ORIGINAL ARTICLE

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

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Background: Although acne is a common follicular inflammatory dermatosis, studies of the relationship between *Malassezia* yeasts and acne have rarely been conducted. **Objective:** We sought to identify *Malassezia* yeasts from acne patients and establish a relationship between specific types of species of *Malassezia* and acne. **Methods:** Sixty acne patients were enrolled. Each strain obtained was identified as one of eleven species by 26S rDNA PCR-RFLP. We then compared these data with those of age- and sex-matched healthy subjects. **Results:** Growth of *Malassezia* was evident in fewer patients with acne (50%) in comparison to controls (70.6%). *M. restricta* was dominant in patients with acne (23.9%), whereas *M. globosa* was most common (26.7%) in healthy controls. In the patients group, the rate was the highest (71.7%) in the twenties and, in terms of body site, the rate was the highest (60%) in the chest. In the control group, the rate was the highest (75.0%) in the thirties and in the forehead (85.0%). **Conclusion:** The detection rate of *Malassezia* yeasts was conspicuously low in the acne patients group. Statistically significant differences were observed between the patient and the control groups in the twenties and thirties, and in terms of body site, in the forehead and chest. **(Ann Dermatol 23(3) 321~328, 2011)**

-Keywords-
Acne, *Malassezia*, 26S rDNA PCR-RFLP

INTRODUCTION

Acne is a chronic, self-limited inflammatory disorder of the pilosebaceous unit that peaks during puberty, the period in which androgen production is suddenly increased. It commonly affects the face, neck, and chest. It is known to be caused by increased sebum production, due to the influence of androgen, hyperkeratosis of the entry site of the follicle, and induction of inflammatory mediators owing to growth of *P. acnes*[1].

Types of resident flora vary widely, depending on location. The infra-infundibulum harbors *P. acnes*, while the acroinfundibulum is populated by *Malassezia* yeasts, and the skin surface around the follicle by aerobic *Staphylococcus epidermidis*[2,3]. Among these, *Malassezia* yeasts are a lipophilic fungus classified as normal flora, and are isolated in 75~80% of healthy subjects[4,5]. However, as morphologic, immunologic, physiologic, and molecular biological studies progressed, the need for new classification of the species was brought forward. Thus, in 1996, Guého et al. re-classified the yeasts into seven species (*M. furfur, M. obtusa, M. globosa, M. slooffiae, M. sympodialis, M. pachydermatis,* and *M. restricta*) based on morphologic, microscopic, physiologic, and molecular biological characteristics[6-10]. Also, recently, in Japan, four new species, *M. dermatis, M. japonica, M. nana,* and *M. yamatoensis,* and in Europe, two additional species, *M. caprae,* and *M. equina,* have been identified; therefore, the yeasts are now classified into thirteen species[11-15].
They are known to be linked to pityriasis versicolor,

seborrheic dermatitis, *Malassezia* folliculitis, and, most recently, atopic dermatitis and psoriasis[16-18]. Although the presence of *Malassezia* yeasts in both the comedon and follicle of acne patients has been confirmed[19], its exact role in the pathogenesis of acne is not clearly defined.

Thus, the authors were motivated to engage in a study of the distribution of *Malassezia* yeasts in patients with acne, using molecular biological techniques. We also sought to determine whether there is any difference in the distribution of *Malassezia* yeasts between patient and control groups, through comparison with sex- and age-matched controls from the previous study.

MATERIALS AND METHODS

Subjects

The patients group consisted of 60 acne patients aged 11 to 40 who visited Konkuk University Hospital, Department of dermatology, between July 2005 and December 2009. The patient group was classified into teens, twenties, and thirties subgroups, with each subgroup consisting of 20 patients. All patients were age- and sex-matched with sixty healthy controls. Subjects with dermatologic problems other than acne were excluded from the study. Those who were on systemic adreno-corticoid therapy, phototherapy, or antifungal agents within the past two months, as well as those who had been treated with topical antifungal agents within the past month, and/or with topical corticosteroid within one week, were also excluded from the study. All subjects were instructed to avoid the use of moisturizers and facial cleansing on the day of examination, and informed consent was obtained from each individual after a thorough explanation of possible physical and psychological adverse outcomes that could arise during the course of the study. This study was conducted in strict abidance with the Helsinki principles.

The forehead, cheek, and chest, which are common locations of acne, were chosen for sampling, and, whenever possible, samples were taken from actual acne lesions.

Culture of clinically isolated species

1) Preparation of the Leeming-Notman medium and washing solution

The LNA (Leeming-Notman agar) medium was prepared by mixing glycerol monoesterate (BDH, Poole, UK) 0.5 g, bacteriological peptone (Oxoid, Hampshire, UK) 20 g, glucose (Oxoid, Hampshire, UK) 5 g, yeast extract (Oxoid, Hampshire, UK) 0.1 g, ox bile (Merck, Darmstadt, Germany) 4 g, agar No.1 (Oxoid, Hampshire, UK) 12 g,

Tween 60 (Yakuri, Osaka, Japan), 0.5 ml, and glycerol (Tedia, Fairfield, USA) 1 ml with one liter of distilled water and sterilizing it for twenty minutes at 121°C. Following the sterilization process, cycloheximide (Sigma, St Louis, MO, USA) 200 mg, and chloramphenicol (Sigma, St Louis, MO, USA) 50 mg were added, followed by 5 ml of non-skim milk treated at a super-high temperature (Konkuk Dairy, Seoul, Korea). After thorough mixing, the solution was spread evenly on a Petri dish and kept refrigerated until use. The washing solution was prepared by dissolving $NaH_2PO_42H_2O$ 1.17 g in 100 ml of distilled water, 85 ml of which was taken and mixed with Na_2HPO_4 10.6 g and 1,000 ml of distilled water. The pH was adjusted to 7.9, and 1 ml of Triton X100 was added to the solution before sterilizing at 121°C for twenty minutes and refrigerating.

2) Specimen sampling

Specimens were harvested from the forehead, cheek, and chest, and were sampled by a scrub-wash technique, based on the method suggested by Williamson and Kligman[20]. A stainless tube, with an interior area of 4.909 cm^2, was set on the selected part of the skin, i.e. forehead, cheek, and chest, and then 1 ml of detergent[16] (0.01% $NaH_2PO_42H_2O$, 1.01% Na_2HPO_4, 0.1% Triton X-100 [pH 7.9]) was added to the tube. After rubbing the skin with a glass rod for one minute, the sample was removed using a pipette and stored in a different container. One ml of the detergent was then added to the stainless tube, and the specimen was sampled repetitively and added to the first sample. One hundred microliters of the sampled specimen was then mixed with 900 μl of the detergent with a 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[21].

Molecular analysis

1) DNA extraction & polymerase chain reaction (PCR)

For DNA extraction and PCR analysis of skin isolates, we adopted the colony PCR analysis[10], which was developed for extraction of DNA directly from a colony of a PCR tube and for amplification of 26S rDNA at the same time, instead of direct genomic DNA extraction methods. A colony of *Malassezia* yeast was removed and transferred to a PCR tube, then warmed 3 times a day for 1 minute, in a double boiler, using a microwave; the tube was then immersed in 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 performed. PCR, using the Mastercycler 5333 (Eppendorf, Hamburg, Germany), was then performed immediate-
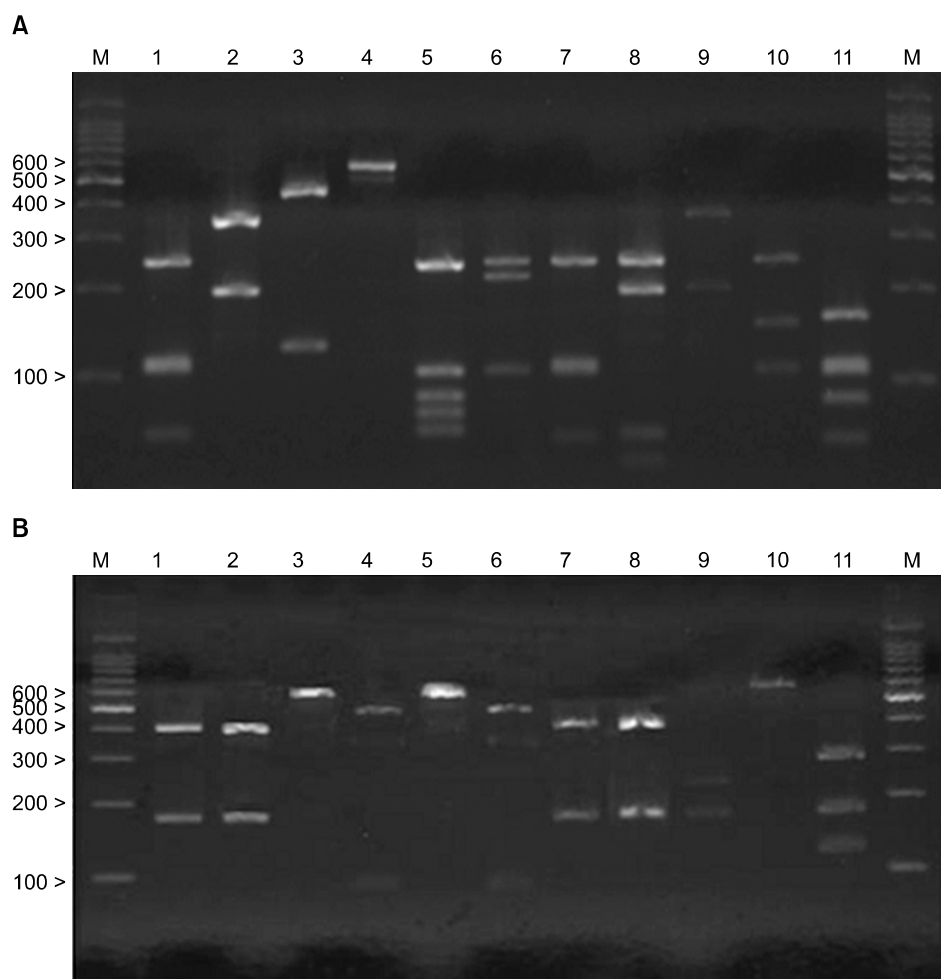
A

B

Fig. 1. PCR-RFLP patterns of 26S rDNA PCR digested with *Hha* I (A), and *Bst*F51 (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).

ly[22,23]. For amplification of 26S rDNA, a primer capable of amplifying all 11 standard strains at once was chosen. The sequence was as follows: forward, 5'-TAACAAGGATT-CCCCTAGTA-3' and reverse, 5'-ATTACGCCAGCATCCT-AAG-3'. Conditions in the early stage of the reaction were at 95°C for 14 minutes for pre-denaturation, 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), using 1X TAE migrating buffer (pH 8.0, 40 mM Tri-acetate 1 mM EDTA).

2) Restriction fragment length polymorphism (RFLP) analysis

After checking the amplified 26S rDNA, the product of PCR was purified using the Accu-Prep PCR purification kit (Bioneer, Daejeon, Korea). For 26S rDNA RFLP analysis of *Malassezia* yeasts, two restriction enzymes were used: *Hha* I (Takara Biomedicals, Otsu, Japan) and *Bts*F51 (SibEnzyme, Novosibirsk, Russia). The restriction enzyme reaction was conditioned with 10X PCR buffer, 10U

restriction enzyme, and reaction mixture, including 7.5 μl of the PCR product to become 20 μl. After three hours of reaction at 37°C, electrophoresis was performed in TAE buffer with 3.5% (w/v) NuSieve GTG agarose gel (FMC, Rockland, ME, USA) by 100 volts. It was then stained with ethidium bromide, and the size and number of DNA fragments were measured by a UV transilluminator for analysis of RFLP patterns[23] (Fig. 1).

Statistical analysis

Chi-squared tests (SPSS) were used to test differences in the distribution of the *Malassezia* species between patients with acne and healthy controls from equivalent age groups. Statistical significance was defined by the *p*-value less than 0.05.

RESULTS

Comparison of positive culture rates of acne patients and healthy controls

Malassezia yeasts were cultured in 90 out of 180 samples

collected from the forehead, cheek, and chest of 60 acne patients, yielding a 50% isolation rate, and a total of four species of *Malassezia* yeasts (i.e. *M. restricta, M. globosa,*

M. sympodialis, and *M. furfur*) were isolated. On the other hand, a total of five species (*M. globosa, M. restricta, M. sympodialis, M. dermatis,* and *M. furfur*) were recovered

Table 1. The recovery rate of the *Malassezia* species according to the age group

Age groups	Male (%)		Female (%)		Total (%)	
	HC (%)	AC (%)	HC (%)	AC (%)	HC (%)	AC (%)
11~20	21/30 (70.0)	15/30 (50.0)	17/30 (56.7)	9/30 (30.0)	38/60 (63.3)	24/60 (40.0)
21~30	24/30 (80.0)	28/30 (93.3)	20/30 (66.7)	15/30 (50.0)	44/60 (73.3)	43/60 (71.7)
31~40	25/30 (83.3)	14/30 (46.7)	20/30 (66.7)	9/30 (30.0)	45/60 (75.0)	23/60 (38.3)
Total	70/90 (77.8)	57/90 (63.3)	57/90 (63.3)	33/90 (36.7)	127/180 (70.6)	90/180 (50.0)

AC: acne patients, HC: healthy controls.

Table 2. The recovery rate of the *Malassezia* species according to the body sites

Body site	Male (%)		Female (%)		Total	
	HC (%)	AC (%)	HC (%)	AC (%)	HC (%)	AC (%)
Forehead	27/30 (90.0)	19/30 (63.3)	24/30 (80.0)	11/30 (36.7)	51/60 (85.0)	30/60 (50.0)
Cheek	21/30 (70.0)	17/30 (56.7)	15/30 (50.0)	7/30 (23.3)	36/60 (60.0)	24/60 (40.0)
Chest	22/30 (73.3)	21/30 (70.0)	18/30 (60.0)	15/30 (50.0)	40/60 (66.7)	36/60 (60.0)
Total	70/90 (77.8)	57/90 (63.3)	57/90 (63.3)	33/90 (36.7)	127/180 (70.6)	90/180 (50.0)

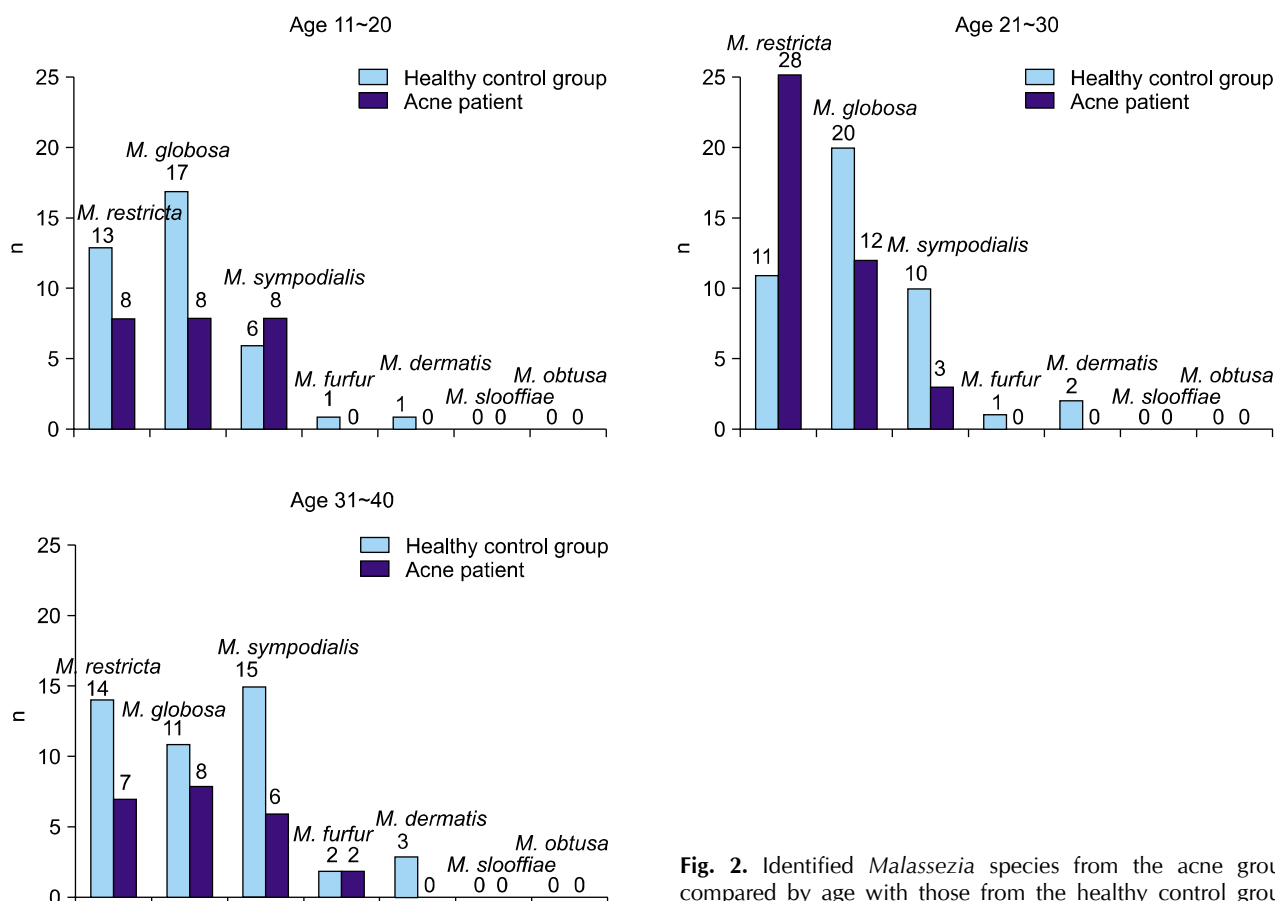AC: acne patients, HC: healthy controls.

Fig. 2. Identified *Malassezia* species from the acne group, compared by age with those from the healthy control group.

in the healthy control group. In terms of age, for the patients group, the culture rate was the highest in the twenties group (i.e. age 21 to 30), with 71.7% (43 of 60), followed by the teens group (24 of 60, 40%), and the thirties group (23 of 60, 38.3%). In the control group, the rate was the highest in the thirties (age 31 to 40), with 75% (45 of 60) (Table 1). In terms of body sites, for the patients group, the rate was the highest in the chest, with 60% (36 of 60), followed by 50% (30 of 60) in the forehead, and 40% (24 of 60) in the cheek. In the controls, the rate was the highest in the forehead, with 85% (51 of 60) (Table 2).

Species of *Malassezia* yeasts by age

Upon stratification of the isolated species of *Malassezia* yeasts according to age in the patients group, *M. restricta*

Table 3. Identified *Malassezia* species from the age groups - 26S rDNA PCR-RFLP analysis

Identified *Malassezia* species	AG 11~20		AG 21~30		AG 31~40		Total	
	HC (%)	AC (%)	HC (%)	AC (%)	HC (%)	AC (%)	HC (%)	AC (%)
M. restricta	13 (21.7)	8 (13.3)	11 (18.3)	28 (46.7)	14 (23.3)	7 (11.7)	38 (21.1)	43 (23.9)
M. globosa	17 (28.3)	8 (13.3)	20 (33.3)	12 (20.0)	11 (18.3)	8 (13.3)	48 (26.7)	28 (15.6)
M. sympodialis	6 (10.0)	8 (13.3)	10 (16.7)	3 (5.0)	15 (25.0)	6 (10.0)	31 (17.2)	17 (9.4)
M. furfur	1 (1.7)	0 (0.0)	1 (1.7)	0 (0.0)	2 (3.3)	2 (3.3)	4 (2.2)	2 (1.1)
M. dermatis	1 (1.7)	0 (0.0)	2 (3.3)	0 (0.0)	3 (5.0)	0 (0.0)	6 (3.3)	0 (0.0)
M. slooffiae	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
M. obtusa	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
No growth	22 (36.7)	36 (60.0)	16 (26.7)	17 (28.3)	15 (25.0)	37 (61.7)	53 (29.4)	90 (50.0)
p-value	0.073		0.006		0.002		<0.001	
Total	60 (100)	60 (100)	60 (100)	60 (100)	60 (100)	60 (100)	180 (100)	180 (100)

AG: age group, AC: acne patients, HC: healthy controls. $p < 0.05$ is considered as statistically significant.
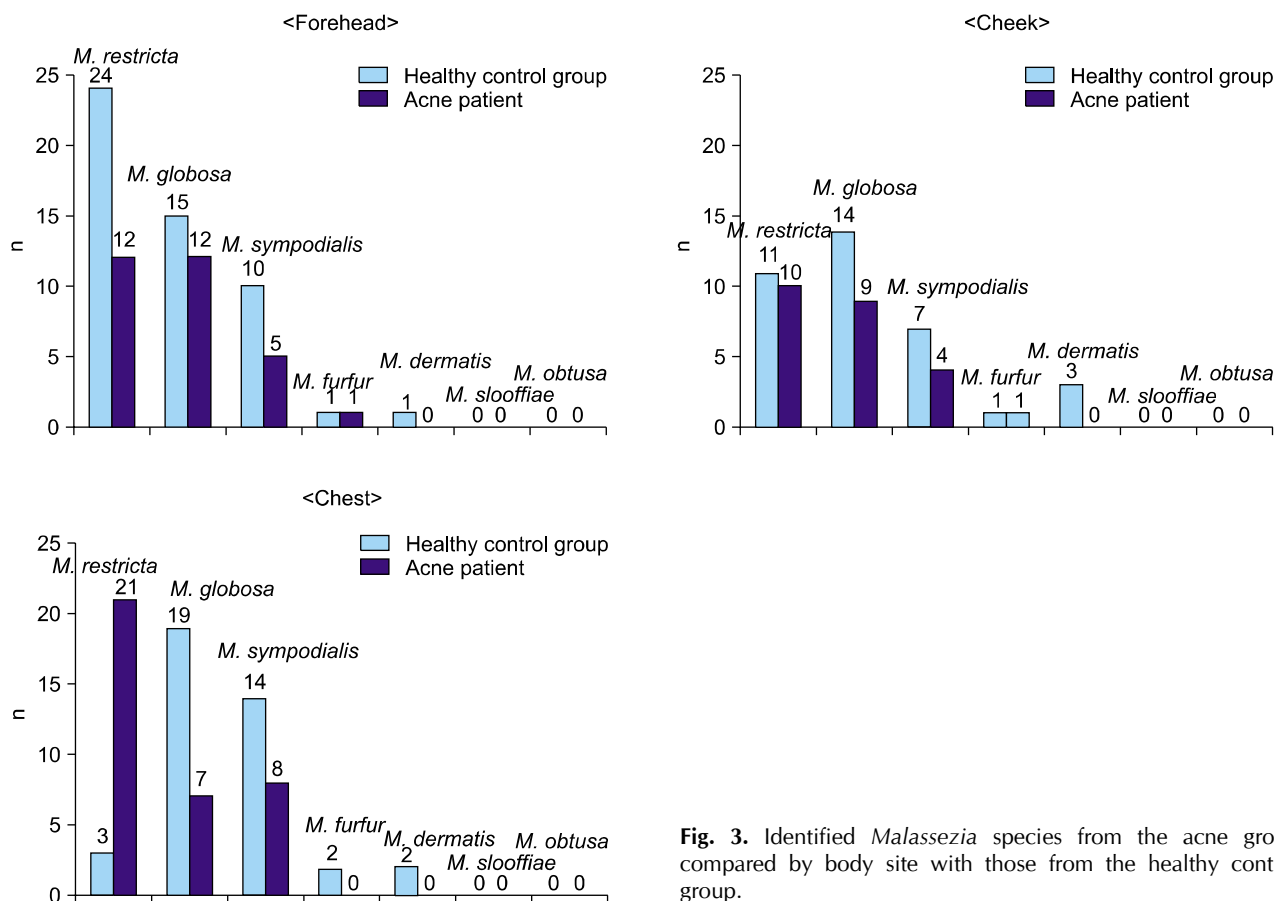

Fig. 3. Identified *Malassezia* species from the acne group compared by body site with those from the healthy control group.

Table 4. Identified *Malassezia* species from the investigated body sites - 26S rDNA PCR-RFLP analysis

Identified *Malassezia* speices	Forehead		Cheek		Chest		Total	
	HC (%)	AC (%)	HC (%)	AC (%)	HC (%)	AC (%)	HC (%)	AC (%)
M. restricta	24 (40.0)	12 (20.0)	11 (18.3)	10 (16.7)	3 (5.0)	21 (35.0)	38 (21.1)	43 (23.9)
M. globosa	15 (25.0)	12 (20.0)	14 (23.3)	9 (15.0)	19 (31.7)	7 (11.7)	48 (26.7)	28 (15.6)
M. sympodialis	10 (16.7)	5 (8.3)	7 (11.7)	4 (6.7)	14 (23.3)	8 (13.3)	31 (17.2)	17 (9.4)
M. furfur	1 (1.7)	1 (1.7)	1 (1.7)	1 (1.7)	2 (3.3)	0 (0.0)	4 (2.2)	2 (1.1)
M. dermatis	1 (1.7)	0 (0.0)	3 (5.0)	0 (0.0)	2 (3.3)	0 (0.0)	6 (3.3)	0 (0.0)
M. slooffiae	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
M. obtusa	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
No growth	9 (15.0)	30 (50.0)	24 (40.0)	36 (60.0)	20 (33.3)	24 (40.0)	53 (29.4)	90 (50.0)
p-value	0.003		0.196		<0.001		<0.001	
Total	60 (100)	60 (100)	60 (100)	60 (100)	60 (100)	60 (100)	180 (100)	180 (100)

AC: acne patients, HC: healthy controls. *p*<0.05 is considered as statistically significant.

was the most frequently isolated species in the twenties, with 46.7%, and *M. globosa* was most commonly identified in the thirties, with 13.3%; in the teens, *M. restricta, M. globosa*, and *M. sympodialis* were recovered in equal proportions (13.3%). In contrast, for the control group, *M. globosa* was the most predominant species in the teens (28.3%) and the twenties (33.3%) subgroups, while *M. sympodialis* was most commonly found in the thirties (25.0%) (Fig. 2). A statistically significant difference was observed between the patient and control groups in the twenties and thirties (Table 3).

Species of *Malassezia* yeasts by body sites

Upon stratification of the species of *Malassezia* yeasts according to body sites in the acne patients, *M. restricta* was most commonly isolated, with 43 cases (23.9%), which was higher than the rate in the control group, with 38 cases (21.1%). In the forehead, *M. restricta* was found in 20%, *M. globosa* in 20%, and *M. sympodialis* in 8.3%. In the cheek, *M. restricta* was isolated in 16.7%, *M. globosa* in 15%, and *M. sympodialis* in 6.7%. In the chest, *M. restricta* was recovered in 35%, *M. sympodialis* in 13.3%, and *M. globosa* in 11.7%. In the control group, *M. globosa* was most commonly isolated overall (48 of 180, 26.7%) (Fig. 3). Also, a statistically significant difference between the two groups was observed in two body sites, but not in the cheek (Table 4).

DISCUSSION

Acne is a chronic, self-limited inflammatory disorder of the pilosebaceous unit that peaks during puberty, when androgen production is suddenly increased. It commonly affects the face, neck, and chest. It is known to be caused by increased sebum production through the influence of

androgen, hyperkeratosis of the entry site of the follicle, and induction of inflammatory mediators due to growth of *P. acnes*[1]. Clinically, it manifests as various non-inflammatory lesions, such as comedon, papule, pustule, and nodule[24]. Although far from life-threatening, its potential consequences should not be underestimated, for it causes embarrassment, depression, low self-esteem, social withdrawal, and poor quality of life.

The organisms found in the acne lesions, alongside *P. acnes*, include *Malassezia*. Given the fact that *Malassezia* yeasts are capable of forming comedones, it is likely that *Malasszia* folliculitis might actually co-exist with deep acnes of the face, and many cases labeled "deep-seated acnes" might in fact have been *Malassezia*-associated lesions[19,25]. Also, medications for treatment of acne decrease the number of *P. acnes*, while the relative number of *Malassezia* yeasts increases; thus, treatment might seemingly result in no improvement[26]. In fact, according to one report, antifungal treatment is effective in cases of deep acnes with known presence of *Malassezia* yeasts[2,27].

Overall, when compared to normal controls, the isolation rate of *Malassezia* yeasts was conspicuously low in the acne patients group. This observation may be attributable to the fact that overgrowth of *P. acnes* may have brought about a relative decrease in the number of *Malassezia* yeasts. In this study, the age of the patients group was limited to the teens, twenties, and thirties. This is because the majority of cases affect ages 12 to 25. The pre-study hypothesis was that the isolation rate of *Malassezia* yeasts would be particularly lower in the teens and twenties, compared to that of the thirties; however, our study revealed that the isolation rate was rather higher in groups of patients in their teens and twenties, a finding that may be attributable to variation in the severity of skin lesions in the patients group.

In general, the most frequent site of acne is the face, followed by the back, chest, and neck. The cheek is the location where acne lesions are most severe, both in adults and adolescents. Also, in adults, the frequency of acne is known to be highest in the chin and around the mouth[28]. This led to speculation that the detection rate would be lower in the cheek, where the number of lesions is relatively higher, compared to the chest. In our study, the rate of isolation was lower in the cheek and the forehead, where the number of acne lesions is relatively high, compared to the chest.

According to the report by Kang and Kim[19], who conducted a direct smear test and culture for identification of the causative organisms on the facial comedones of patients diagnosed with acne vulgaris, the most frequently isolated organism in acne is *M. restricta* (28%), followed by *M. globosa* (13%). The results of the present study were very similar, with *M. restricta* being recovered in 23.9% and *M. globosa* in 15.6%. In particular, in the case of *M. restricta*, the rate was conspicuously higher in the twenties group, and on the chest in the patients group, and the difference was statistically significant. This cautiously brings up the possibility of *M. restricta* having a relationship with acne lesions of the chest. However, to this point, there has not been a sufficient number of studies on the relationship between development of acne and species of *Malassezia* yeast, which would support the results of this study. Also, this might be due to technical errors while sampling specimens, e.g. isolation of the strains from lesions that may be clinically confused with acnes (e.g., *Malassezia* folliculitis, miliaria pustulosa etc.). *Malassezia* folliculitis might be clinically confused with acne, for the main clinical manifestations are papules and pustules, with pruritus on the chest and back[2]. *Malassezia* folliculitis might coexist with acne lesions because the areas are equally populated by *Malassezia* yeasts, although the facial areas, such as the cheek, forehead, and chin, are the main locations of acne vulgaris lesions induced by *P. acnes*; furthermore, it may also be related to the report by Jacinto-Jamora et al.[29] that lesions in the forehead and the chin are in fact *Malassezia*-associated.

Given the outcome of the study, we have found a few clues. However, to this point, there has been no report clearly stating the relationship between acne and *Malassezia* yeasts, nor was this study successful in identifying the relationship. Therefore, further studies comparing inflammatory from non-inflammatory lesions in the distribution of *Malassezia* yeasts, and using large-scale quantitative analysis, will hopefully guide us to elucidation of the relationship between acne and *Malassezia* yeasts.

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