

ORIGINAL ARTICLE

Characterization of the skin fungal microbiota in patients with atopic dermatitis and in healthy subjects

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ABSTRACT

Patients with atopic dermatitis (AD) are highly susceptible to viral, bacterial, and fungal skin infections because their skin is dry and this compromises the barrier function of the skin. Therefore, the skin microbiota of patients with AD is believed to be different from that of healthy individuals. In the present study, the skin fungal microbiota of nine patients with mild, moderate, or severe AD and ten healthy subjects were compared using an rRNA clone library. Fungal D1/D2 large subunit analysis of 3647 clones identified 58 species and seven unknown phylotypes in face scale samples from patients with AD and healthy subjects. *Malassezia* species were predominant, accounting for 63%–86% of the clones identified from each subject. Overall, the non-*Malassezia* yeast microbiota of the patients was more diverse than that of the healthy individuals. In the AD samples 13.0 ± 3.0 species per case were detected, as compared to 8.0 ± 1.9 species per case in the samples taken from healthy individuals. Notably, *Candida albicans*, *Cryptococcus diffluens*, and *Cryptococcus liquefaciens* were detected in the samples from the patients with AD. Of the filamentous fungal microbiota, *Cladosporium* spp. and *Toxicocladosporium irritans* were the predominant species in these patients. Many pathogenic fungi, including *Meyerozyma guilliermondii* (anamorphic name, *Candida guilliermondii*), and *Trichosporon asahii*, and allergenic microorganisms such as *Alternaria alternata* and *Aureobasidium pullulans* were found on the skin of the healthy subjects. When the fungal microbiota of the samples from patients with mild/moderate to severe AD and healthy individuals were clustered together by principal coordinates analysis they were found to be clustered according to health status.

Key words skin, microbiota, atopic dermatitis, rRNA.

Atopic dermatitis, a chronic disease caused by hypersensitivity to dry skin manifesting as dermatitis with pruritus, typically alternates between remission and deterioration. Because the barrier function of the skin is reduced and the amounts of filaggrin and ceramide, which protect the skin, are small, the skin is dry and susceptible to dermatitis, which is an allergic response to various external stimuli, including skin microorganisms (1). As a result, specific

IgE antibodies against skin microorganisms are present in the serum of AD patients.

The human body is colonized by various microorganisms, including bacteria and fungi. Among the human cutaneous fungal microbiota, lipophilic yeasts and *Malassezia*, are predominant. Because *Malassezia* requires lipids for growth, they colonize sebum-rich areas such as the head, face, or neck rather than the limbs or trunk.

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List of Abbreviations: AD, atopic dermatitis; *C. diffluens*, *Cryptococcus diffluens*; *C. liquefaciens*, *Cryptococcus liquefaciens*; *C. parapsilosis*, *Candida parapsilosis*; LSU, large subunit; *M.*, *Malassezia*; PCA, principal coordinates analysis; *R. glutinis*, *Rhodotorula glutinis*; *R. mucilaginosa*, *Rhodotorula mucilaginosa*; *S.*, *Staphylococcus*; *T. asahii*, *Trichosporon asahii*; *T. washingtonensis*, *Tilletiopsis washingtonensis*.

This fungus is believed to be an exacerbating factor in AD because specific IgE antibodies against *Malassezia* are present in the serum of patients with AD, and antifungal therapy can improve the symptoms of AD while decreasing the degree of colonization by *Malassezia* (2, 3). Currently, 14 species are recognized within the genus *Malassezia*, five of which (*M. caprae*, *M. cuniculi*, *M. equina*, *M. nana*, and *M. pachydermatis*) show affinity for nonhuman animals. Recent molecular-based culture-independent studies have revealed the presence of *M. globosa* and *M. restricta* in almost all patients with AD; other species were found in approximately 10%–60% of cases, suggesting that these two *Malassezia* species play a major role in AD (4–6). In fact, the degree of production of IgE antibodies against both species is greater than that against other *Malassezia* species (7). Comprehensive analyses of skin bacteria have produced dramatic results over the last few years, but few of these have included comprehensive analyses of the fungal microbiota of skin. Fungal microbiota, other than *Malassezia*, are detectable using comprehensive analyses, such as pyrosequencing or rRNA clone libraries.

In the present study, we investigated the skin microbiota of AD patients and healthy individuals using a rRNA gene clone library method, and elucidated the relationship between pathological conditions and skin microbiota in AD patients.

MATERIALS AND METHODS

Subjects and sample collection

Nine outpatients with AD (three each with mild, moderate, and severe disease) at Tokyo Medical University Hospital were involved in this study (Supplemental file S1). The patients had been treated by intermittent topical application of medium to strong steroid ointments in a petrolatum base. None of the subjects had received systemic or topical antibiotics, or antifungal agents. Adult healthy Japanese males and females (five of each) in their 20s, 30s, 40s, and 50s were analyzed. Daily skin care and the use of makeup were not prohibited prior to sampling. This study was approved by our institutional review board. Scale samples were obtained from the face (lesional sites in the patients) by stripping with OpSite, a transparent dressing (Smith & Nephew, Hull, UK) according to the method of Sugita *et al.* (4). A 7 cm × 9 cm piece of OpSite was firmly applied to the lesional skin of the patients and non-lesional skin of healthy individuals, and then peeled off. Each site was sampled three times.

DNA extraction

Fungal DNA was extracted from the tape immediately after scale collection. The removed OpSite dressing was

placed in a 1.5 mL Eppendorf tube containing 1000 μ L of lysing solution (100 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0], and 0.5% sodium dodecyl sulfate). The procedure was repeated three times with freezing–thawing using liquid nitrogen, then the tube and contents were incubated for 15 min at 100°C. The OpSite dressing was then removed from the tube, and the suspension extracted with phenol–chloroform–isoamyl alcohol (25:24:1, [vol/vol/vol]). Next, the samples were extracted with chloroform–isoamyl alcohol (24:1, [vol/vol]) and the DNA precipitated with ethanol using Ethachinmate (Nippon Gene, Toyama, Japan) as a precipitation activator. The DNA pellet was resuspended in 50 μ L of TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA [pH 8.0]). An unused OpSite dressing was used as a negative control.

Polymerase chain reaction amplification of D1/D2 LSU rDNA, cloning, and sequencing of the product

D1/D2 LSU rDNA was amplified by PCR with fungal universal primers (NL1 [5'-GCATATCAATAAGCGGAGGAA AAG-3'] and NL4 [5'-GGTCCGTGTTTCAAGACGG-3']) (8). Extracted DNA (3 μ L) from each sample was added to 27 μ L of the PCR master mix, which consisted of 3 μ L of 10 × PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, and 15 mM MgCl₂; Takara, Shiga, Japan), 2.4 μ L of 200 μ M deoxynucleoside triphosphates (an equimolar mixture of dATP, dCTP, dGTP, and dTTP; Takara), 20 pmol of each primer, and 2.5 U of Ex *Taq* DNA polymerase (Takara). PCR was performed in a thermocycler (model 9700; Applied Biosystems, Foster City, CA, USA) with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. The products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. The positive clones were sequenced using a 3730x/DNA analyzer (Applied Biosystems) and the primers M13RV (5'-GGAAACAGCTATGACCATG-3') and M13FW (5'-GTAAAACGACGGCCAGT-3').

DNA sequence analysis

A species was defined as organisms sharing >99% identity in their D1/D2 LSU rDNA sequences (8) using a BLAST search (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Principal coordinates analysis

To compare the phylogenetic distribution of the patients with AD and healthy individuals, the UniFrac method (9, 10) was used. A tree file was constructed based on all of the sequences using ClustalW (11); the UniFrac distance metric was determined using an online

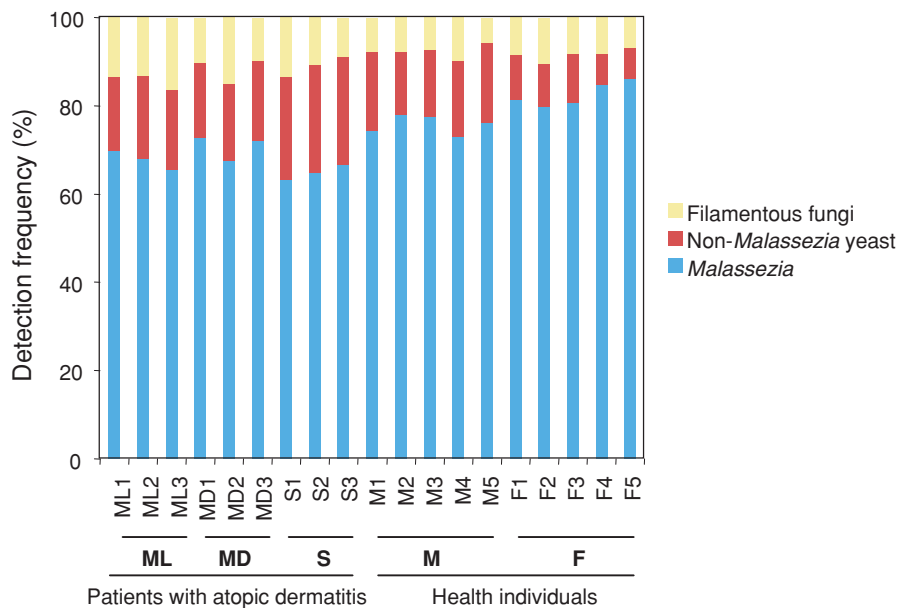


Fig. 1. Distribution of taxonomic groups (*Malassezia*, non-*Malassezia* yeast species, and filamentous fungi). *Malassezia* was the primary microorganism of the skin microbiota in both patients and healthy individuals. F, female; M, male; ML, mild; MD, moderate; S, severe.

resource (<http://bmf2.colorado.edu/unifrac/>). These distances were used as input for PCA.

RESULTS

Features of the human skin clone libraries

In total, 3647 clones from the face samples of the patients with AD and healthy subjects were sequenced. Of them, 2.1% of the clones were putative chimeric D1/D2 LSU rDNA sequences and therefore removed from analysis. Thus, 1797 and 1850 clones from nine patients with AD and ten healthy individuals, respectively, were analyzed. For each patient and healthy subject, 179–228 clones (average, 199.7 ± 16.1) and 144–229 clones (average, 185.0 ± 31.6), respectively, were analyzed (Supplemental file S1). A BLAST search was conducted using GenBank to identify the sequences of the clones corresponding to D1/D2 LSU rDNA (DNA sequence similarity > 99%). An analysis of the DNA sequences was performed using three taxonomic categories: *Malassezia* microbiota, yeast microbiota excluding *Malassezia*, and filamentous fungal microbiota. Of 1797 clones from patients with AD, 1215 (68.7%), 360 (19.9%), and 222 (12.3%) belonged to the genus *Malassezia*, non-*Malassezia* yeasts, and filamentous fungi, respectively. Of the 1850 clones from the healthy subjects, the proportion of clones belonging to the genus *Malassezia* was greater than that for the patients with AD (1455 clones, 79.2%), while the proportion of clones be-

longing to the non-*Malassezia* yeasts (247 clones, 12.8%) and filamentous fungi (148 clones, 8.1%) was smaller.

Malassezia microbiota

Malassezia was the major microorganism of the skin microbiota in both patients and healthy individuals. Their clones accounted for 63.2–72.7% (mean, 68.7 ± 3.2) of the 1797 clones analyzed, three to six species (mean, 4.6 ± 0.9) being detected in the patients (Fig. 1, supplemental file S1). No remarkable correlations between the number of clones or detected species and disease severity were found. The groups (mild, moderate, and severe) accounted for 67.8 ± 2.2 , 70.7 ± 2.8 , and $64.9 \pm 1.8\%$ of the clones, respectively, and 4.3 ± 0.6 , 4.0 ± 1.0 , and 5.3 ± 0.6 species per case, respectively. *M. globosa* and *M. restricta* were the predominant species, regardless of disease severity, with a detection rate of 57.5%–70.4% in all clones analyzed; however, the ratio of *M. globosa* to *M. restricta* was different in each severity group. In patients with mild and moderate disease, the ratio of these species was nearly identical (*M. restricta*/*M. globosa*: 3.1–3.4 for mild and 2.1–4.1 for moderate vs. 1.1–1.4 for severe). In addition to *M. globosa* and *M. restricta*, *M. dermatitis*, *M. obtusa*, *M. slooffiae*, and *M. sympodialis* were detected in the patients' samples (Fig. 2).

M. globosa and *M. restricta* were the predominant species among both healthy men and women; the former accounted for $13.6 \pm 8.0\%$, while the latter accounted for

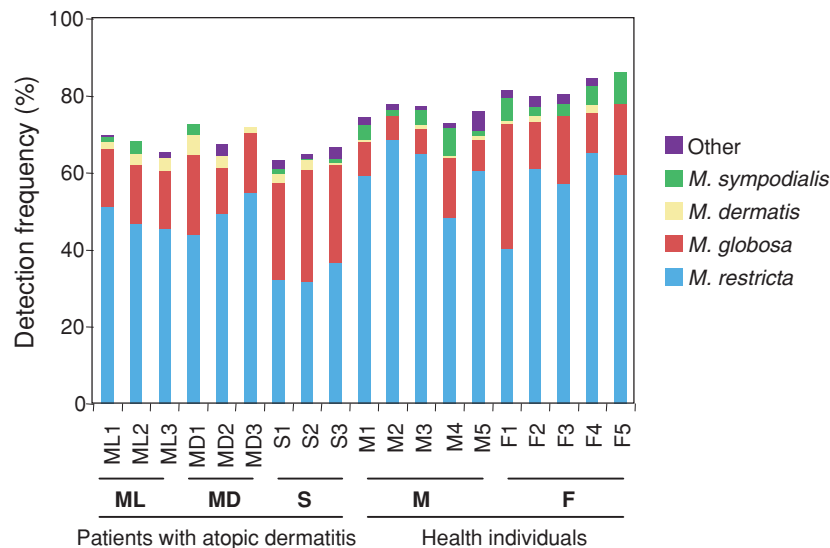


Fig. 2. Distribution of *Malassezia* species.

M. globosa and *M. restricta* were the predominant species, regardless of disease severity, with a detection rate of 63.2%–72.7% in all analyzed clones. F, female; M, male; ML, mild; MD, moderate; S, severe.

58.5 ± 8.4%. No remarkable difference was found between men and women.

Fourteen clones constituted a cluster within the genus *Malassezia* but did not show >99% DNA sequence similarity to 14 recognized species from samples M1, M2, and M5. They are described as *Malassezia* phylotypes 1, 2, and 3 (Supplemental file S2).

Non-*Malassezia* yeast microbiota

Fifteen genera (*Candida*, *Clavispora*, *Cryptococcus*, *Debaryomyces*, *Meyerozyma*, *Mrakia*, *Rhodospiridium*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, *Symphodiomyces*, *Trichosporon*, *Tilletiopsis*, *Yarrowia*, and *Wickerhamomyces*) were detected in 607 clones from scale samples obtained from patients with AD and healthy individuals (Fig. 3, Supplemental file S2). Of these, 32 and 22 yeast species were identified from the patients and healthy individuals, respectively. The yeast microbiota of the patients was more diverse than that of the healthy individuals; 13.0 ± 3.0 and 8.0 ± 1.9 species per case were detected in the patients and healthy individuals, respectively (Supplemental file S1). Also, the number of detected species decreased with increasing severity of AD (i.e. 16.3 ± 1.2, 13.0 ± 0, and 9.7 ± 1.5 for mild, moderate, and severe, respectively). The yeast species that accounted for >1% of the total number of clones from the patient samples were *Candida albicans*, *C. parapsilosis*, *Cryptococcus diffluentis*, *C. liquefaciens*, *Trichosporon asahii*, *Meyerozyma guilliermondii* (anamorphic name, *Candida guilliermondii*), *Rhodotorula glutinis*, and *R. mucilaginosa* (Supplemental file S2). *C. albicans*, *C. diffluentis*, and *C. liquefaciens* were unique to the patients with AD, while

C. parapsilosis, *Meyerozyma guilliermondii*, *R. glutinis*, *R. mucilaginosa* and *T. asahii* were detected in both the patients and healthy individuals. Among the healthy individuals, one unique species (*T. washingtonensis*) was identified in 1.2% of the samples.

Filamentous fungal microbiota

Sixteen genera (*Alternaria*, *Apioplagiostoma*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Davidiella*, *Exophiala*, *Fusarium*, *Gibberella*, *Gibellulopsis*, *Penicillium*, *Persiciopsis*, *Phialophora*, *Toxicocladosporium*, *Trametes*, and *Wallemia*) were detected in a total of 370 clones from scale samples obtained from patients and healthy individuals (Fig. 4, supplemental file S2). In total, 5.2 ± 0.8 and 4.3 ± 0.8 species per case were detected in the patients and healthy individuals, respectively (Supplemental file S2). Filamentous fungi present in >1% of the total number of clones from the patient samples included *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium* spp, and *Toxicocladosporium irritans*. The latter two species were unique to the patients with AD, while the former two were detected in both sets of samples.

Change in microbiota with disease severity

Patients with mild or moderate symptoms constituted a single cluster, whereas patients with severe disease were in a separate cluster. Similarly, the healthy individuals were clustered independently (Fig. 5). Thus, the fungal microbiota were clustered according to health status.

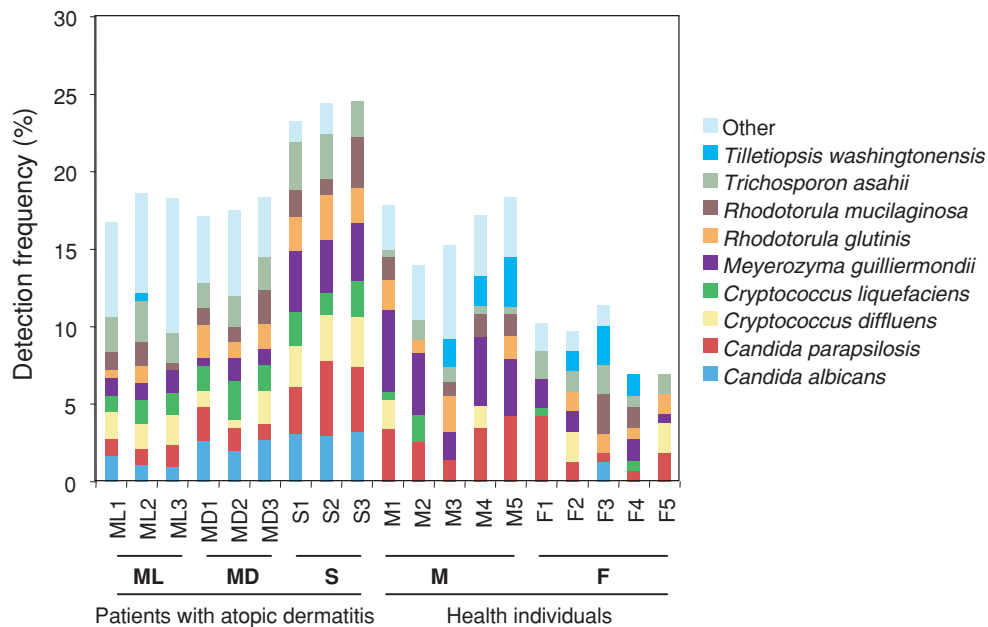


Fig. 3. Distribution of non-*Malassezia* yeast species. The yeast microbiota of the patients was more diverse than that of healthy individuals; 13.0 ± 3.0 and 8.0 ± 1.9 species per case were detected in the patients and healthy individuals, respectively. F, female; M, male; ML, mild; MD, moderate; S, severe.

DISCUSSION

The human skin microbiota has been extensively analyzed. The complexity of the bacterial community is dependent on the specific characteristics of the skin site; *Propionibacterium* and *Staphylococcus* species predominate at sebaceous sites, *Corynebacterium*, *Staphylococcus*,

and beta-Proteobacteria predominate at moist sites, and β -Proteobacteria and Flavobacteriales predominate at dry sites (12). Comprehensive studies on fungal microbiota are remarkably few in number compared to studies on bacterial microbiota. A lipophilic yeast, *Malassezia* constitutes most of the fungal microbiota of human skin (13, 14). Although *Malassezia* species colonize healthy skin, they may

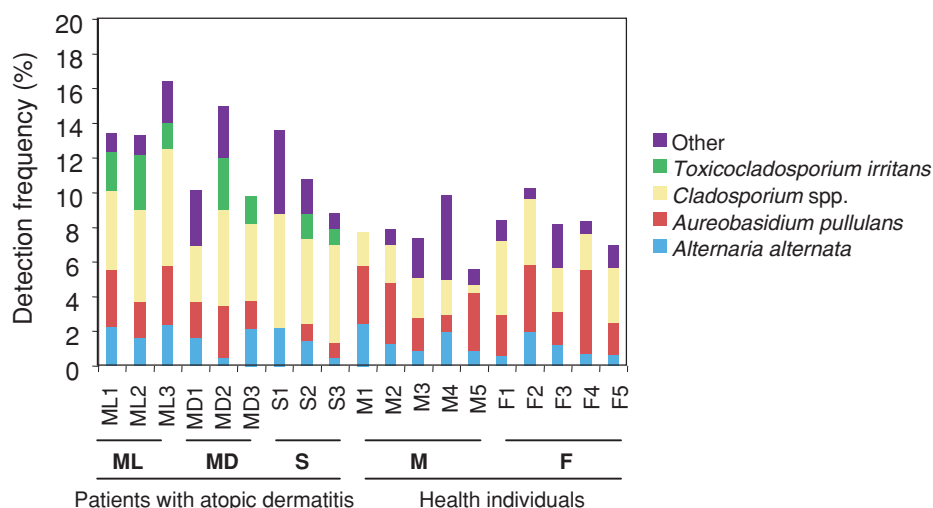


Fig. 4. Distribution of filamentous fungi. 5.2 ± 0.8 and 4.3 ± 0.8 species per case were detected in patients and healthy individuals, respectively. F, female; M, male; ML, mild; MD, moderate; S, severe.

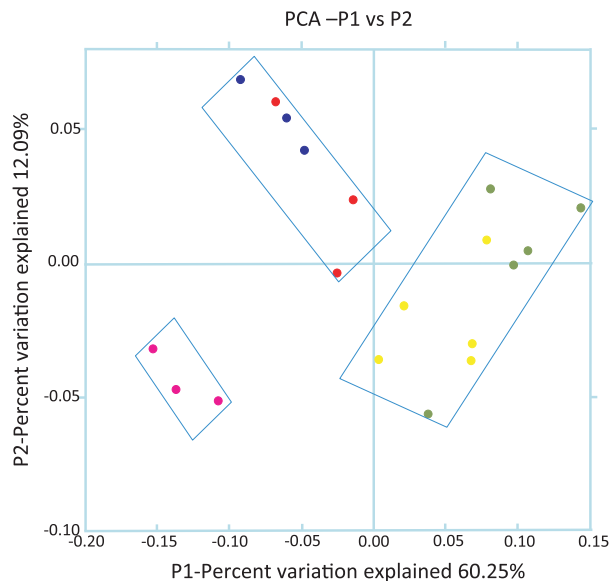


Fig. 5. PCA score plot of the sequence profiles for the predominant skin fungi. Patients with mild or moderate symptoms, patients with severe disease, and healthy individuals were clustered independently. Blue, patients with mild symptoms; green, female healthy individuals; purple, patients with severe symptoms; red, patients with moderate symptoms; yellow, male healthy individuals.

cause or exacerbate seborrheic dermatitis, pityriasis versicolor, folliculitis, or AD (15–17). Regardless of the clinical severity of the symptoms, *M. globosa* and *M. restricta* are the major representatives of the genus *Malassezia*; however, the ratio of the two microorganisms is quantitatively different. *M. globosa* is the predominant species in the lesions of patients with pityriasis versicolor, whereas *M. restricta* is predominant in patients with seborrheic dermatitis and AD (17, 18).

In healthy individuals, *Malassezia* species account for 53%–80% of all fungi at different body locations (forehead, forearm, behind the ear, inner elbow, foreleg, and axial region), the highest proportions being found behind the ears (14). Our rRNA clone library analysis also revealed that these microorganisms account for 72.9%–86.1% in healthy individuals and 63.2%–72.7% in patients with AD. The ratio of the two major components was different in the severe AD group (19). *M. restricta* was predominant over *M. globosa* in patients with mild or moderate disease, whereas the ratio was close to 1 in patients with severe disease. These results suggest that, among patients with AD, the fungal microbiota of the skin is different in patients with severe disease. When the species detected in this study were classified into three taxonomic categories (*Malassezia*, non-*Malassezia* yeast species, and filamentous fungi), the yeasts other than *Malassezia* were more diverse in the patients with AD compared to the healthy

individuals; 13.0 ± 3.0 species were detected in the AD samples while 8.0 ± 1.9 species were detected in the healthy individuals. *Candida albicans*, *Wickerhamomyces anomalus* (formerly, *Pichia anomala*), *T. asahii*, *Cryptococcus diffluentis*, and *C. liquefaciens* were detected more often in patients with AD than in healthy individuals. The former three can cause opportunistic infections in immunocompromised hosts, while the latter two were recently identified as an exacerbating factor in AD (20, 21). Of the 122 AD serum samples tested, 43 (35.2%) and 50 (41.0%) were positive for IgE antibodies against *C. diffluentis* and *C. liquefaciens*, respectively. Fungi that are related to allergies and commonly found in dwelling environments were detected in the skin samples of both the patients and healthy subjects, including *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, *Aureobasidium*, *Wallemia*, and *Rhodotorula*. These microorganisms are distributed in indoor air and house dust (22–25).

Principal coordinates analysis revealed that the fungal microbiota were clustered according to health status. Differences in microbiota are thought to be due to differences between patients with AD and healthy individuals in the physiological condition of the skin. First, skin pH and transepidermal water content may affect fungal microbiota, and these factors are significantly different in patients with AD and healthy individuals (26). Although *Staphylococcus aureus* is not part of the typical microbiota of the skin in healthy individuals, this microorganism is thought to be a trigger for AD. The degree of colonization by *S. aureus* increases with the severity of AD. In contrast, the degree of colonization by *S. epidermidis* decreases gradually with increasing severity of AD. Healthy skin is weakly acidic, whereas the near neutral pH of the skin in patients with AD facilitates the invasion of exogenous microorganisms, including *S. aureus* (27, 28). Second, the degree of expression of antimicrobial peptides may also affect fungal microbiota. Antimicrobial peptides, including defensins and cathelicidins, are produced in healthy skin to inhibit invasion by external pathogens (29). However, they are deficient in the skin of patients with AD, explaining in part why the fungal microbiota of these patients is different from that of healthy individuals. Third, the lipid composition of the skin may also affect the fungal microbiota. Human sebum consists of squalene, cholesterol esters, wax esters, triglycerides, free fatty acids, cholesterol, ceramides, cholesterol sulfate, and phospholipids. Of these, the proportion of ceramide 1, which is a carrier of linoleate and responsible for the water-barrier function of skin, is significantly smaller in patients with AD (30). These skin environmental conditions also differ according to body site, sex, and age. The skin bacterial microbiota are highly dependent on skin environmental conditions;

however, in the case of fungal microbiota, *Malassezia* is predominant at all body sites (13, 14).

In conclusion, we have revealed a difference in skin fungal microbiota between patients with AD and healthy individuals. This difference was due to the physiological and environmental conditions of the skin in each subject. Although few samples were examined, the skin fungal microbiota were clustered according to health condition rather than the subject. To our knowledge, this is the first comprehensive investigation of fungal microbiota in patients with AD.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

S1 Number of fungal clones analyzed and species detected in skin samples obtained from patients with atopic dermatitis and healthy individuals.

S2 Distribution of the fungal species detected in the samples.

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