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ORIGINAL ARTICLE

Epidemiologic Study of *Malassezia* Yeasts in Seborrheic Dermatitis Patients by the Analysis of 26S rDNA PCR-RFLP

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Background: This case-control study concerns a molecular biological method based on the data gathered from a group of Korean subjects to examine the distribution of *Malassezia* yeasts in seborrheic dermatitis (SD) patients. Cultures for *Malassezia* yeasts were taken from the foreheads, cheeks and chests of 60 patients with SD and in 60 healthy controls of equivalent age. **Objective:** The purpose of this study is to identify the relationship between certain species of *Malassezia* and SD. This was done by analyzing the differences in the distribution of *Malassezia* species in terms of age and body parts of the host with healthy controls. **Methods:** 26S rDNA PCR-RFLP, a fast and accurate molecular biological method, was used to overcome the limits of morphological and biochemical methods. **Results:** The positive *Malassezia* culture rate was 51.7% in patients with SD, which was lower than that of healthy adults (63.9%). *M. restricta* was dominant in patients with SD (19.5%). Likewise, *M. restricta* was identified as a common species (20.5%) in healthy controls. In the ages 31～40, *M. restricta* was found to be the most common species (31.6%) among SD patients. **Conclusion:** According to the results of the study, the most frequently isolated species was *M. restricta* (19.5%) in patients with SD. There was no statistically significant difference in the distribution of *Malassezia* species between the SD patients and healthy control groups. **(Ann Dermatol 22(2) 149～155, 2010)**

-Keywords-
26S rDNA PCR-RFLP, *Malassezia* yeasts, Seborrheic dermatitis

INTRODUCTION

Seborrheic dermatitis (SD) is a sub-acute or chronic superficial eczematous dermatitis, characterized by erythematous plaques with a dry or oily scale[1]. It develops in the areas of increased activity of sebaceous glands, usually occuring on the scalp, face, ears, chest, and axillary areas. The causes of SD have not yet been completely elucidated, however hypersecretion of sebaceous glands is seen as the major cause, as it primarily occurs where sebaceous glands are abundant. There are also other hypotheses, including a relationship with the lipophilic *Malassezia* yeasts as resident flora of the skin, and abnormalities of neurotransmitters and epidermal hyperplasia. Furthermore, there are several studies stating that clinical symptoms showed improvement along with decreased amounts of yeasts when treating SD with azole antimycotics[2,3]. In an animal experiment, when *Malassezia* yeasts are applied to the skin, a lesion similar to SD appeared, indicating a need for further study into the relationship between SD and *Malassezia* yeasts[4].

In this research of subjects with SD, 26S rDNA PCR-RFLP was applied to identify and classify *Malassezia* yeasts. In light of the group of healthy controls, this study reveals how the frequency and distribution of the *Malassezia* yeasts changes depending on both age and which body parts are affected, thus clarifying the relationship between the *Malassezia* yeasts and SD.

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MATERIALS AND METHODS

Study participants

There were 60 healthy adults and 60 patients with SD (30 males, 30 females) who participated in this study. The patients were chosen among those who had visited the dermatology outpatient clinic at Konkuk University Medical Center, from July 2005 to June 2008. The healthy adults were grouped depending on their age (20 patients in each age group: 31～40 years, 41～50 and 51～60). Excluded patients included those who had been administered adrenocortical hormones, those who had undergone light therapy or antimycotic treatment within 2 months of the examination date, as well as those who had been treated with topical antifungals within 1 month and/or with topical corticosteroids within 1 week. All participants were told not to wash their face or use moisturizer (not to apply any products) on the examination day. According to the Declaration of Helsinki, the participants were informed of the physical and mental risks that could happen during the study, and written consents were obtained before their participation.

Specimen sampling

Specimens were gathered from forehead, cheek and chest, and were sampled by scrub-wash technique, based on the method that Williamson and Kligman suggested[5,6]. A stainless tube, which has an interior area of 4.909 cm^2, was set on the selected skin: forehead, cheek and chest, and then 1 ml of detergent[7] (0.01% $NaH_2PO_4H_2O_2$, 1.01% Na_2HPO_4, 0.1% Triton X-100 [pH 7.9]) was put into the tube. After rubbing the skin by a glass rod for 1 minute, the sample was taken out by a pipette and stored in a different container. Then, 1 ml of the detergent was put into the stainless tube, and the specimen was repetitively sampled and added to the first sample. 100 μl of the sampled specimen was then mixed with 900 μl of the detergent with 50% concentration, and 100 μl was taken from the mixture, evenly applied on the Leeming-Notman medium, and cultured at 34°C for 14 days[8].

DNA extraction & PCR

For DNA extraction and PCR analysis of skin isolates, this study adopted colony PCR analysis which was developed to extract DNA directly from a colony of a PCR tube and to amplify 26S rDNA at the same time instead of direct genomic DNA extraction methods. A colony of *Malassezia* yeast was taken and transferred to a PCR tube and warmed up 3 times a day for 1 minute, in a double boiler by using a microwave, and then the tube was moved into ice water. The PCR reaction mixture (0.25 mM deoxynucleoside triphosphate, 10X PCR buffer, 5X Q buffer, 0.5 μM primers, 1.25 U Hot StarTaq polymerase, 20 mM $MgSO_4$) was added and vortex mixing was done. Then, PCR, using Mastercycler 5333 (Eppendorf, Hamburg, Germany), was performed immediately. To amplify 26S rDNA, a primer that can amplify all 11 standard strains at once was chosen. The sequence was: forward, 5'-TAACAAGGATTCCCCTAGTA-3' and reverse, 5'-ATTACGCCAGCATCCTAAG-3'[9]. The conditions in the early stage of the reaction were at 95°C for 14 minutes for predenaturation, at 94°C for 45 seconds for denaturation, at 55°C for 45 seconds for annealing, at 72°C for 1 minute for extension of 40 cycles, and at 72°C for 7 minutes for the last extension. Amplified DNA was visualized by electrophoresis on a 1.5% (w/v) agarose gel with Ethidium bromide (0.5 μg/ml) by using 1 X TAE migrating buffer (pH 8.0, 40 mM Tri-acetate 1 mM EDTA).

Restriction fragment length polymorphism (RFLP) analysis

After checking the amplified 26S rDNA, the PCR product was purified using the Accu-Prep PCR purification kit (Bioneer, Daejeon, Korea). For the 26S rDNA RFLP analysis of *Malassezia* yeasts, 2 restriction enzymes were used: *Hha I* (Takara Biomedicals, Otsu, Japan) and *BtsC I* (SibEnzyme, Novosibirsk, Russia). The restriction enzyme reaction was conditioned with 10X PCR buffer, 10 U restriction enzyme, and reaction mixture including 7.5 μl of the PCR product to become 20 μl. After 3 hours of reaction at 37°C, electrophoresis was performed in the TAE buffer with 3.5% (w/v) NuSieve GTG agarose gel (FMC, Rockland, ME, USA) by 100 volts. Then, it was stained with ethidium bromide, and the size and number of DNA fragment were checked by a UV transilluminator to analyze RFLP patterns[10].

Statistical analysis

Chi-squared tests (SPSS) were used to find the difference in the distribution of the *Malassezia* species between patients with SD and healthy adults from equivalent age groups. The results are considered to be statistically significant when the *p*-value is less than 0.05.

RESULTS

Comparison of identification rates of *Malassezia* yeasts between patients with SM and healthy adults

Specimens were collected from 3 body sites: forehead, cheek, and chest, in 60 SD patients and 60 healthy adults. *Malassezia* yeasts of the patient group were cultured in 93 out of 180 specimens, and thus the identification rate was

51.7%. Five *Malassezia* species (*M. sympodialis, M. restricta, M. furfur, M. globosa, M. dermatis*) were identified in the group of SD patients (Fig. 1, 2). The healthy control group, on the other hand, showed 7 *Malassezia* species (*M. globosa, M. restricta, M. sympodialis, M. furfur, M. dermatis, M. slooffiae, M. obtusa*), and the identification rate was 63.9% (115/180) (Table 1, 2). Within age groups, a high identification rate was shown in of the 30s (31～40 years) in both patient and healthy adult groups: 66.7% (40/60) in the patient group, 71.7% (43/60) in the healthy adult group. Within body parts, in both patient and healthy adult groups, a high identification rate was shown in the forehead: 56.7% (34/60) in the patient group and 66.7% (40/60) in the healthy adult group.

Comparison of the *Malassezia* species by different ages

Identifying and classifying the *Malassezia* yeasts cultured on each body part according to ages, in the patients in their 30s and 40s, *M. restricta* (30s: 31.6%, 40s: 16.6%) was identified as the major species, and in their 50s, *M. sympodialis* (20.0%) was identified as the major species. In the healthy adult groups, *M. sympodialis* (25.0%), *M. restricta* (21.6%) and *M. globosa* (21.7%) were identified to be predominant in the 30s, 40s, and 50s, respectively (Table 1). Comparing the difference in the distribution of the *Malassezia* species between the patient and control groups by equivalent ages, there was no statistically significant difference (Fig. 3).

Comparison of the *Malassezia* species by different body sites

Identifying the *Malassezia* yeasts cultured from patients' body parts, in whole parts *M. restricta* was identified more than other species (19.5%, 35 cases). Both in the experiment and control groups by different body parts, *M. restricta* was identified frequently in the forehead and cheek. In the chest, on the other hand, *M. sympodialis* (16.7%) and *M. globosa* (26.7%) were identified as common species in both the patient and healthy adult groups, respectively (Table 2). The results showed no significant difference in the distribution of the *Malassezia* species in different body parts in either the patient or healthy control groups (Fig. 4).

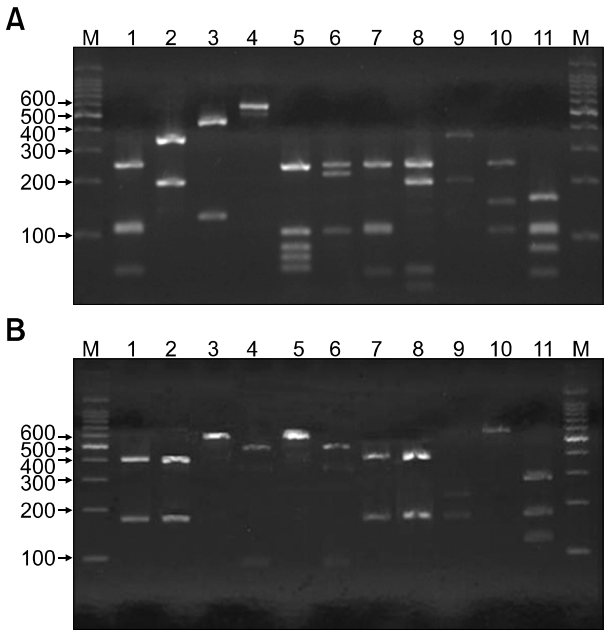

Fig. 1. PCR-RFLP patterns of 26S rDNA PCR digested with *Hha* I (A), *BtsC* I (B) of 11 *Malassezia* standard strains. Lanes: M: molecular marker, 1: *M. furfur* (KCTC 7743), 2: *M. sympodialis* (KCTC 7985), 3: *M. globosa* (CBS 7966), 4: *M. restricta* (KCTC 7848), 5: *M. slooffiae* (KCTC 17431), 6: *M. pachydermatis* (KCTC 17008), 7: *M. japonica* (CBS 9432), 8: *M. nana* (JCM 12085), 9: *M. dermatis* (JCM 11348), 10: *M. obtusa* (KCTC 7847), 11: *M. yamatoensis* (CBS 9725).

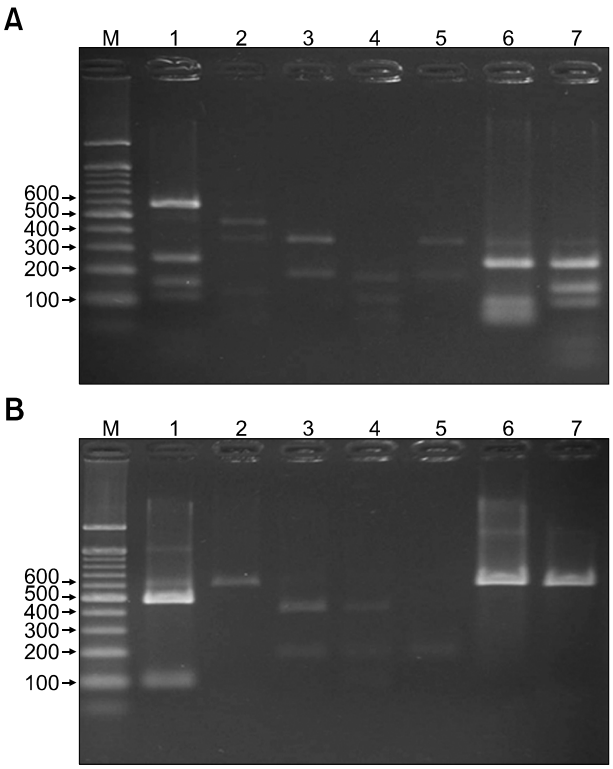

Fig. 2. PCR-RFLP patterns of 26S rDNA PCR digested with *Hha* I (A), *BtsC* I (B) of *Malassezia* yeasts from seborrheic dermatitis patients and healthy controls. Lanes: M: molecular marker, 1: *M. restricta*, 2: *M. globosa*, 3: *M. sympodialis*, 4: *M. furfur*, 5: *M. dermatis*, 6: *M. slooffiae* (from healthy control), 7: *M. obtusa* (from healthy control).

Table 1. Identified *Malassezia* species by ages - 26S rDNA PCR-RFLP analysis

Identified *Malassezia*	31～40		41～50		51～60		Total	
	HC (%)	SD (%)	HC (%)	SD (%)	HC (%)	SD (%)	HC (%)	SD (%)
Total recovery rate of *Malassezia* yeasts	43/60 (71.7)	40/60 (66.7)	32/60 (53.3)	25/60 (41.7)	40/60 (66.7)	28/60 (46.7)	115/180 (63.9)	93/180 (51.7)
M. restricta	12/60 (20.0)	19/60 (31.6)	13/60 (21.6)	10/60 (16.6)	12/60 (20.0)	6/60 (10.0)	37/180 (20.5)	35/180 (19.5)
M. globosa	10/60 (16.6)	9/60 (15.0)	9/60 (15.0)	4/60 (6.6)	13/60 (21.7)	8/60 (13.3)	32/180 (17.8)	21/180 (11.7)
M. sympodialis	15/60 (25.0)	7/60 (11.7)	8/60 (13.3)	7/60 (11.7)	10/60 (16.6)	12/60 (20.0)	33/180 (18.3)	26/180 (14.4)
M. furfur	2/60 (3.3)	4/60 (6.6)	2/60 (3.3)	2/60 (3.3)	2/60 (3.3)	2/60 (3.3)	6/180 (3.3)	8/180 (4.4)
M. dermatis	3/60 (5.0)	1/60 (1.6)	0/60 (0.0)	2/60 (3.3)	2/60 (3.3)	0/60 (0.0)	5/180 (2.8)	3/180 (1.7)
M. slooffiae	1/60 (1.6)	0/60 (0.0)	0/60 (0.0)	0/60 (0.0)	0/60 (0.0)	0/60 (0.0)	1/180 (0.6)	0/180 (0.0)
M. obtusa	0/60 (0.0)	0/60 (0.0)	0/60 (0.0)	0/60 (0.0)	1/60 (1.6)	0/60 (0.0)	1/180 (0.6)	0/180 (0.0)
No growth	17/60 (28.3)	20/60 (33.3)	28/60 (46.7)	35/60 (58.3)	20/60 (33.3)	32/60 (54.2)	65/180 (36.1)	87/180 (48.3)
p-value	0.239		0.104		0.112		0.188	

HC: healthy controls, SD: seborrheic dermatitis. $p<0.05$ is considered as significant.

Table 2. Identified *Malassezia* species by body sites - 26S rDNA PCR-RFLP analysis

Identified *Malassezia*	Forehead		Cheek		Chest		Total	
	HC (%)	SD (%)	HC (%)	SD (%)	HC (%)	SD (%)	HC (%)	SD (%)
Total recovery rate of *Malassezia* yeasts	40/60 (66.7)	34/60 (56.7)	37/60 (61.7)	30/60 (50.0)	38/60 (63.3)	29/60 (48.3)	115/180 (63.9)	93/180 (51.7)
M. restricta	19/60 (31.7)	17/60 (28.3)	15/60 (25.0)	12/60 (20.0)	3/60 (5.0)	6/60 (10.0)	37/180 (20.5)	35/180 (19.5)
M. globosa	7/60 (11.7)	5/60 (8.3)	9/60 (15.0)	7/60 (11.7)	16/60 (26.7)	9/60 (15.0)	32/180 (17.8)	21/180 (11.7)
M. sympodialis	8/60 (13.3)	6/60 (10.0)	11/60 (18.3)	10/60 (16.7)	14/60 (23.3)	10/60 (16.7)	33/180 (18.3)	26/180 (14.4)
M. furfur	3/60 (5.0)	4/60 (6.7)	1/60 (1.6)	1/60 (1.6)	2/60 (3.3)	3/60 (5.0)	6/180 (3.3)	8/180 (4.4)
M. dermatis	2/60 (3.3)	2/60 (3.3)	1/60 (1.6)	0/60 (0)	2/60 (3.3)	1/60 (1.6)	5/180 (2.8)	3/180 (1.7)
M. slooffiae	1/60 (1.6)	0/60 (0)	0/60 (0)	0/60 (0)	0/60 (0)	0/60 (0)	1/180 (1.6)	0/180 (0)
M. obtusa	0/60 (0)	0/60 (0)	0/60 (0)	0/60 (0)	1/60 (1.6)	0/60 (0)	1/180 (1.6)	0/180 (0)
No growth	20/60 (33.3)	26/60 (43.3)	23/60 (38.3)	30/60 (50.0)	22/60 (36.7)	31/60 (51.7)	65/180 (36.1)	87/180 (48.3)
p-value	0.265		0.147		0.102		0.141	

HC: healthy controls, SD: seborrheic dermatitis. $p<0.05$ is considered as significant.

DISCUSSION

Malassezia yeasts, found in 75～80% of healthy adults, are lipophilic fungi regarded as normal flora of the skin[11-13]. The genera *Pityrosporum* and *Malassezia* yeasts had long been considered as morphologically heterogeneous and thus are classified and distinguished from one another[14].

Though, since the early days, morphologic diversity has been pointed out in previous studies of *Malassezia* yeasts,

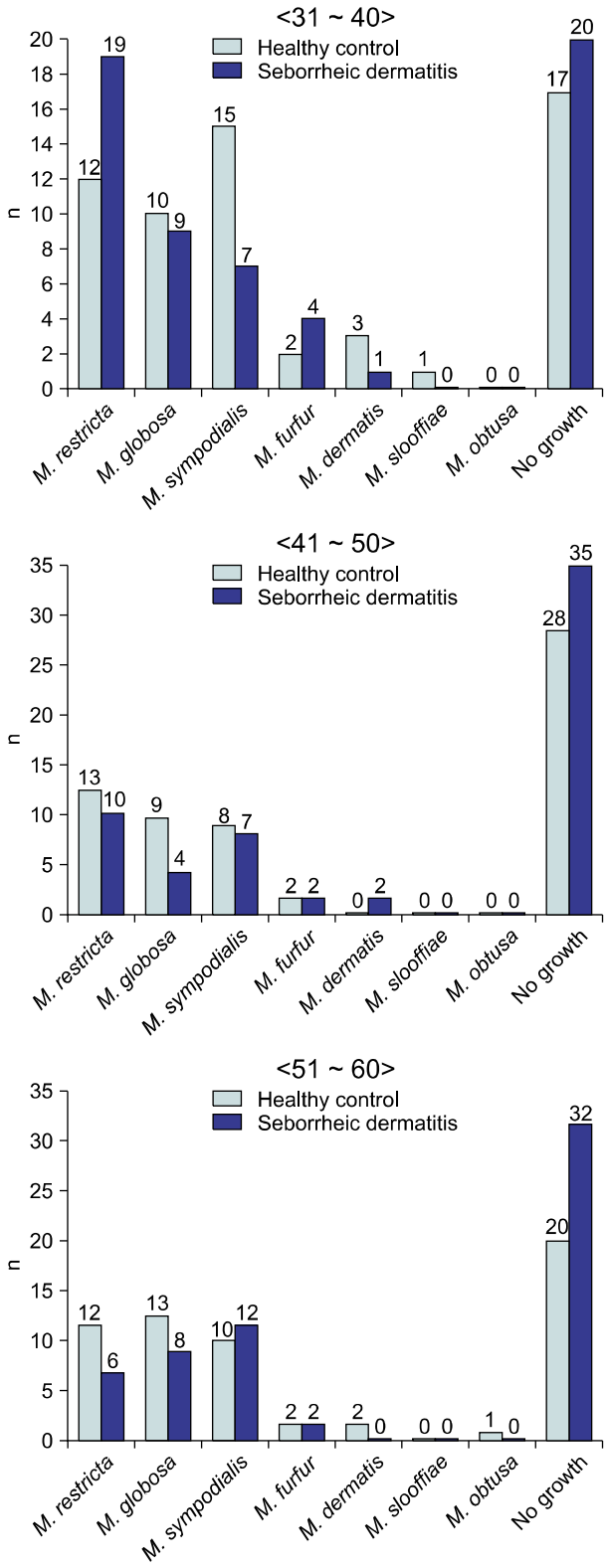

Fig. 3. Identified *Malassezia* species from seborrheic dermatitis group compared with healthy control group, by ages.

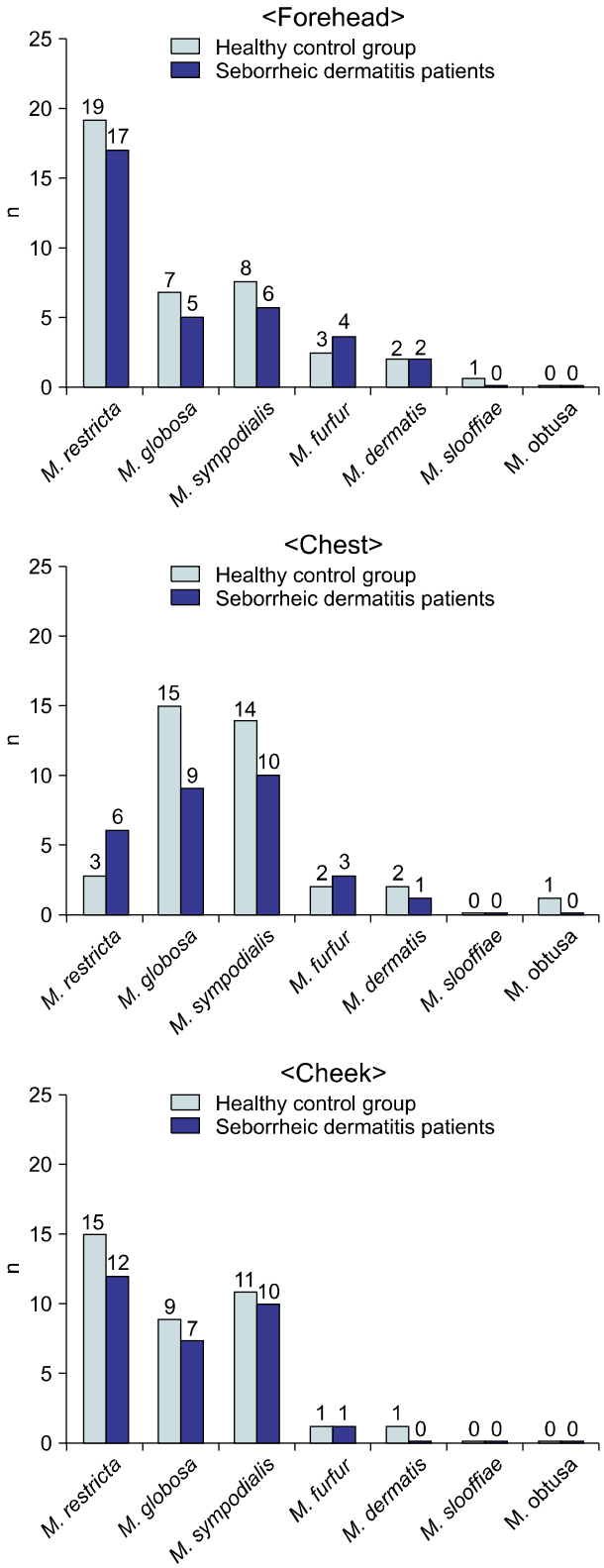

Fig. 4. Identified *Malassezia* species from seborrheic dermatitis group compared with healthy control group, by body sites.

besides *M. furfur*, only the lipid-independent *M. pachydermatis* was classified as *Malassezia* for a long time[15]. As morphological, immunological, physiological, and molecular biological research on *Malassezia* yeasts continued,

a need was developed for the re-classification of the genus *Malassezia*. Thus, Gueho et al. isolated *M. furfur* and re-classified into 7 species, considering the morphology, microstructure analysis, physiology, and molecular biology of each: *M. furfur, M. pachydermatis, M. sympodialis, M. globosa, M. obtusa, M. restricta, M. slooffiae*[15-20]. Moreover, 4 more *Malassezia* species in Japan; *M. dermatis, M. japonica, M. nana*, and *M. yamatoensis*, and 2 more species in Europe; *M. caprae, M. equina* have been recently identified, thus *Malassezia* yeasts are classified into 13 species at present[9,19,21-25].

It has been reported that *Malassezia* yeasts are associated with dermatoses such as pityriasis versicolor, SD, and *Malassezia* folliculitis. Recently reported is the implication of *Malassezia* yeasts in atopic dermatitis and psoriasis[12,13,21,26,27]. Furthermore, confluent and reticulated papillomatosis[28,29] and onychomycosis have been identified as being related to *Malassezia* yeasts[30,31]. The pathogenicity of *Malassezia* yeasts is therefore becoming significantly understood to be an underlying factor in certain generalized infections, with the yeasts occurring in premature infants who have received fluids containing fatty acids through vein catheter, as well as in adults with immune deficiency[32,33].

SD occurs on sites with increased activity of sebaceous glands, and is characterized by erythematous plaques accompanied with dry or oily scales. Thus, it is indicated that SD may result from either: abnormalities of the skin's immune response to normal lipophilic *Malassezia* yeasts, from distribution changes, or from abnormal proliferation of certain *Malassezia* yeasts (the underlying cause unknown at this time). These are etiological theories and have not yet been proven.

In this research, 26S rDNA PCR-RFLP, a relatively fast and accurate molecular biological method, was used to identify *Malassezia* yeasts and also to examine the differences in the distribution of *Malassezia* yeasts between patients with SD and healthy controls, according to age and body parts. Compared with healthy adults, the group of patients with SD had 5 *Malassezia* yeasts (*M. sympodialis, M. restricta, M. furfur, M. globosa, M. dermatis*). Seven *Malassezia* yeasts (*M. globosa, M. restricta, M. sympodialis, M. furfur, M. dermatis, M. slooffiae, M. obtusa*) were identified in the healthy controls. In both groups, *M. restricta* was predominant. And in the healthy controls and SD patients, the sum of the identification rates of 3 *Malassezia* species, *M. restricta, M. sympodialis*, and *M. globosa*, was 88.6% (102/115) and 88.1% (82/93) respectively, constituting a majority of the identified *Malassezia* species. Moreover, according to findings regarding the frequency of *Malassezia* by different ages and body parts, no statistically significant difference was found in either SD patients or healthy controls.

In clarifying how normal lipophilic *Malassezia* yeasts could lead to SD, the results indicate that the difference of the *Malassezia* species is not the cause but rather the increased colonies of certain *Malassezia* yeasts, due to the destruction of skin barriers and to abnormalities of the immune system. Previous studies have examined lesions of patients with SD by using real-time PCR assay, which enables a quantitative analysis on *Malassezia* yeasts[34]. The finding is that lesions of patients with SD had more *M. restricta* than did the skin of the normal patients. This implies that a hyperproliferation of specific *Malassezia* yeasts may induce inflammation and result in SD.

In clarifying the relation between SD and *Malassezia* yeasts, further studies are needed. This is necessary not only to compare the qualitative difference of the *Malassezia* yeasts by body sites but also to etiologically analyze the quantitative difference. Studies may have to cover immunological features as well as abnormalities of skin barrier functions which result in proliferation of specific *Malassezia* yeasts.

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