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Analysis of the skin mycobiome in adult patients with atopic dermatitis

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ABSTRACT

With the recent availability of culture-independent sequencing methods, studies have been conducted to analyze skin microorganisms present in patients with atopic dermatitis (AD). However, the database on the skin fungal communities, “mycobiome,” has been relatively restrictive compared with the bacterial world. We aimed to comparatively analyze the overall skin mycobiome between patients with AD and healthy individuals in the Korean population. We analyzed skin swab samples obtained from the antecubital fossae of 8 patients with AD and 8 healthy controls. Using sequencing method followed by direct DNA extraction and molecular PCR, taxonomic compositions of fungi at stepwise level ranks were analyzed. The phylogenetic marker used was internal transcribed spacer 2 regions of DNA. We observed the tendency of higher intra- and inter-personal taxonomic diversity at genus and species levels in AD samples. Non-*Malassezia* fungal diversity was also noticeable in the patient group compared with healthy controls. *Malassezia globosa* and *Malassezia restricta* were prevalent in all samples across both study groups, and some *Malassezia* species, including *Malassezia slooffiae* and *dermatis* characterized AD. Our data might provide a new insight into the mycobiome of adult AD, which contributes to building a systemic mycobiome database in AD.

Key words: atopic dermatitis, fungi, *Malassezia*, mycobiome

INTRODUCTION

Atopic dermatitis (AD) is a representative multifactorial skin disease, which is affected by both genetically determined host immune status and environmental factors following skin barrier destruction. AD is characterized by immune dysregulation predisposing to IgE production, and

various environmental factors, including microorganisms, might act as allergens.^(1, 2) Patients with AD are vulnerable to microbial infection due to skin barrier fragility and often undergo concomitant infections by viruses, bacteria, and fungi. Antiviral, antimicrobial, or sometimes antifungal treatments are included in treatment options if needed. For this reason, studies have actively been conducted to analyze the microorganism composition on the AD skin.

Because of the development of culture-independent sequencing technology in the past decade, the insight into the skin microbial communities has much expanded. However, the understanding of the skin microbiome has been limited within the bacterial world. The majority of fungi on the skin are part of the normal flora, whereas some fungal species are pathogens of occasional infections and a range of skin conditions.⁽³⁻⁵⁾ Altered skin fungal communities have been associated with compromised cutaneous immunities and changes in skin physiology.⁽⁶⁾ Fungi sometimes modulate the physiology and virulence of other microorganisms.^(5, 7) Although the skin fungal communities have an essential role in understanding the disease pathogenesis and development of treatments, sufficient database has not been constructed on the skin “mycobiome” profile in AD yet. Moreover, the lack of skin mycobiome researches with non-Western populations has obstructed the determination of a skin “pan-mycobiome.” a worldwide collection of mycobiome database. The mycobiome might vary across different racial groups, similar to previous bacterial studies.^(8, 9) Advanced investigations to elucidate the characteristics and roles of skin fungal communities in Korean patients with AD are currently needed.

In this study, we aimed to comparatively analyze the overall skin mycobiome between Korean patients with AD and healthy controls. We assessed the relative abundance of each constituent and community diversity at various taxonomic ranks. Furthermore, we also focused on the *Malassezia* species composition to elucidate the role of *Malassezia* in AD pathophysiology.

METHODS

Subjects and sample collection

We enrolled 20 participants, 10 patients with AD and 10 healthy controls. All patients with AD were diagnosed with AD by a dermatologist before the registration in Konkuk University Hospital, Seoul, Korea. They were all confirmed to have a typical AD skin lesion on the antecubital fossae through careful physical examination. All subjects in the patient group had chronic AD on the antecubital fossa that had persisted for more than 10 years (14–20 years). We also recruited a negative control group, consisting of subjects without AD and therefore constituted a “normal” state. History taking and physical examination confirmed that they did not have any evidence of AD. Patients who had any active skin diseases, exclusive of AD, were excluded. We also excluded patients with a history of receiving concomitant medical or topical treatments that could affect the study results, particularly the following: oral antifungal agents, anti-inflammatory drugs, immunomodulators, including steroids (used within four weeks of study enrollment), and topical antifungal agents, steroids, or calcineurin inhibitors (used within two weeks of study enrollment). The presence of any systemic immune-associated disease was also an exclusion criterion. All the participants provided the International Review Board (IRB)-approved written informed consent for enrollment in this study.

Skin swab samples from all subjects were obtained based on a protocol approved by the IRB of Konkuk University Hospital (KUH 1120070, Analysis of the mycobiome in patients with atopic dermatitis). No bathing or moisturizing was permitted for 24 h before sample collection. Cotton swabs were premoistened with sterilized saline before sample collection. We swabbed the lesional sites of the antecubital fossae with repeated rubbing for at least 50 times to improve the yield. The cotton swab heads used for skin scale sample collection were separated and stored with 500-μL lysing solution (100 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) in conical tubes at –80 °C. Subsequently, the DNAs of the obtained samples were isolated and processed similarly to those described previously.^(10–12)

Polymerase chain reaction (PCR) amplification and sequencing

Universal PCR primers for fungi were used to amplify the internal transcribed spacer 2 (ITS2) regions of the extracted DNAs: UTS3-Mi (forward primer [5'-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-GCATCGATGAAGAACGCAGC-3'; underlining sequence indicates the target region primer]) and ITS4-Mi (reverse primer [5'-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-TCCTCCGCTTATTGATATGC-3']). The PCRs were performed using 2.5 µL of 10× buffer, 2.5 µL of each deoxynucleoside triphosphate, 10 pmol of each primer, 2.5 µL Taq DNA polymerase (Dr.Max DNA polymerase, 500 U), and 5 µL of extracted DNA in the final 25-µL volume. The PCR amplifications were performed under the following conditions: initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final elongation at 72 °C for 5 min. The PCR products were confirmed by 2% agarose gel electrophoresis and visualized under a Gel Doc system (BoiRad, Hercules, CA, USA). Subsequently, secondary amplification to attach the Illumina NexTera barcode was performed with the primary PCR products using i5 (forward primer [5'-AATGATACGGCGACCACCGAGATCTACAC-CTCTCTAT-TCGTCGGCAGCGTC-3'; X indicated the barcode region] and i7 (reverse primer [CAAGCAGAAGACGGCATACGAGAT-TCGCCTT-AGTCTCGTGGGCTCGG]). The condition of the secondary amplification is identical to the former, except for the primary PCR products as template DNAs and eight amplification cycle sets. The amplified products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) after being confirmed under 2% agarose gel electrophoresis and visualized. Equal concentrations of purified products were pooled together, and short fragments (non-target products) were removed using an Ampure beads kit (Agencourt Bioscience, MA, USA). The quality and product size were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Mixed amplicons were prepared using emulsion PCR and then deposited on Picotiter plates. Sequencing was performed at Chunlab, Inc. (Seoul, Korea), with Illumina MiSeq Sequencing system (Illumina, USA) based on the manufacturer's instructions.

Taxonomic identification and diversity analyses

Quality check (QC) and filtering of low-quality (mean quality value, $Q < 25$) reads by using trimmomatic 0.321 preceded the process of raw reads. After QC pass, paired-end sequence data were merged together by PandaSeq2. Primers were trimmed with Chunlab's in-house program at a similarity cut-off of 0.8. Subsequently, the noise of sequences was removed using Mothur's pre-clustering program. It merges, sequences, and extracts unique sequences, allowing up to 2 bp difference between sequences.

The Ezfungi database was used for taxonomic assignment by BLAST 2.2.224. The database was derived from the public database, covering sequences of both the ITS1 and ITS2 regions. The sequences of the database were grouped for validation and taxonomic identification. The names of the phylotypes were assigned on the basis of the rule of the widely used EzTaxon database.⁽¹³⁾ CD-Hit7 and UCLUST8 clustered sequence data, and alpha diversity analysis were performed. Beta diversity was assessed using the Fast Unifrac algorithm developed by Rob Knight's group.⁽¹⁴⁾

RESULTS

To investigate whether skin fungal communities of patients with AD differed from those of healthy controls, the collected samples from the antecubital fossa of each subject were sequenced at the ITS2 region. Among the specimens, 2 of 10 samples from AD patients and healthy controls were excluded, respectively, owing to negative PCR results. We analyzed 8 AD samples and 8 healthy control samples. The basic characteristics of each subject group are shown in Table S1. The female-to-male ratio was 1:1. The age distributions were not statistically different between the patients with AD and the healthy controls ($P = 0.3883$). The detailed clinical information, including the severity of AD, and the number of generated sequence reads of each sample are described in Table S2.

Taxonomic composition analysis

We investigated the taxonomic compositions of all samples at various taxonomic ranks. First, the relative abundance of fungal phyla was presented in Figure 1. Overall, *Basidiomycota* represented a major proportion at 73.39% (range, 28.82%–98.54%) and 89.13% (range, 97.80%–99.72%) in AD samples and healthy controls, respectively. *Ascomycota* followed *Basidiomycota* with proportion of 22.84% (range, 0%–66.18%) and 7.57% (range, 0%–15.81%) in AD samples and healthy controls, respectively. Two AD samples (AD7 and AD8) interestingly showed unusual patterns with *Ascomycota* more predominantly than *Basidiomycota*. When analyzed at class, order, and family level, *Exobasidiomycetes*, *Malasseziales*, and *Malasseziaceae* represented the highest abundance. The genus *Malassezia* was the most common among the 15 genera identified, accounting for 53.84% (range, 5.02–97.96%) in AD samples and 80.44% (range, 65.97%–99.40%) in healthy controls. However, various fungal genera, including *Alternaria*, *Aureobasidium*, *Aspergillus*, *Cladosporium*, *Trichosporon*, and *Irpex*, replaced the genus *Malassezia* in two AD samples (AD7 and AD8). Other yeast genera, including *Candida* (10.75% in AD4), *Cryptococcus* (5.75% in HC8), and *Filobasidiella*, the sexual state of *Cryptococcus* (7.18% in AD1) were also identified. *Rhodotorula* was also identified in HC6 sample at 7.26% (Fig. 2A). Figure 2B shows the taxonomic compositions at the species level.

Diversity assessment of fungal communities

We analyzed and compared the diversity of identified sequence reads in each sample rank by rank using Shannon diversity index (SDI), calculated by the equation below.

$$H' = -\sum p_i \ln(p_i), \text{ the SDI is expressed as } e^{H'}.$$

$p_i = N_i/N$; the relative abundance of each group of organisms

The results are shown in Figure 3A–C. The SDIs of AD (1.74 ± 0.45) and control group (1.47 ± 0.36) were nearly identical at the phylum level. At the genus and species levels, the differences in SDIs became apparent between the two study groups. As shown as circular and square dots in each

graph, some AD samples showed much higher genus and species-level diversity than healthy controls. However, we could not comparatively analyze the mean SDIs between the two study groups because of prominent interpersonal variation of the SDIs in the AD group at both genus and species levels.

Subsequently, we conducted the Fast unifracs analysis to investigate the similarity and difference of community structures among samples based on a phylogenetic tree. Blue and red dots indicate AD samples (AD1–AD8) and healthy controls (HC1–HC8), respectively. The three major axes (PC1, PC2, and PC3) were presented with their respective percentage of explained variance. The results showed that AD samples tended to be more differentiated in fungal communities compared with healthy controls (Fig. S1).

Colonization by the fungus *Malassezia*

This study identified nine *Malassezia* species. The detection frequencies of each species in the AD and control groups are shown in Figure 4A. In both study groups, *M. globosa* and *M. restricta* were detected from all samples. *M. sympodialis* was also detected from all the AD samples. It was the second most frequent species in the control group (75% of healthy individuals). *M. japonica* was an AD-dominant species. Three species, *M. slooffiae*, *M. obtusa*, and *M. yamatoensis*, were detected exclusively in the AD samples. *M. furfur* was detected more frequently in the healthy controls than in the AD group (Fig. 4A).

In the taxonomic composition analyses in each sample, *M. globosa* accounted for 42.4%–86.7% and 48.0%–94.2%, and *M. restricta* accounted for 8.1%–48.5% and 0.13–41.9% of the AD samples and healthy controls, respectively. Among the AD samples, two had *M. dermatis* as the third most abundant species (16.74% in AD1 and 0.85% in AD3). By contrast, *M. furfur* accounted for 11.55% in HC8, being the second most abundant *Malassezia* species over *M. restricta*. *M. sympodialis* was detected in both AD and healthy control samples with a small proportion of <5%. *M. japonica* and *M. obtusa* were detected in AD samples, particularly AD3, but not in any healthy control (Fig. 4B).

Subsequently, we investigated the diversity of *Malassezia* species by calculating the SDIs of the *Malassezia* components in each sample. The SDIs of the AD samples and healthy controls are marked as a circle and rectangle, respectively, in Figure 4C. The mean SDIs of the two study groups were not significantly different. The intrapersonal variation of SDIs in patients with AD was not as prominent as in the analysis with all species, including non-*Malassezia* species.

DISCUSSION

The number of studies profiling microorganisms of the skin is increasing with recent availability of culture-independent sequencing methods. They have improved our insight into bacterial communities, “microbiome,” and its impact in human health and disease. AD is the representative disease, having been an objective of the microbiome studies. AD is known to be the result of dry hypersensitive skin condition, which hinders the protective barrier function of the horny layer. The comprised skin barrier makes the skin readily develop inflammation in response to external stimuli, including microorganisms. By applying culture-independent methodology, identification of microbe compositions on the AD skin, which was unsuccessful in approximately 80% of species by conventional culturing method,⁽¹⁵⁾ has broadly been described. *Staphylococcus aureus* is a well-known microorganism associated with AD exacerbation.⁽¹⁶⁾

Not only bacteria, but also fungi are implicated in AD. However, studies on the fungal communities have been relatively restrictive due to lack of interest and optimal methodology compared with the bacterial world. Genomic methodologies available for fungi have been limited compared with tools for bacteria. Most of the studies investigating the fungi on human skin have been conducted based on the traditional culture-dependent methods. The efficiency of culture-dependent methods is, however, prone to vary with the isolation techniques and culturing conditions. The isolation medium used and the slow growth of some species, such *M. obtusa* and *M. restricta*, influence the accuracy of the study results.^(17, 18) To overcome these limitations, Sugita et al.⁽¹⁹⁾ first conducted culture-independent, molecular-based method to analyze the *Malassezia* microbiota of

skin. They directly extracted *Malassezia* DNA from samples and investigated community structure using PCR with species-specific primers or rRNA clone methods. Since then, the reliability of the non-culture approach has been verified through several analogous reports.⁽¹⁹⁾⁻⁽²⁰⁾

We report here the results of skin mycobiome analyses based on the culture-independent sequencing methods across 8 adult patients with AD and 8 healthy subjects. We aimed to present the differences in the structure of skin fungal communities between two study groups and suggest correlational patterns between fungal colonizers and AD pathophysiology in the Korean population.

In this study, we first investigate the relative abundance of each constitute, or the taxonomic composition at all level ranks from phylum to species. Two AD samples (AD7 and AD8) interestingly had unusual taxonomic compositions with *Ascomycota* as a major fungal phylum. As described in previous studies, *Basidiomycota* is a dominant phylum of a healthy skin surface except at a specific body site or age group, such as the popliteal crease in children.^(21, 22) The overall assessments of intra- and interpersonal diversities via phylum-level SDI calculation showed no significant differences between AD and healthy control groups.

At the genus- and species-level classification, more complicated and characteristic patterns were expected in patients with AD. Previous studies have revealed that the change of host immunity state broke the balance in fungal communities mainly detectable at the genus level. As a typical example, mycobiome alteration by the presence of *Candida* and *Aspergillus* genera in patients with primary immunodeficiency was reported by Oh et al.⁽²³⁾ Findely et al.⁽²⁴⁾ described more diverse fungal genera detected on the skin of patients with antifungal treatment. Overall, the difference in mean SDI between two subject groups tended to increase at the genus and species levels compared with the phylum level. A higher interpersonal variation in the AD samples was observed. The analyzed fungal flora of healthy skin in this study almost exclusively comprised *Malassezia*, which was parallel to previous studies.^(25, 26) In contrast, *Alternaria*, *Aureobasidium*, *Aspergillus*, and *Cladosporium* family members, in part, replaced *Malassezia* in some AD samples, suggesting high non-*Malassezia* fungal diversity of AD skin, as previously described in a systemic review by Dybboe

et al. They reported more diverse non-*Malassezia* species in AD compared with healthy controls.⁽²⁾ *Alternaria* and *Ascomycota* fungi identified in the AD7 sample are well known as major plant pathogens. Only few cases of morbid skin infection by *Alternaria* species have been reported worldwide.⁽²⁷⁾ *Aspergillus* was also detected on the AD7 sample. The mold *Aspergillus* has been well known to play a role as a pathogen of primary or secondary cutaneous aspergillosis in patients with immunodeficiency, which is rare but life-threatening⁽²⁸⁾ It is interesting that *Alternaria* and *Aspergillus* are rare pathogens, but are both well-known mold allergens that have been targeted in skin prick or specific IgE tests.⁽²⁹⁾ *Aureobasidium* was another atypical genus detected in the sample. Considering at the species level, *Aureobasidium pullulans* is a polymorphic fungus ubiquitously isolated from the natural environment. Pathogenic strains of *A. pullulans* have been actively studied as an opportunistic pathogen isolated from the skin of an immunocompromised patient.⁽³⁰⁾ Despite the presence of these uncommon fungal members on the skin surface, the patient providing the AD7 sample was a 23-year-old man without any disease history except AD. In addition, the lesional sites where the samples were obtained were assessed as “mild.” Any specific finding was not observed, except pinkish erythematous scaly patches. AD8, another sample showing interesting results with *Cladosporium*, was from a 20-year-old male without any unusual history. We considered the results as being from environmental or behavioral factors we missed. Meanwhile, in 2006, Kallawicha et al.⁽³¹⁾ investigated the association between ambient bioaerosol exposure and allergic skin diseases, including AD, contact dermatitis, and other eczema by database analyses. They reported that exposure to total fungal spores was significantly associated with atopic dermatitis, particularly in males, after adjustment for air pollution and sociodemographic factors. Overall, some species of *Alternaria*, *Aspergillus*, and *Cladosporium* might have played a role as mold allergens and had an effect on the course of AD, while others, such as *Aureobasidium* species, might have been transit microorganisms or contaminants.

Among the fungi, *Malassezia* species have been mainly studied associated with AD. Several studies have suggested evidence supporting the role of *Malassezia* species as aggravating factors in patients with AD. One of them confirmed that patients with AD reacted more frequently to

Malassezia extract and recombinant *Malassezia* antigens than healthy controls.⁽³²⁾ In another prior clinical study, antifungal treatment with itraconazole significantly reduced Severity Scoring of Atopic Dermatitis (SCORAD) index, particularly in yeast-sensitized patients with AD.⁽³³⁾ However, the results of studies based on *Malassezia* taxonomy are still controversial. The results vary with characteristics of the subject group, such as age, sex, and race. The methodologies, including sampling methods (swabbing, tape stripping, etc.) and site sampled (lesional or nonlesional skin, head and neck, trunk, limbs, etc.) also seem to influence the discrepancy between the study results.⁽⁴⁾ Thus, more preliminary data on the relationship between AD and *Malassezia* taxonomic composition in diverse conditions are needed for systemic database construction and application into clinical practice. For these reasons, we performed specific analyses on the data from the *Malassezia* species in this study. We have identified nine *Malassezia* species, considered as all the species detectable on the human skin. A total of 14 species were currently recognized within the genus *Malassezia*. Among them, the five species (*M. caprae*, *M. cuniculi*, *M. equine*, *M. nana*, and *M. pachydermatis*) are known to show affinity for animals. Considering the results of *Malassezia* analyses (Fig. 4A and B), species distinctive of AD were *M. slooffiae*, *M. dermatis*, *M. japonica*, *M. yamatoensis* and *M. obtusa*. *M. dermatis* was a newly isolated member of the genus *Malassezia* in 2002. Sugita et al. first discovered the isolates on the skin surface of patients with AD and named it as *M. dermatis*.⁽³⁴⁾ Since then, *M. dermatis* has been regarded as a representative species in AD together with *M. globosa*, *M. restricta*, and *M. sympodialis*.⁽¹⁷⁾ With regard to other species, including *M. sympodialis* and *M. furfur*, from which allergens are officially registered in the nomenclature list,⁽³⁵⁾ the data quite differed across studies.⁽⁴⁾ With respect to the relationship between *Malassezia* and clinical severity, we cannot provide noticeable conclusions through this study. The distribution of SDIs calculated in terms of *Malassezia* species showed a different pattern from that calculated in terms of all species, including non-*Malassezia* species. The interpersonal variance of SDIs in the AD samples, which was distinct when analyzed at species level, disappeared, resulting in a Gaussian distribution in both study groups despite the small sample sizes. These results indicate that the changes of non-*Malassezia* community structures cause differences between patients with AD and healthy controls. Moreover, they suggest the involvement of non-*Malassezia* diversity in AD pathophysiology, as described by previous

reports.⁽²⁾ In one of these studies, Zhang et al.⁽³⁶⁾ described more-diverse non-*Malassezia* fungal microbiota in patients with AD than in healthy controls, obtaining scale samples from the face (lesional sites) of the patients. Our results could reinforce and enrich the verification. We consider the quantitative demonstration of the degree of diversity using SDI as one of the strengths of our study. In addition, the body site where we obtained scale samples was different from where Zhang et al. did, which contributes to building multifaceted pictures of the mycobiome.

Malassezia might influence AD development or aggravation in another way. As an example, the specific genotypes of *M. globosa* and *M. restricta* might also play a role in AD because they commonly colonize in both patients with AD and healthy populations in large amounts regardless of disease severity. The number of short sequence repeats in the intergenic spacer (IGS) 1 region is thought to determine variant genotypes.^(37, 38)

The selection of the antecubital fossae as a sampling site might have contributed to the discrepancy from other study results. The overall fraction of the genus *Malassezia* was lower than that in other mycobiome researches across patients with AD and healthy controls, suggesting the influence of sampling site on the study results. Most of the *Malassezia* species are prone to colonize the head and neck rather than the trunk and limbs because they require lipid components to grow. *M. globosa* was more prevalent than *M. restricta* in almost all samples, which also supports the intervention of site sampled. *M. globosa* is found more frequently on the chest and arms, whereas *M. restricta* is found on the scalp and forehead.⁽⁴⁾ The head and neck are known to be the most often involved sites in adult AD as well as the most *Malassezia*-prevalent sites. For these reasons, the head and neck have been preferred as a sampling site in a number of studies covering healthy population or patients with AD. However, large skin folds, such as the antecubital and popliteal fossae, are also frequently involved sites in adult patients. Actually, the enrolled patients with AD in this study commonly had typical lesions on their antecubital fossae, whereas some had no definite skin lesions on the face and neck. We selected the antecubital fossae with a consideration of risk that the colonization on the

surface of the head and neck was hardly kept intact due to frequent tampering; washing, peeling, make-up, massage, etc. Moreover, because we found that the mycobiome of the antecubital fossae had rarely studied in adult patients with AD, we supposed that this study could provide a new insight into the mycobiome of adult AD.

Mycobiome researches in patients with AD are often challengeable. Obtaining unified conclusions from different groups, in comparison with studies covering other skin diseases, seems difficult. The first reason is that fungal colonization can be inhibited by antifungal activities of inflammatory cells and mediators present in AD skin lesion.⁽³⁹⁾ The vague correlation between overall fungal diversity and clinical factors, including AD severity can be accounted for by this. The second is that lipid components needed for *Malassezia* growth decrease due to destruction of barrier function in AD skin.⁽⁴⁰⁾ Finally, delicate clinical correlations considering temporal dynamics and all, which can alter the mycobiome structure, seem to be difficult.

This study has some limitations. First, some isolates might be “transit” microorganisms that do not colonize skin. The presence of these microorganisms shows instability and diversity of the overall mycobiome; however, it is difficult to say whether they have an effect on AD pathophysiology. Second, because of the small sample size, statistical analysis was impossible. This study was not focused on reliable quantification, but instead on comparative observation of overall characteristics between two groups.

In summary, despite some limitations, we detected a tendency for higher intra- and interpersonal skin fungal diversities in populations with AD, compared with those in healthy populations. Simultaneously, non-*Malassezia* diversity was more noticeable in AD populations. These results suggest a correlation between colonized fungi alternation and AD pathophysiology. Besides, we obtained valuable information on the *Malassezia* distribution of AD skin from new body site of newly set study subjects: the antecubital fossae of adult patients with antecubital lesion. This would be a preliminary study that contributes to building a systemic mycobiome database, which covers a broad spectrum of AD; infantile, juvenile, and adult AD with various involved body sites. A massive

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database should be accumulated from individual mycobiome data under diverse conditions. Systemic analyses of the database will contribute to fully understanding AD pathophysiology and setting new treatment guideline for refractory AD.

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AUTHOR CONTRIBUTIONS

Lee YW and Jung WH designed the study. Han SH, Cheon HI, Hur MS and Kim MJ performed the research. Han SH analysed the data and wrote the paper. Choe YB and Ahn KJ contributed essential reagents and tools. All authors substantially contributed to the research and were involved in drafting the paper or revising it critically, and all authors approved the submitted and final versions.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

SUPPORTING INFORMATION

Additional supporting data may be found in the supplementary information of this article.

Table S1. Basic characteristics of study groups

Table S2. Clinical and demographic status of samples and number of generated sequence reads

Figure S1. Fast unifracs analysis to similarity and difference of fungal community structures between each sample based on a phylogenetic tree.

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Figure 1 Relative abundance of fungal phyla. Internal transcribed spacer 2 (ITS2) sequences of AD samples (AD1–AD8) and healthy controls (HC1–HC8) are taxonomically classified to the phylum level. The relative abundance of fungal phyla in each sample is described as a percentage (Y-axis).

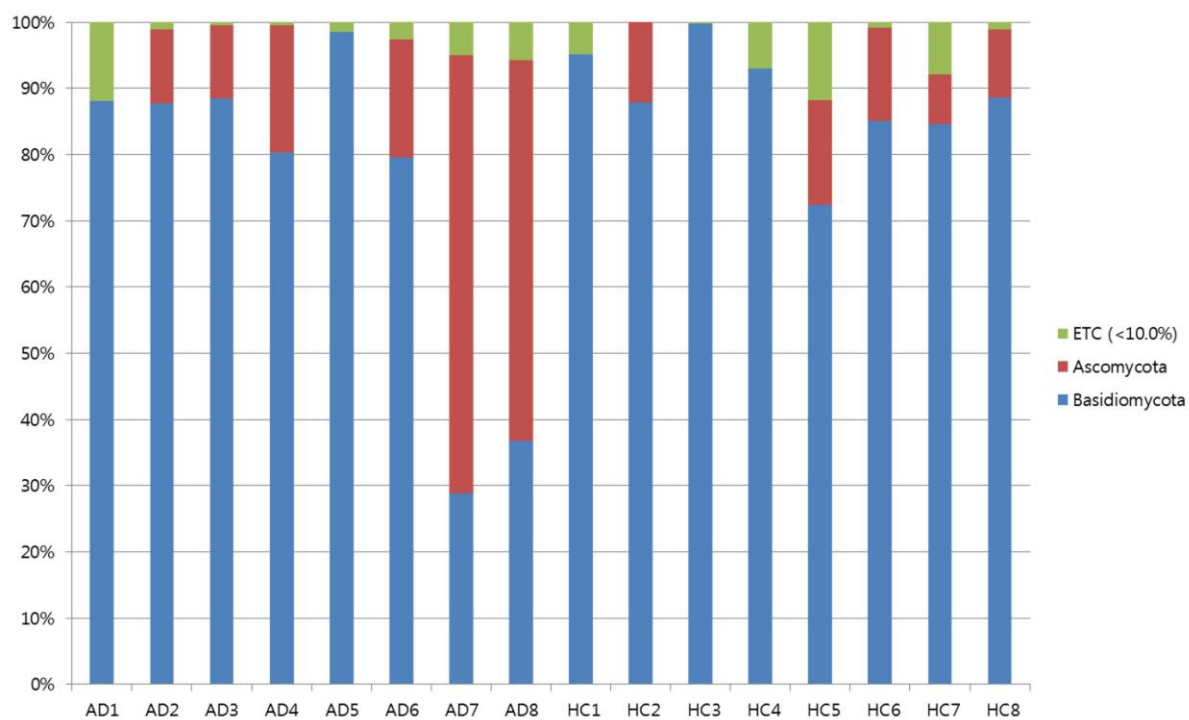
AD: atopic dermatitis; HC: healthy control

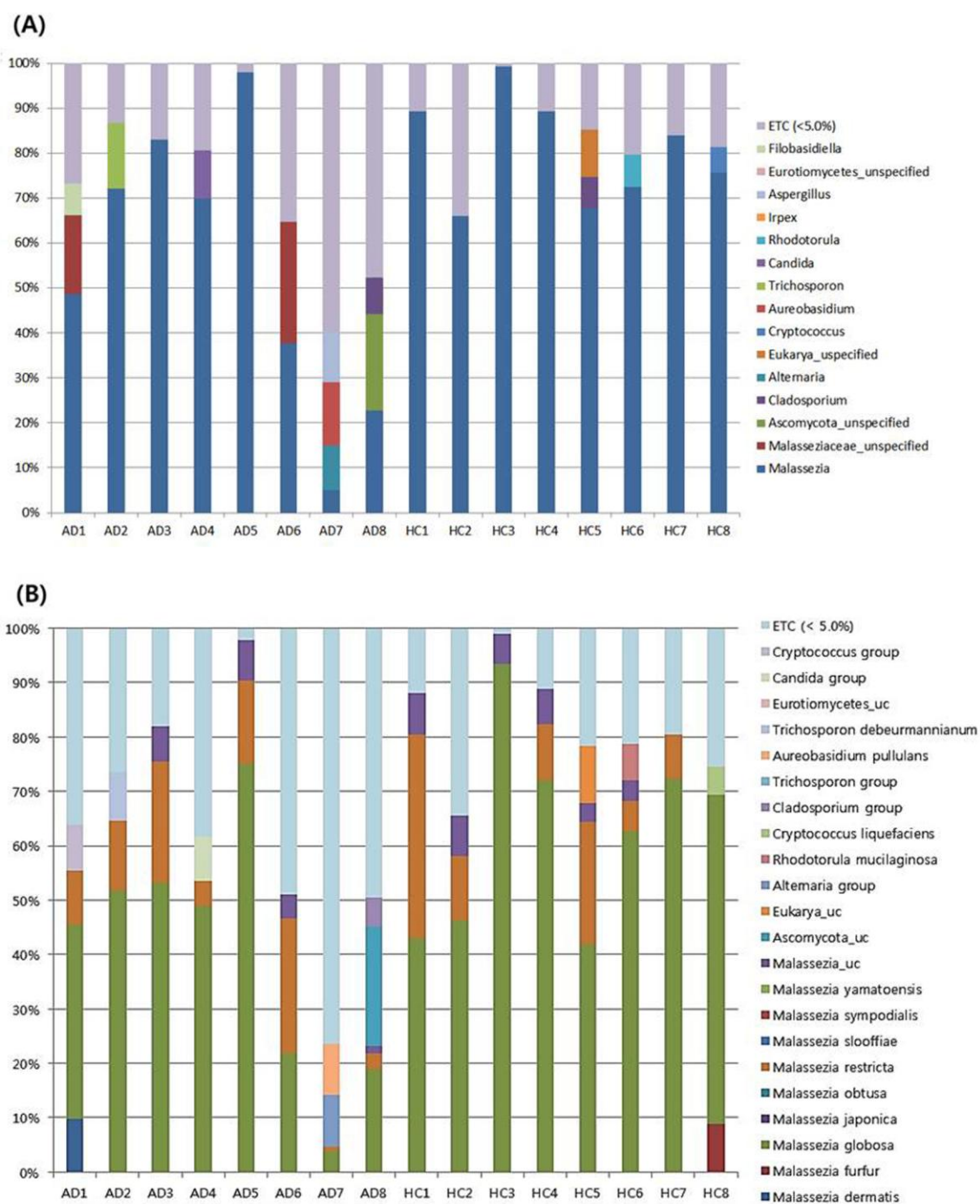
Figure 2 Relative abundance of fungal genera and species. Sequences of internal transcribed spacer 2 (ITS2) regions are taxonomically classified to the genus and species levels. The relative abundance of fungal genera (A) and species (B) in each AD sample (AD1–AD8) and healthy controls (HC1–HC8) are described as a percentage (Y-axis). AD: atopic dermatitis; HC: healthy control

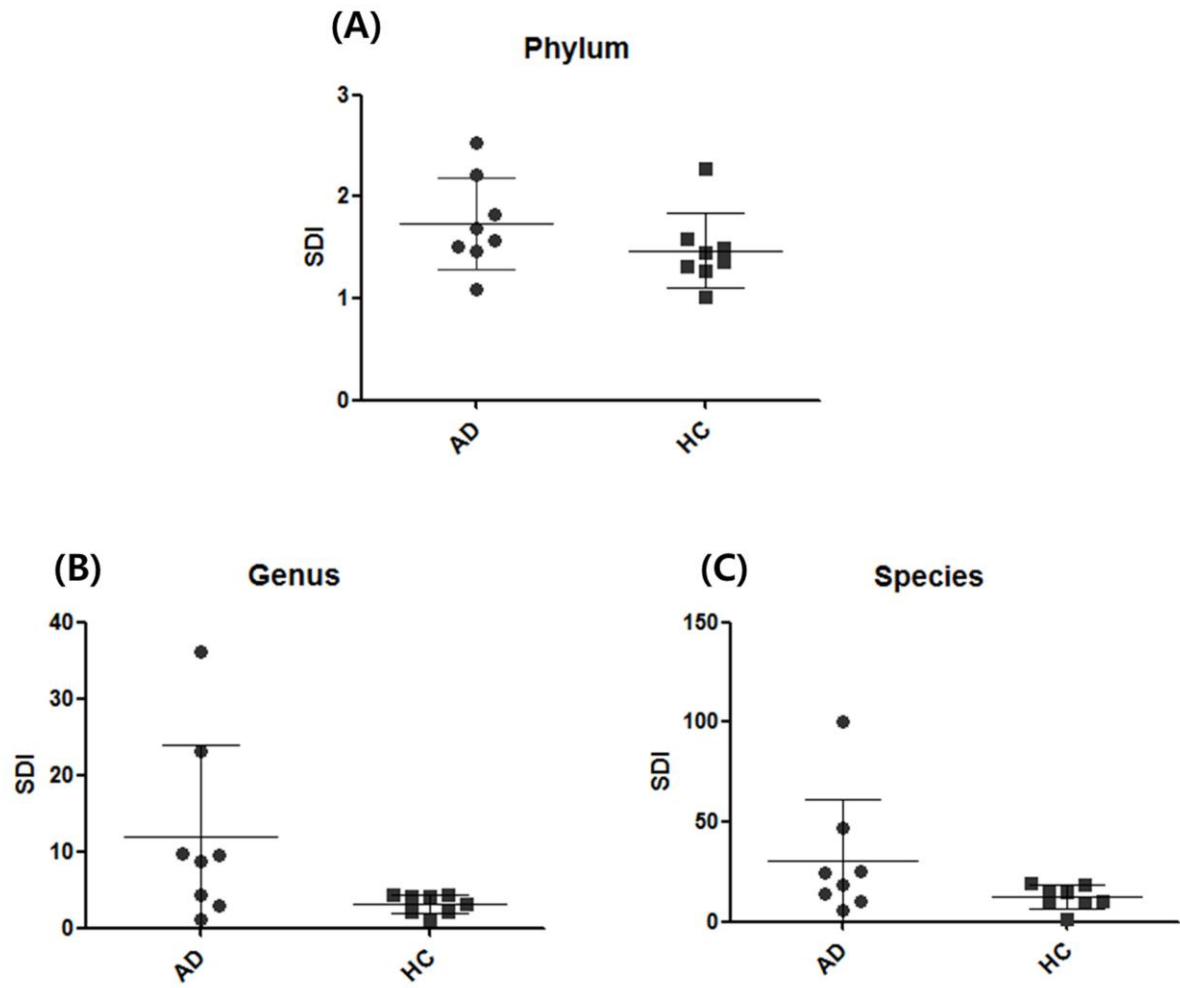
Figure 3 Diversity analyses using Shannon diversity index (SDI). SDIs of each sample at phylum (A), genus (B), and species (C) levels are marked as circular (AD1–AD8) and square dots (HC1–HC8).

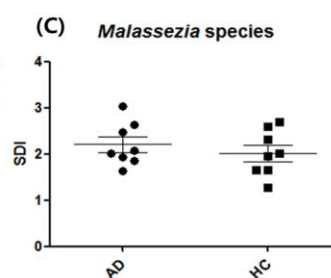
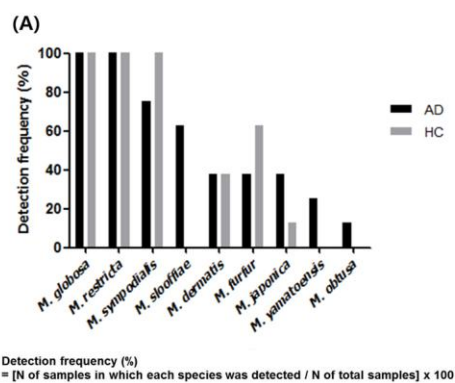
AD: atopic dermatitis; HC: healthy control

Figure 4 *Malassezia* distribution analyses. The sequences classified into *Malassezia* species are analyzed. Detection frequency (%) of each *Malassezia* species in the AD group and healthy controls is depicted (A). The relative abundance of *Malassezia* species in each sample is expressed as a percentage (Y-axis) (B). Diversity analysis in terms of *Malassezia* species was also conducted using calculated SDI. The SDIs of each sample are marked as circular (AD1–AD8) and square dots (HC1–HC8) dots. The differences between the AD group and healthy controls were analyzed using unpaired *t* test, as the SDI values of the samples in each study group followed a Gaussian distribution (C). AD: atopic dermatitis; HC: healthy control









AD1
AD2
AD3
AD4
AD5
AD6
AD7
AD8
HC1
HC2
HC3
HC4
HC5
HC6
HC7
HC8

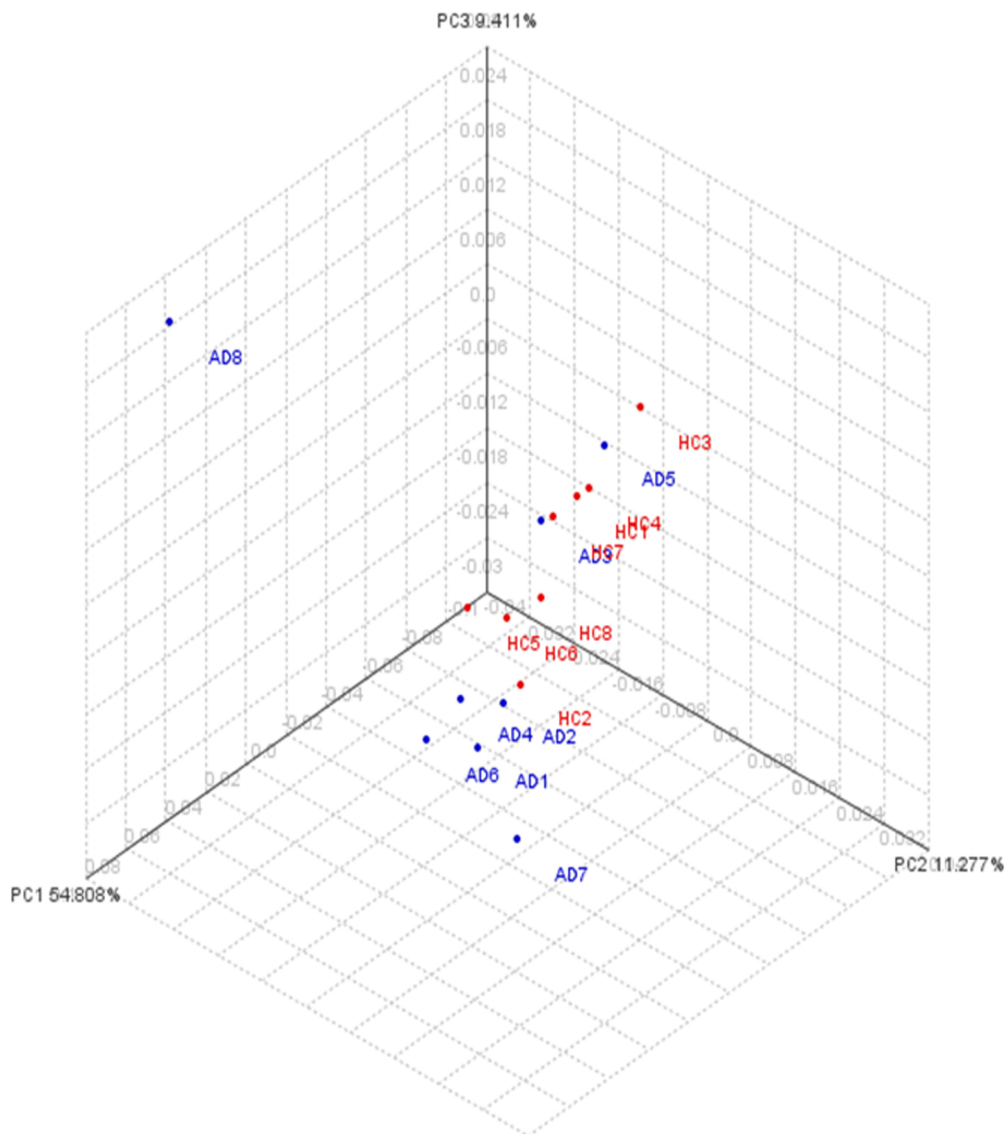


Table S1 Basic characteristics of study groups

	Atopic dermatitis (AD)	Healthy controls (HC)	P value*
Total subjects analyzed, n	8	8	-
Male:female, ratio	1:1	1:1	-
Mean age , years (SD)	27.00 (9.55)	30.75 (7.13)	0.3883

***Statistical tests used: unpaired *t* test**

Table S2 Clinical and demographic status of samples and number of generated sequence reads

Sample ID	Gender	Age	Atopic dermatitis				Number of sequence reads
			Duration	IGA	EASI	SCORAD	
AD1	Female	20	20 yr	2	3.4	45.6	19,986
AD2	Female	22	15 yr	3	6.4	50.7	19,491
AD3	Male	27	20 yr	4	8.8	41.6	19,949
AD44	Female	42	20 yr	3	1	20	19,961
AD5	Female	42	20 yr	2	0.5	11.7	17,345
AD6	Male	20	14 yr	4	15	52.3	19,984
AD7	Male	23	20 yr	1	0.8	8.3	19,988
AD8	Male	20	15 yr	3	2.7	24.1	19,973
HC1	Male	27			No		19,981
HC2	Female	29			No		19,979
HC3	Male	45			No		15,565
HC4	Male	34			No		15,091
HC5	Female	28			No		19,979
HC6	Female	22			No		19,938
HC7	Female	26			No		19,971
HC8	Female	35			No		19,991

AD: atopic dermatitis; HC: healthy control; IGA: investigator's global assessment; EASI: eczema area and severity index; SCORAD: scoring atopic dermatitis

Figure S1 Fast unifrac analysis to similarity and difference of fungal community structures between each sample based on a phylogenetic tree. Blue and red dots indicate AD samples (AD1–AD8) and healthy controls (HC1–HC8), respectively.