is shown in Fig. 3. The number of SSRs in the IGS 1 region of samples from healthy subjects was more variable than in samples from AD patients for $(CT)_n$. The number of sequence repeats in the IGS 1 region ranged from 4 to 11 for $(CT1)_n$, 3 to 10 for $(CT2)_n$, and 3 to 11 for $(CT3)_n$, and there were 4 in 50%, 8 in 60%, and 9 to 11 in 80% of the samples from AD patients. For $(GT)_n$, the respective numbers of repeats in 70 to 80% of the SSRs in the IGS 1

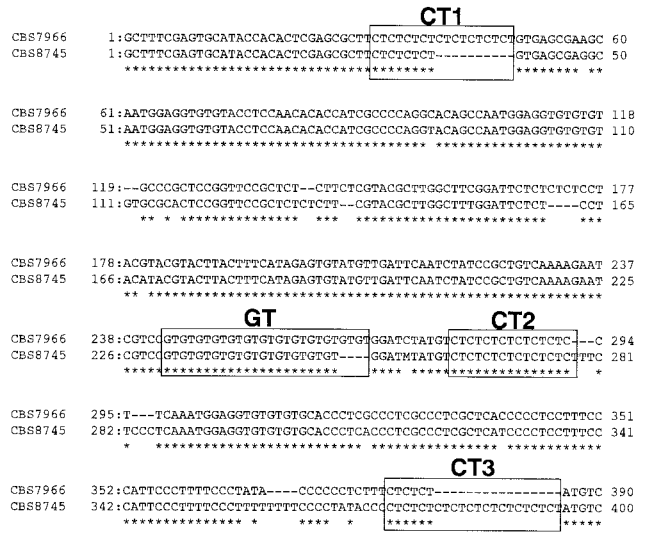


FIG. 2. Alignment of the DNA sequences of the IGS 1 region of *M. globosa* CBS 7966 and CBS 8745.

region derived from AD patients and healthy subjects were 9 to 11 and 15 to 19, respectively.

**(iv) Phylogenetic analysis.** In each patient or healthy subject, three clone sequences were determined, and they were identical without exception. A phylogenetic tree constructed from 52 IGS 1 sequences is shown in Fig. 4. The tree consists of four major groups, which correspond to the sources of the samples (AD patients or healthy subjects). Two groups were from AD patients, and one was from healthy subjects. The remaining group included samples from both AD patients and healthy subjects. The IGS 1 sequences derived from the healthy subjects were more diverse than those from AD patients. The levels of IGS 1 sequence similarity within samples collected from AD patients and from healthy subjects were 94.5% ± 3.5% and 89.9% ± 3.5%, respectively.

**Formation of chimeric molecules.** Thirty clones were chosen at random and sequenced. No clone was identified as a chimeric molecule.

## DISCUSSION

One member of the genus *Malassezia*, *M. globosa*, commonly colonizes the skin of both AD patients and healthy subjects.

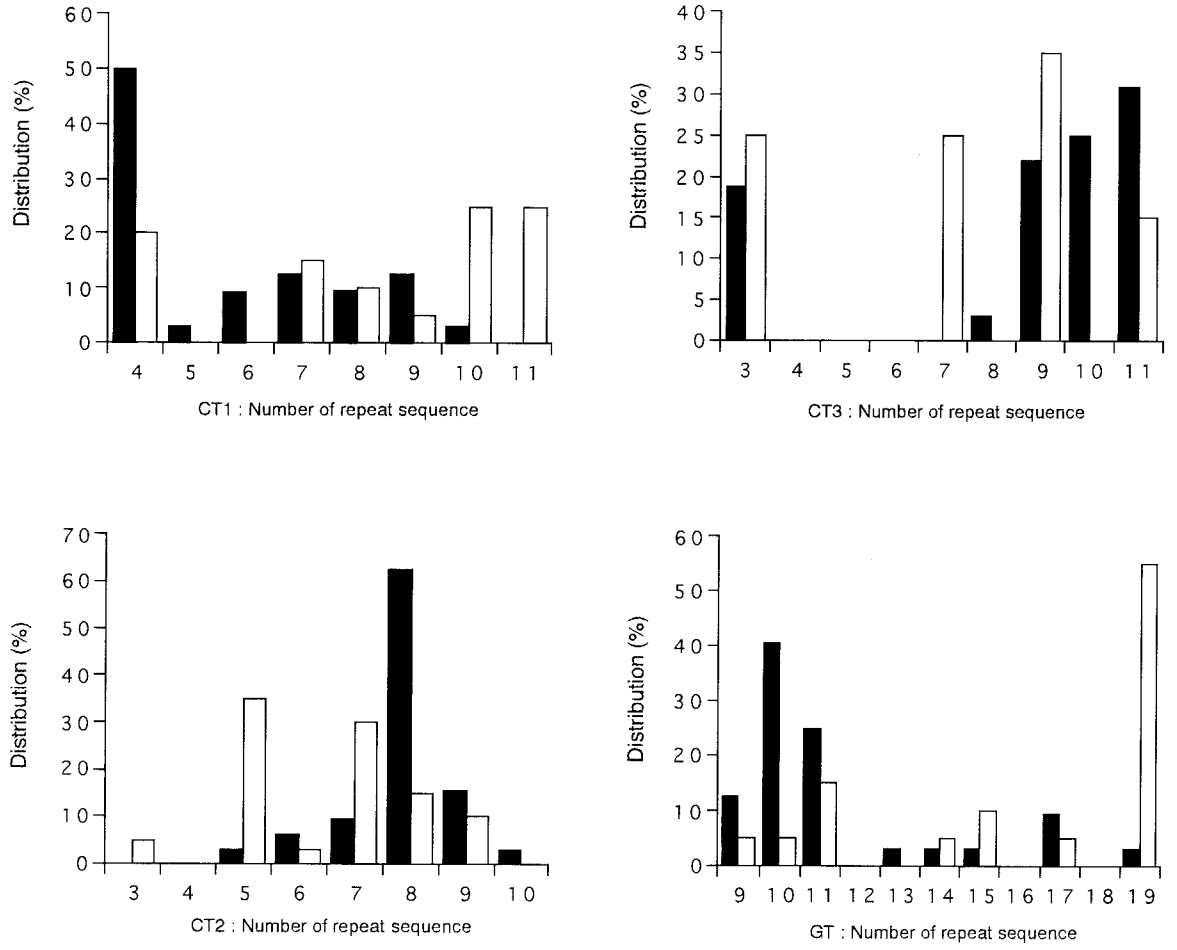


FIG. 3. Distribution of SSRs in the IGS 1 region of the *M. globosa* rRNA gene. Solid bars, *M. globosa* obtained from AD patients; open bars, *M. globosa* obtained from healthy subjects.

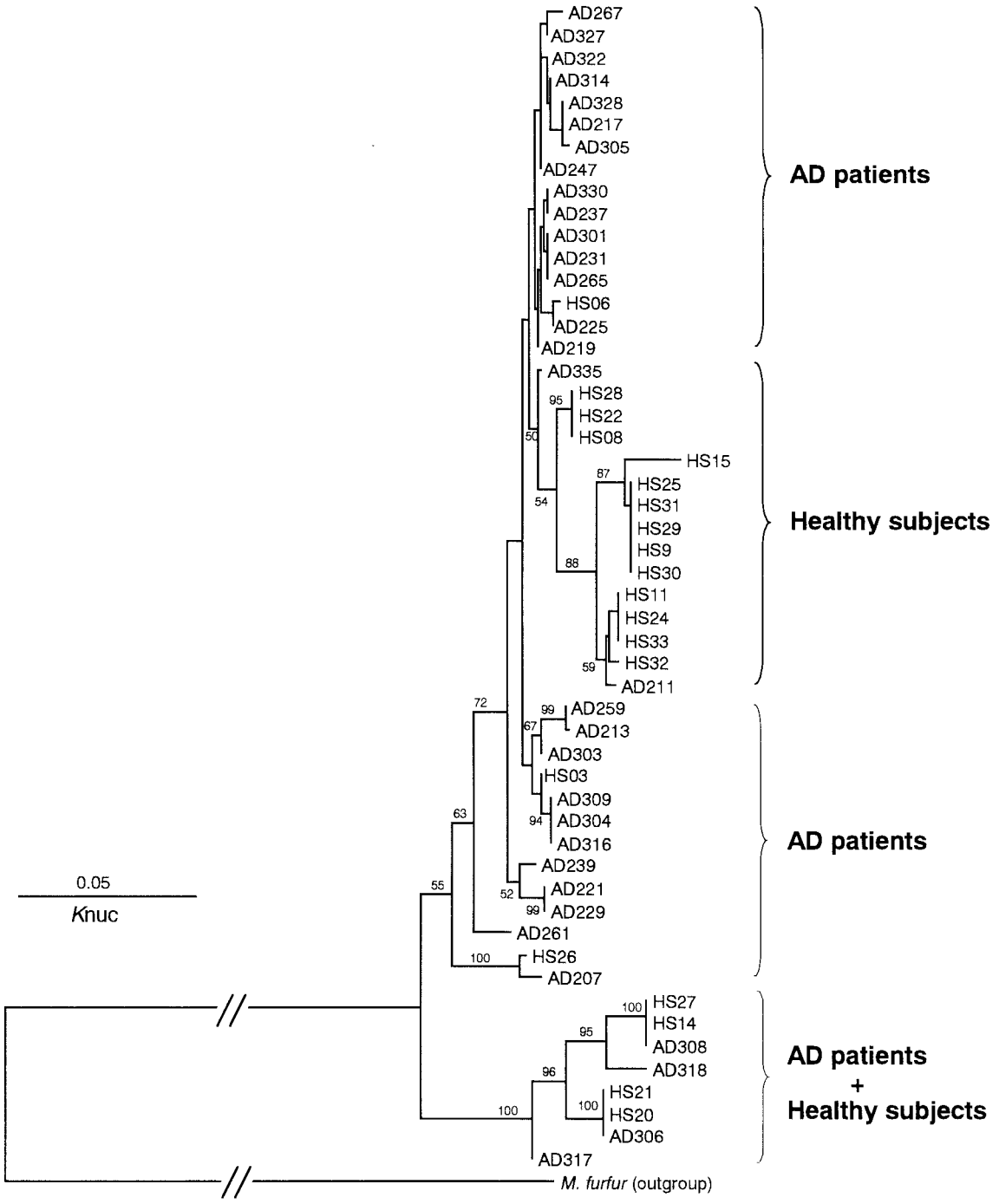


FIG. 4. Phylogenetic tree of *M. globosa* colonizing the skin surface of AD patients and healthy subjects based on DNA sequences of the IGS 1 region. AD, patient with atopic dermatitis; HS, healthy subject. The numbers show the confidence level from 100 replicate bootstrap samplings (frequencies of <50% are not shown). *K*nuc, Kimura's parameter (10).

The major antigen for IgE antibodies in AD patients is a glycoprotein (Malg46b) from *M. globosa* (14, 15). This paper describes differences between the IGS 1 genotypes of *M. globosa* colonizing the skin surface of both populations. The rRNA gene is a marker that reflects the phylogenetic evolution of microorganisms and has been widely used for taxonomy and identification (17, 23). While the taxonomic significance of the 18S and 26S rDNA and ITS regions is known, that of the IGS region is unclear. Previously, we demonstrated that the DNA sequence of the IGS region showed remarkable intraspecies diversity in the pathogenic yeasts *Cryptococcus neoformans* and *Trichosporon asahii* (24, 26). While analyzing the IGS sequences of several yeasts from humans, we found SSRs in the *M. globosa* IGS sequence. Due to their high variability, SSRs

are widely used to study the molecular epidemiology of pathogenic microorganisms (3, 4, 19, 29). As far as we know, *M. globosa* is the only yeast from humans that has these SSRs. The IGS 1 sequences of *M. globosa* isolates obtained from AD patients and healthy individuals were almost identical in the two groups, with the exception of the four SSRs, which could be used to distinguish between microorganisms from AD patients and those from healthy individuals. When a phylogenetic tree was constructed from IGS sequences excluding the SSRs, the *M. globosa* sequences obtained from AD patients and healthy subjects intermingled.

We used a PCR-based nonculture method to analyze the genotypes of *M. globosa* colonizing the skin surface of patients with AD, since *M. globosa* is difficult to isolate by culture methods. When using PCR-based approaches, the generation of chimeric sequences must also be considered, because pseudosequences may generate nonexistent genotypes of this microorganism. Wang and Wang (30) found that chimeric sequences occurred at a rate of 32% after 30 cycles of PCR amplification targeting the consensus sequence of the bacterial 16S rRNA gene by using mixed genomic DNA from eight bacterial species. We used *M. globosa* species-specific oligonucleotide primers targeting the IGS sequence, which is the most variable region in the rRNA gene. The primers used in this study did not amplify the DNA of other *Malassezia* species (data not shown). Although chimera molecules should not be generated theoretically, we also confirmed that no chimera molecules formed under our experimental conditions with genomic DNA from the eight known *Malassezia* species.

The *M. globosa* organisms originating from AD patients were phylogenetically different from the *M. globosa* organisms obtained from healthy subjects with respect to their IGS sequences, although *M. globosa* colonized both AD patients and healthy individuals at high frequency. Why do the genotypes in each population differ? The reason is unclear, but the genotypes might correspond to the physiological characteristics of this microorganism. First, we considered the possible influence of skin surface lipids, a mixture of secretions from the sebaceous glands and epidermal cells, consisting mainly of triglycerides, squalene, wax esters, cholesterol, ceramides, and free fatty acids (6). Although the lipid composition in AD patients is generally no different from that of healthy subjects, a significant decrease in ceramide 1 and differences in the concentrations of the related molecules linoleate and oleate have been reported (12, 31). Such differences in composition may affect the colonization of strains with different lipid requirements. Moreover, therapeutic agents used to treat AD may affect the selective colonization of the microorganism. The base ingredients in these ointments affect the growth of *Malassezia* species (16). While *M. furfur* can utilize white petrolatum, hydrophilic ointment, and heparinoid in hydrophilic ointment, *M. globosa* cannot utilize these ingredients. Therefore, active ingredients such as steroids and tacrolimus might affect the selective colonization of *M. globosa*. The antifungal drug susceptibility of this microorganism should also be considered. Since no patient in this study received antifungal therapy, this possibility can be excluded. Since the analysis of *M. globosa* genotypes in this study is based on a nonculture method, the significance of genotype differences will be elucidated by investigating their phenotypic and physiological characteristics with viable cells.

In conclusion, our IGS sequence analysis revealed differences in the genotypes of *M. globosa* colonizing the skin surface of AD patients and healthy subjects, suggesting that genotype should be taken into consideration when studying the relationship between *M. globosa* and AD.

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