

# Comparison of Nested PCR and RFLP for Identification and Classification of *Malassezia* Yeasts from Healthy Human Skin

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**Background:** *Malassezia* yeasts are normal flora of the skin found in 75~98% of healthy subjects. The accurate identification of the *Malassezia* species is important for determining the pathogenesis of the *Malassezia* yeasts with regard to various skin diseases such as *Malassezia* folliculitis, seborrheic dermatitis, and atopic dermatitis. **Objective:** This research was conducted to determine a more accurate and rapid molecular test for the identification and classification of *Malassezia* yeasts. **Methods:** We compared the accuracy and efficacy of restriction fragment length polymorphism (RFLP) and the nested polymerase chain reaction (PCR) for the identification of *Malassezia* yeasts. **Results:** Although both methods demonstrated rapid and reliable results with regard to identification, the nested PCR method was faster. However, 7 different *Malassezia* species (1.2%) were identified by the nested PCR compared to the RFLP method. **Conclusion:** Our results show that RFLP method was relatively more accurate and reliable for the detection of various *Malassezia* species compared to the nested PCR. But, in the aspect of simplicity and time saving, the latter method has its own advantages. In addition, the 26S rDNA, which was targeted in this study, contains highly conserved base sequences and enough sequence variation for inter-species identification of *Malassezia* yeasts. (**Ann Dermatol 21(4) 352 ~ 357, 2009**)

## -Keywords-

26S rDNA PCR-RFLP, *Malassezia* yeasts, Molecular biological classification, Nested PCR

## INTRODUCTION

The *Malassezia* species are considered part of the normal flora of the skin and are associated with pityriasis versicolor, *Malassezia* folliculitis, seborrheic dermatitis, dandruff, atopic dermatitis and psoriasis<sup>1</sup>. In 1996 Gueho et al.<sup>2</sup> classified them into seven species: *M. furfur*, *M. pachydermatis*, *M. sympodialis*, *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae*. Recently, on the basis of common DNA sequences and molecular biology testing methods, four new species have been added: *M. dermatis*, *M. japonica*, *M. nana* and *M. yamatoensis*<sup>3-7</sup>.

Many investigators involved in *Malassezia* yeast research still use the morphological analysis of size, surface, color, and shape of the cultured colony and biochemical analysis for identification of this organism<sup>8-11</sup>. However, the morphological analysis is usually a time-consuming, multi-step process necessitating several experimental techniques; in addition, this approach does not take the taxonomic component into consideration, and thus the genetic link between species cannot be determined by conventional methods. Therefore, the morphological and biochemical methods currently used for the study of this yeast limit the identification and classification of new species.

To overcome the limits of morphological assessment, recent studies have used a variety of molecular methods such as the nested polymerase chain reaction (PCR)<sup>12</sup>, real-time PCR<sup>13</sup>, pulsed field gel electrophoresis (PFGE)<sup>14</sup>,

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amplified fragment length polymorphism (AFLP)<sup>15,16</sup>, denaturing gradient gel electrophoresis (DGGE)<sup>16</sup>, random amplification of polymorphic DNA (RAPD)<sup>16,17</sup>, single strand conformation polymorphism (SSCP)<sup>18</sup>, terminal fragment length polymorphism (tFLP)<sup>19</sup>, restriction fragment length polymorphism (RFLP)<sup>20-23</sup>, and sequencing analysis<sup>24</sup>.

To investigate a more accurate and rapid molecular approach to the identification and classification of *Malassezia* yeasts, we compared the accuracy and efficacy of the RFLP and nested PCR methods.

## MATERIALS AND METHODS

### Subjects and sample collection

Normal subjects included 110 healthy volunteers (60 males, 50 females); 0~80 years of age without any dermatoses of the examined regions. Sterile cotton swabs were moistened with wash fluid containing 0.1% Triton X-100 in 0.075 M phosphate buffer (pH 7.9), and rubbed gently, with rotation on the skin. Swabbing was performed for 10 sec on sites including: the scalp, forehead, cheek, chest, and thighs. The swabs were immediately placed in Leeming and Notman agar media. The investigations were conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from each subject before the procedure.

### Culture of samples

The yeast was cultured on agar plates with Leeming and Notman agar (LNA) - 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, glycerol (Tedia, Fairfield, USA) 1 ml, 10 ml of whole-fat cow's milk per liter that contained cycloheximide (Sigma, St Louis, MO, USA) 200 mg, and chloramphenicol (Sigma, St Louis, MO, USA) 50 mg. The plates were incubated at 34°C for 14 days.

### DNA extraction and PCR

The yeasts grown in agar were harvested and resuspended in 0.4 ml of lysis buffer (100 mM Tris-HCl pH 8.0, 1.0% SDS, 2.0% Triton X-100, 10 mM EDTA, 100 mM NaCl). Equal volumes of phenol/chloroform/isoamyl alcohol (phenol: chloroform: isoamyl alcohol=25:24:1, v/v) and glass beads (0.5 mm) were added, and the mixture was vortexed for 10 min. The samples were centrifuged at 12,000×g for 15 min. Total DNA was precipitated from

the aqueous fraction with isopropanol and centrifuged at 12,000×g for 20 min at 4°C. The DNA pellet was washed in 70% ethanol and resuspended in sterile water.

### 26S rDNA restriction fragment length polymorphism (26S rDNA RFLP analysis)

To amplify 26S rDNA from genomic DNA, the reaction mixture contained 25 mM of each dNTP, 10X PCR buffer, 5X Q buffer, 0.5 μM primer, 0.4 μM forward primer (5'-TAACAAGGATCCCCCTAGTA-3'), reverse primer (5'-ATTACGCCAGCATCCTAAG-3'), and 1.25 U Hot StarTaq polymerase in 50 μl reaction volume. Thirty five cycles with the following protocol were programmed: denaturation for 45 sec at 94°C; annealing for 45 sec 55°C; extension for 1 min at 72°C. After the confirmation of amplified 26S rDNA, the PCR products were purified using an Accu-Prep PCR purification kit (Bioneer, Daejeon, Korea). The absorbance at 260 nm and the 260/280 ratio (as a measure of DNA quality) were obtained for each purified PCR product sample using the NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies) and the estimated concentration of DNA (ng/μl). The 26S rDNA was confirmed by running 50 ng of 26S rDNA on a 2% agarose gel. Two restriction enzymes, *Hha*1 (Takara Biomedicals, Otsu, Japan) and *Bst*F51 (SibEnzyme, Novosibirsk, Russia) were used to perform the 26S rDNA-RFLP study of *Malassezia*. In this experiment, the restriction enzyme digestion was performed with 10 X PCR buffer, 10 U of the restriction enzyme, and PCR products 7.5 μl, of each sample was examined, from which the maximum expected concentration was 50 ng/μl (1 μg DNA in 20 μl recovery solution), which added up to 20 μl. After the reaction at 37°C for 3 hours, the electrophoresis was performed with a 3.5% (w/v) NuSieve GTG agarose gel (FMC, Rockland, ME, USA) with 100 volts and stained with ethidium bromide. The restriction fragments were analyzed by the size and number of the DNA fragments under UV transillumination (Fig. 1).

### Genomic DNA nested PCR

To amplify rDNA from genomic DNA, a nested PCR was carried out using primers for *Malassezia*. The first amplification mix was carried out in a 20 μl reaction consisting of 2× pre mix (2 μl 10× buffer, 1.6 μl of 25 mM MgCl<sub>2</sub>, 0.25 μl of 10 mM dNTPs, nTaq-Hot DNA polymerase -Enzynomics-Korea), 1 μl of 2.5 μM primer and 10 ng of purified DNA. Primer pairs selecting for the *Malassezia nana* genes for ITS1, 5.8S rRNA, ITS2 are shown after an initial 5 min denaturation at 95°C, 20 cycles of 95°C for 30 sec, 53.5°C for 30 sec and 72°C for 1 min were carried out, followed by a 7 min extension at

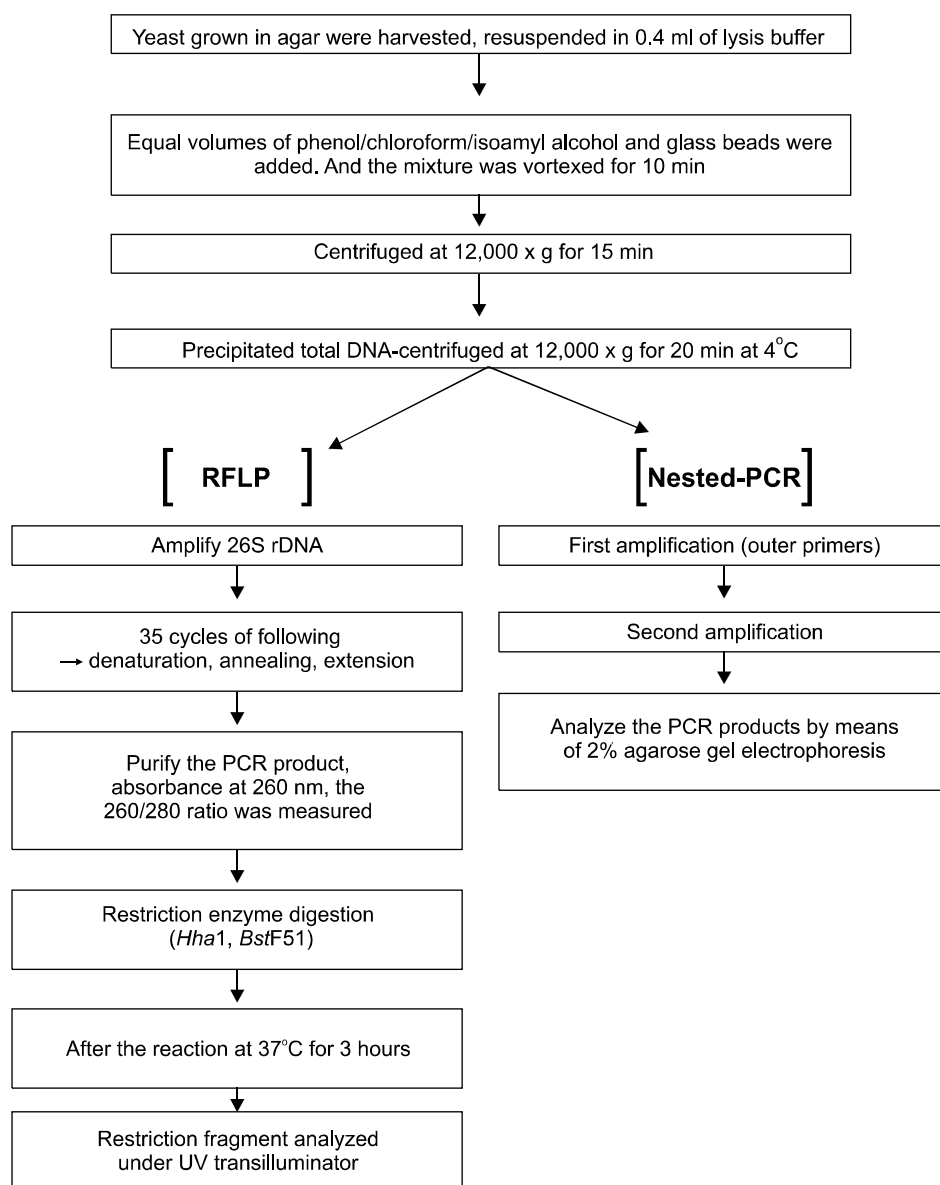


Fig. 1. Flow chart of the nested PCR and RFLP methods.

Table 1. Comparison of RFLP and nested PCR for identification of specific *Malassezia* yeasts from healthy human skin

Subtypes	RFLP (%) [A]	Nested PCR (%) [B]	Different sample [A-B]	Consistent rate (%) [B/A]
<i>M. globosa</i>	116 (33.1)	111 (31.7)	5*	95.6%
<i>M. sympodialis</i>	55 (15.6)	54 (15.4)	1 <sup>†</sup>	98.1%
<i>M. furfur</i>	39 (11.1)	39 (11.1)	0	100%
<i>M. restricta</i>	106 (30.3)	105 (30.0)	1 <sup>‡</sup>	99.1%
<i>M. slooffiae</i>	5 (1.4)	5 (1.4)	0	100%
<i>M. dermatis</i>	6 (1.6)	6 (1.7)	0	100%
Total	327	320	7	98.8%

\*3 *M. obtusa* (131 bp), 2 *M. nana* (114 bp) different from *M. globosa* (126 bp), <sup>†</sup>*M. nana* (114 bp) different from *M. sympodialis* (99 bp), <sup>‡</sup>*M. obtusa* (131 bp) different from *M. restricta* (137 bp).

72°C using a thermal cycler (Gene Amp PCR System 2400-Applied Biosystem, Monza, Italy). The reaction mixture for the second amplification round was the same as for the first, except for the "inner" primers [forward primer (5-GTCTCTGGCGCCTAACCCACTATA -3), reverse primer (5-TCCACGTACATACAACCATACGA-3)] were used instead of the "outer" primers [forward primer (5-GC-CATACGGACGATAA-3), reverse primer (5-AGCAAATGACGTATCATGCCATGC-3)]. For the second amplification round 49 µl of amplification mix and 1 µl of the first amplification round PCR product were used. The thermal cycling was repeated as for the first amplification round using 30 cycles after the initial 2 min denaturation. Each amplification run contained a negative control, consisting of water and a positive control. Analysis of the PCR products was performed by 2% agarose gel electrophoresis followed by visualization with ethidium bromide (0.5 µg/ml) staining and UV illumination to confirm the expected products (Fig. 1).

### Comparison of RFLP and nested PCR

Among the cultured samples of *Malassezia*, we first identified the species of the *Malassezia* yeasts using a 26S rDNA RFLP, and based on these findings, we conducted

the nested PCR and compared the results of the 26S rDNA RFLP and nested PCR.

## RESULTS

### Culture rate of *Malassezia* yeasts

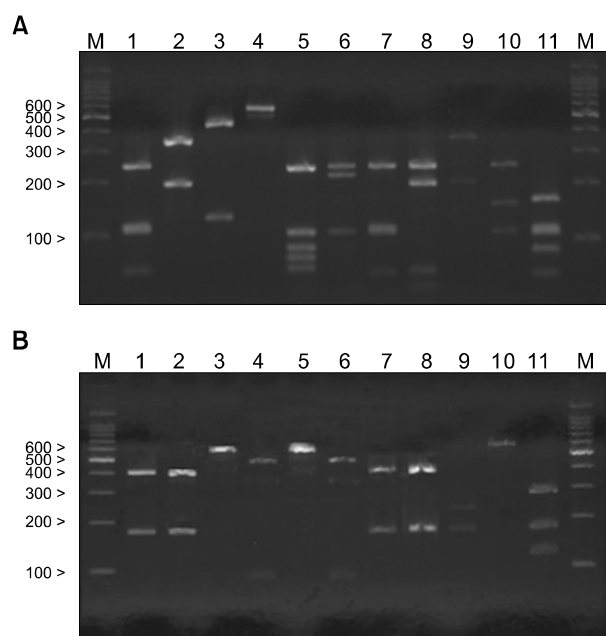
The overall positive culture rate of the *Malassezia* yeasts samples from different body sites of 110 persons was 63.6%, with 350 positive samples out of 550 samples.

### Molecular biological identification of *Malassezia* species using 26S rDNA PCR-RFLP analysis

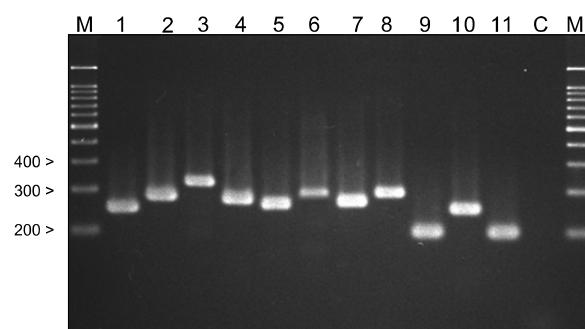
Using 26S rDNA PCR-RFLP analysis, we successfully identified 11 species of *Malassezia* yeasts from the standard *Malassezia* species (Fig. 2). In addition, we identified six species of *Malassezia* yeasts from 350 positive samples. The results showed that *M. globosa* was identified most frequently in 116 samples (33.1%); *M. restricta* in 106 samples (30.3%); *M. sympodialis* in 55 samples (15.6%); *M. furfur* in 39 samples (11.1%); *M. slooffiae* in 5 samples (1.4%), and *M. dermatitis* in 6 samples (1.6%). As for the 23 samples with co-identification of more than two *Malassezia* species, they were excluded from the analysis for a more direct comparison between the RFLP and nested PCR methods (Table 1).

### Molecular biological identification of *Malassezia* species using nested PCR

Using nested PCR analysis, we successfully identified 11 species of *Malassezia* yeasts from the standard *Malassezia* species (Fig. 3). In addition, 6 species of *Malassezia* yeasts were identified from 327 positive samples that were already confirmed by RFLP methods. These results



**Fig. 2.** 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. dermatitis* (JCM 11348); 10. *M. obtusa* (KCTC 7847); 11. *M. yamatoensis* (CBS 9725).



**Fig. 3.** Nested PCR products of standard *Malassezia* species. Lanes: M. molecular marker; 1. *M. dermatitis* (JCM 11348); 2. *M. furfur* (KCTC 7743); 3. *M. globosa* (CBS 7966); 4. *M. japonica* (CBS 9432); 5. *M. nana* (JCM 12085); 6. *M. obtusa* (KCTC 7847); 7. *M. pachydermatis* (KCTC 17008); 8. *M. restricta* (KCTC 7848); 9. *M. slooffiae* (KCTC 17431); 10. *M. sympodialis* (KCTC 7985); 11. *M. yamatoensis* (CBS 9725); C. negative control.

showed that *M. globosa* was identified most frequently in 111 samples (31.7%); *M. restricta* in 105 samples (30.0%); *M. sympodialis* in 54 samples (15.4%); *M. furfur* in 39 samples (11.1%); *M. slooffiae* in 5 samples (1.4%), and *M. dermatitis* in 6 samples (1.7%) (Table 1).

### Comparison of RFLP and nested PCR

The results of re-identifying and classifying *Malassezia* species using nested PCR among the 327 positive samples, where cases with two or more yeasts identified were excluded, showed that 320 out of 327 (98.8%) samples were identical with the results of the RFLP method. In cases with *M. globosa*, 111 out of 116 samples were identical, showing a 95.6% concordance rate, and among the 5 non-identical samples, 3 were later identified as *M. obtusa* (131 bp) and 2 as *M. nana* (114 bp) by the nested PCR. In addition, 105 out of 106 *M. restricta* samples identified by the RFLP method were identical, and the other 1 case was confirmed as *M. obtusa* (131 bp) by the nested PCR. As for *M. sympodialis*, 54 out of 55 samples were identical, and the other 1 case was confirmed to be *M. nana* (114 bp) by the nested PCR (Table 1).

## DISCUSSION

*Malassezia* yeasts are normal flora found on the skin of 75~98% of healthy persons, and has also been cited as a causative organism in pityriasis versicolor and as an aggravating factor in various skin diseases, including atopic dermatitis and seborrheic dermatitis<sup>1</sup>. Especially in atopic dermatitis, *Malassezia* yeast act as an allergenic aggravating factor rather than as infectious agents. Among the *Malassezia* species, sensitization to *M. sympodialis* is highly specific for patients with atopic eczema and does not occur in patients with only inhalant allergies, urticaria, or allergic contact dermatitis<sup>25</sup>. Therefore, the accurate identification of the *Malassezia* species is important to determine the pathogenesis of *Malassezia* yeasts in a variety of skin diseases.

Many different morphological and molecular methods have been used for the identification and classification of *Malassezia* yeasts. Most prior studies have used morphological and biochemical methods of analysis, which are time-consuming and subject to controversy, due to ambiguous objective criteria. Among the molecular methods, each method has different diagnostic accuracy for identifying *Malassezia* yeasts, and can also be inaccurate when used for the investigation of *Malassezia* yeast pathogenesis. Therefore, it is important to ascertain the efficacy of molecular methods including accuracy and

cost-efficacy.

In this study, we compared the accuracy and efficacy of RFLP and nested PCR, both relatively accurate methods for the identification of *Malassezia* yeasts. We already reported that 26S rDNA RFLP method was a sensitive and rapid method for identification system for *Malassezia* species, which showed more than 99% consistent rate with genebank homology of *Malassezia* yeasts when analyzing clinical isolates and performing 26S rDNA sequencing<sup>24</sup>. In the results of this study, although both methods showed relatively rapid and reliable results for identification, nested PCR method showed faster and time saving advantages, but some additional different *Malassezia* species (7 species: 1.2%) were identified by the nested PCR compared to the RFLP method. This can be explained by the fact that the nested PCR resulted in a single band differences in the electrophoresis of the *Malassezia* species from genomic DNA, which may have biased the analysis because of small differences among single band patterns. On the other hand, the RFLP method showed multiple band differences in the electrophoresis, which could help with a more precise identification of *Malassezia* species compared to the nested PCR method.

The RFLP method used in this study enables the examination of genetic variations by cleaving the amplified DNA with restriction enzymes and analyzing the patterns of the fragments. The improved accuracy and rapid diagnosis make this a desirable approach. The nested PCR has the advantages of simplicity and rapid turn around for results, but may be less accurate.

The 26S rDNA, which was targeted in this study, contains highly conserved base sequences and enough sequence variation for inter-specific identification. In addition, it is compatible with morphological methods and appropriate for the identification currently known *Malassezia* species; it requires only two restriction enzymes, *Hha* I, *BtsC* I, and has been proven to be technically easier to perform than other molecular techniques.

Our results show that RFLP method was relatively more accurate and reliable for the detection of various *Malassezia* species than the nested PCR method. But, in the aspect of simplicity and time saving, the nested PCR method has its own advantages. New, more rapid and precise, molecular methods for the identification and classification of *Malassezia* yeasts are needed; further studies will likely involve the quantitative analysis of *Malassezia* microflora using a real-time PCR assay.

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