

# Identification of *Malassezia* Species from Pityriasis Versicolor Lesions with a New Multiplex PCR Method

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**Abstract** Despite the fact that a range of molecular methods have been developed as tools for the diagnosis of *Malassezia* species, there are several drawbacks associated with them, such as inefficiency of differentiating all the species, high cost, and questionable reproducibility. In addition, most of the molecular methods require cultivation to enhance sensitivity. Therefore, alternative methods eliminating cultivation and capable of identifying species with high accuracy and reliability are needed. Herein, a multiplex polymerase chain reaction (PCR)-based method was especially developed for the detection of eleven *Malassezia* species. The multiplex PCR was standardized by incorporating a consensus forward primer, along with *Malassezia* species-specific reverse primers considering the sizes of the PCR products. In the method, the multiplex-PCR primer content is divided into three parts to circumvent the problem of increased nonspecific background resulting from the use of a large number of primers. DNA extraction protocol described by Harju and colleagues was modified using liquid nitrogen instead of  $-80^{\circ}\text{C}$  to break down the yeast membrane. By a modified extraction procedure followed by multiplex PCR and electrophoresis, the

method enables identification and differentiation of *Malassezia* species from both of the samples obtained directly from skin and yeast colonies grown in culture. Fifty-five patients who were confirmed with pityriasis versicolor were enrolled in the study. Multiplex PCR detected and differentiated all 55 samples obtained directly from the patients' skin. However, 50 out of 55 samples yielded *Malassezia* colony in the culture. In addition, eight of 50 colonies were misdiagnosed or not completely differentiated by conventional methods based on the sequence analysis of eight colonies. The method is capable of identifying species with high accuracy and reliability. In addition, it is simple, quick, and cost-effective. More importantly, the method works efficiently for the diagnosis of *Malassezia* species obtained directly from patient samples.

**Keywords** *Malassezia* · Pityriasis versicolor · Multiplex PCR

## Introduction

The *Malassezia* species are implicated in the pathogenesis of common skin diseases such as pityriasis versicolor (PV), atopic dermatitis, seborrheic dermatitis, and psoriasis. Additionally, the species *M. pachydermatis* and *M. furfur* can cause systemic infections [1, 2]. The major antifungal agents are the main choice treatment for *Malassezia* infection. However, there are variations in the susceptibilities of *Malassezia* spp. to

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major antifungal drugs, including tacrolimus, and the azole agents itraconazole and ketoconazole [3, 4].

On the basis of common DNA sequences and molecular methods, *Malassezia* species expanded to 14 species [5]. Remarkably, 10 of them including *M. globosa*, *M. restricta*, *M. sympodialis*, *M. furfur*, *M. obtusa*, *M. slooffiae*, *M. pachydermatis*, *M. dermatis*, *M. japonica*, and *M. yamatoensis* were found to be related to human diseases [2, 5]. Several investigators involved in *Malassezia* yeast research still use the culture-based techniques based on their morphology and biochemical features [6–8]. The phenetic analyses, however, are usually a time-consuming, multistep process, necessitating several experimental techniques [5].

To overcome this limitation, studies have used a variety of molecular methods such as the nested polymerase chain reaction (PCR), real-time PCR, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), denaturing gradient gel electrophoresis (DGGE), random amplification of polymorphic DNA (RAPD), single-strand conformation polymorphism, terminal fragment length polymorphism, restriction fragment length polymorphism (RFLP), and sequence analysis [5, 9]. Despite the usefulness of these molecular techniques, there are several drawbacks associated with them, such as inefficiency of differentiating all the species, requirement of technical devices, high cost of analysis, and questionable reproducibility. In addition, most of the methods mentioned above require cultivation to enhance sensitivity, thereby increasing both the potential for culture bias and the turnaround time for analysis [5].

The discrepancies between molecular methods and culture-based techniques showed that there is the need for well-controlled, comparative PCR studies of samples taken directly from the skin as well as the same samples following in vitro culturing. Therefore, objectives of this study were to increase the sensitivity of the method to eliminate the need for cultivation and thereby increase the detection rate and eliminate cultural bias in the results. In this investigation, an alternative multiplex PCR-based method developed for the detection of 11 *Malassezia* species is presented. By a modified extraction procedure followed by multiplex PCR and electrophoresis, the method enables identification and differentiation of 11 *Malassezia* species from both of the samples obtained directly from skin and yeast colonies grown in culture.

## Materials and Methods

### Standard Strains

Eleven reference strains, including *M. dermatis* (CBS 9,145), *M. furfur* (CBS 7,019), *M. japonica* (CBS 9,432), *M. globosa* (CBS 7,966), *M. nana* (CBS 9,561), *M. obtusa* (CBS 7,876), *M. pachydermatis* (CBS 1,879), *M. slooffiae* (CBS 7,956), *M. sympodialis* (CBS 7,222), *M. yamatoensis* (CBS 9,725), and *M. restricta* (CBS 7,877), were purchased from the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands.

### Patients

A total of 62 patients clinically suspected of PV, referred to the Mycology Laboratory of the Ankara University of School of Medicine, were recruited. Fifty-five patients were confirmed with PV, based on the presence of both hyphae and yeasts in direct microscopy; the *Malassezia* spp. were isolated from 50 patients. The samples from the 55 patients and 50 *Malassezia* spp. grown in the culture were used in the tests.

### Collection and Culture of Sample

Samples were collected by scraping the skin with a sterile blunt scalpel blade in sterile petri dishes. The amount of samples is around 5–10 mg. The only inclusion criterion was the presence of a sufficient amount of material for investigation by DNA extraction and culture analysis. Half of the samples were used directly for DNA extraction, and the other half was inoculated in modified Leeming–Notman agar (MLNA) (1 % w/v peptone, 1 % w/v glucose, 0.2 % w/v yeast extract, 0.8 % desiccated ox bile, 0.1 % v/v glycerol, 0.05 % w/v glycerol monostearate, 0.5 % v/v Tween 60, 2 % v/v oleic acid, and 1 % w/v agar in distilled water) supplemented with cycloheximide (0.5 %) and chloramphenicol (0.05 %) as recommended by Sugita et al. [10]. The culture was incubated at 32 °C for 2 weeks.

### Morphological and Physiological Characteristics

Growth in Sabouraud dextrose agar (SDA) (Becton, Dickinson and Company, NJ, USA), morphological

examination on MLNA after incubation at 32 °C for 7 days, Tween 20, 40, 60, and 80 utilization, and catalytic reactions were performed [11–13].

#### Designing Primers for M-PCR

The forward primer assigned as ITSANA-F was chosen from highly conserved regions within 5.8S ribosomal RNA of *Malassezia* species using a multiple DNA sequence alignment program (<http://www.ebi.ac.uk/clustalw>). Reverse primers for the individual *Malassezia* species were chosen from the *Malassezia* strains' DNA sequences considering specificity to individual strains' DNA sequences and size of the PCR product. The primers for the strains and the expected size of the PCR products are shown in Table 1. The GenBank accession numbers of the sequences used in the study are as follows: *M. dermatitis* AY390285, *M. furfur* HM014469, *M. japonica* EF140669, *M. globosa* KC152884, *M. nana* EF140667, *M. obtusa* GU291269, *M. pachydermatis* FJ545240, *M. slooffiae* EU915458, *M. sympodialis* AY743650, *M. yamatoensis* AB125262, and *M. restricta* EU400587. In addition to these, the sequences of the other strains of the individual species were also considered during the primer selection. The accuracy of the designed primers was confirmed by NCBI-BLAST program. Primers were synthesized by the company Integrated DNA Technologies, Coralville, IA.

#### DNA Extraction

For DNA extraction from both the scraped skin samples and the fresh yeast colonies grown in culture, methods of Harju et al. [14] with some modification were used. Skin samples or colonies were resuspended in 200 µL of lysis buffer [2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA (pH 8.0)]. The tubes were placed in liquid nitrogen until they were completely frozen and thawed at boiling water for 30 s. The process was repeated two times, and the tubes were vortexed vigorously for 30 s at each repetition. Two hundred microliters of phenol-chloroform was added, and the tubes were vortexed for 2 min and then centrifuged for 3 min at room temperature at 10,000×g. The aqueous layer was transferred to a tube containing 500 µL of ice-cold 100 % ethanol. The samples were centrifuged for 12 min at 4 °C at 12,000×g. Supernatants were discarded, and DNA pellets were washed with 1 mL of 70 % ethanol followed by drying at room temperature. DNA was resuspended in 50 µL double-distilled H<sub>2</sub>O. The whole DNA extraction procedure takes 30–40 min.

#### DNA Amplification

DNA amplification was carried out in sterile ultrapure water with PCR buffer, with 1.5 mmol/L MgCl<sub>2</sub> (Fermentas-Life science technologies,

**Table 1** Primers, sequences, and the expected sizes of PCR products

Primer name	Sequence	Expected sizes of PCR product when used with ITSANA-F (bp)
ITSANA-F	CGAAACGCGATAGGTAATGTG	
RestrictaR	ATC CGC CAA GCC GTT TCA	150
SympoR	ATTCGGGCGAGCCT GGT ACA	204
JapR	CTC TGC CGC CCT CCC TTC G	256
GlobosaR	ACC AAATGCTCT CCT ACA GCA	332
PachyR	CGC ACA GTACCT TTC GGC AA	140
ObtusR	TTTCGG GCG AGC CTG TTT AGC	207
FurR	TCC CTT TCA GAG CGG TTT GC	252
DermatisR	GACCAGAATAAAAAGGGCTGTAT	345
SloffiaeR	TCCGCCGCCGCGTAGCGT CCT A	134
NanaR	GCG AGC CTA GCG GTC CAA GC	204
YamaR1	ATAGCC GACCAAGGC CAA GTC	250
ITSANA-R	CAAATGACGTATCATGCCATGC	340

Vilnius, Lithuania), 100  $\mu\text{mol/L}$  each of dNTPs (Fermentas-Life science technologies), 0.5  $\mu\text{mol/L}$  each of oligonucleotides, and 2.5 U of Taq DNA polymerase (Fermentas-Life science technologies), in a 0.2-mL sterile thin-walled PCR tube, with a total volume of 50  $\mu\text{L}$ . Using an Eppendorf Mastercycler personal thermocycler, template DNAs were initially denatured at 94 °C for 2 min. After this cycle, 39 thermocycles, each consisting of 30 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C, were performed. To ensure complete extension, the reaction mixtures were further incubated for 10 min at 72 °C. Amplified DNA was analyzed by gel electrophoresis using 2.0 % (w/v) agarose in a Tris–borate buffer containing ethidium bromide for detection of DNA under ultraviolet transillumination. One-kilobase marker was used to confirm the size of resolved DNA.

#### Subcloning of PCR Products and PCR Sensitivity Assay

To obtain well-characterized positive controls and to determine the further sensitivity of the PCR method, we cloned the PCR products of each of the *Malassezia* strains into the pBSK vector. Briefly, individual virus DNA sequences were amplified with the primers and control yeast DNAs described herein above, followed by gel extraction and cleaning of PCR product using the gene clean kit (GeneMark, Hopegen Biotechnology, Dali City, Taiwan). For subcloning of the PCR product into pBSK vector plasmid, first, pBSK-T PCR cloning vector was prepared. Briefly, pBSK vector was cut with EcoRV enzyme followed by the T nucleotide added to the ends of vector using dTP, DNA polymerase, and appropriate buffer (Fermentas-Life science technologies). The individual *Malassezia* PCR products were subcloned into pBSK-T vector using T4 ligation enzyme (Fermentas-Life science technologies). Prepared pBSK-*M. dermatitis*, pBSK-*M. furfur*, pBSK-*M. japonica*, pBSK-*M. globosa*, pBSK-*M. nana*, pBSK-*M. obtusa*, pBSK-*M. pachydermatis*, pBSK-*M. slooffiae*, pBSK-*M. sympodialis*, pBSK-*M. yamatoensis*, and pBSK-*M. restricta* plasmids were used to define the M-PCR sensitivity. To determine the sensitivity of the PCR assay, we diluted individual plasmid stock preparations at  $1 \times 10^4$  copies/ $\mu\text{L}$  in tenfold steps to  $1 \times 10^{-1}$  copies/ $\mu\text{L}$  and completed the PCR.

#### M-PCR

Multiplex PCR was performed in three PCR tubes considering the sizes of the PCR products to differentiate 11 *Malassezia* species. According to their sizes, the PCR products were categorized into four groups: Sizes of the *M. restricta*, *M. pachydermatis*, and *M. slooffiae* PCR products are between 135 and 150 bp; sizes of the *M. sympodialis*, *M. obtusa*, and *M. nana* PCR products are between 204 and 207 bp; sizes of the *M. japonica*, *M. furfur*, and *M. yamatoensis* PCR products are between 250 and 256 bp; and sizes of the *M. globosa* and *M. dermatitis* PCR products are between 330 and 345 bp as shown in Table 1. Primers for the three multiplex PCR tubes were arranged such that the first multiplex PCR tube contains primers for *M. restricta*, *M. sympodialis*, *M. japonica*, *M. globosa*, and ITSANA-F; the second multiplex PCR tube contains primers for *M. pachydermatis*, *M. obtusa*, *M. furfur*, *M. dermatitis*, and ITSANA-F; and the third multiplex PCR tube contains primers for *M. slooffiae*, *M. nana*, *M. yamatoensis*, and ITSANA-F.

#### DNA Sequencing

DNA sequencing was performed with BigDye terminator sequencing chemistry on an automatic DNA sequencer ABI 377 (Applied Biosystems, Foster City, CA, USA). DNAs of the seven *Malassezia* species which were unidentified or identified different with the conventional method were sequenced to verify the specificity of the multiplex PCR. PCR products were obtained using primers ITSANA-F and ITSANA-R from the DNAs of the individual colonies, and DNA sequencing was performed using the same primers. The n-BLAST program was used for sequence analysis.

#### Results

##### The Evaluation and the Efficacy of the Modified DNA Extraction Protocol

DNA extraction protocol described by Harju and colleagues was modified using liquid nitrogen instead of  $-80$  °C to break down the yeast membrane. Ten skin samples and 10 grown colonies were subjected to DNA extraction methods of Harju et al. and modified

method. Clear and strong bands were obtained from 20 PCRs in which DNAs were obtained using modified extraction method. No bands or weak bands were obtained from 20 PCRs in which DNAs obtained using DNA extraction protocol were described by Harju and colleagues.

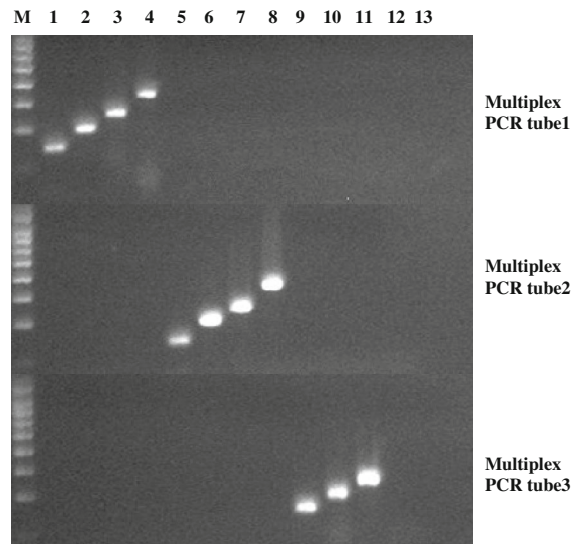
### Analysis of M-PCR Products

The sensitivity of combinations of primer pairs is tested with simple PCR and the consensus, and reverse primers that give the best results were selected. In this step, the sensitivity evaluation of the primer sets was made according to the density of the PCR products in agarose gel. The further sensitivity of multiplex PCR was determined using different dilution of individual pBSK plasmids containing PCR product of the individual *Malassezia*. We found that multiplex PCR can detect as low as 10–100 DNA copies in 50 µL of PCR tube depending on plasmids described in “Materials and Methods.” The next step includes performing multiplex PCR in three PCR tubes as described in “Materials and Methods.” The multiplex PCR can recognize and differentiate all individual DNAs of the strains in each PCR tube (Fig. 1). The sizes of the PCR products were observed around the range of 150, 200, 250, and 340 bp in the agarose gel as expected (Table 1).

### Specificity of Multiplex PCR

In the next experiment, the DNAs of the individual standard *Malassezia* strains were tested. As seen in Fig. 1, the first multiplex PCR tube identified and differentiated the DNAs of *M. restricta*, *M. sympodialis*, *M. japonica*, and *M. globosa*, but not the DNAs obtained from the other strains. The second multiplex PCR tube identified and differentiated the DNAs of *M. pachydermaditis*, *M. obtusa*, *M. furfur*, and *M. dermatis* individual DNAs, but not the DNAs obtained from the other strains, and the third multiplex PCR tube identified and differentiated the DNAs of *M. slooffiae*, *M. nana*, and *M. yamatoensis*, but not the DNAs obtained from the other strains.

In addition to DNAs of the *Malassezia* strains, DNAs of clinically isolated microorganisms, including *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Trichophyton rubrum*, *Microsporum canis*, and *Aspergillus fumigatus*, and human DNA were



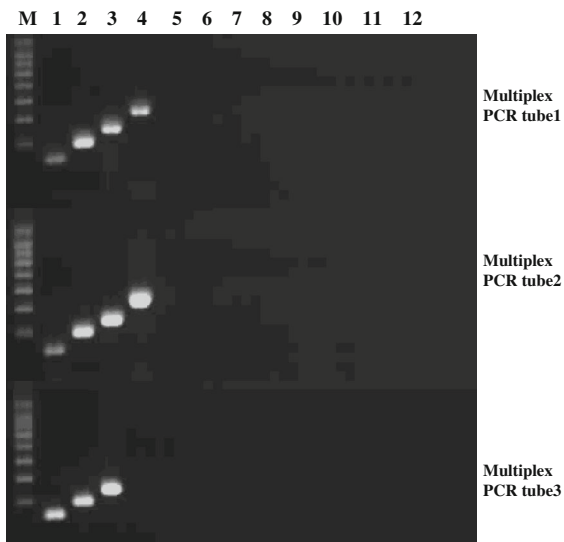
**Fig. 1** Results of M-PCRs using the first, second, and third M-PCR tubes with template DNAs obtained from standard *Malassezia* strains. *M* molecular size marker, 1 *M. restricta*, 2 *M. sympodialis*, 3 *M. japonica*, 4 *M. globosa*, 5 *M. pachydermaditis*, 6 *M. obtusa*, 7 *M. furfur*, 8 *M. dermatis*, 9 *M. slooffiae*, 10 *M. nana*, 11 *M. yamatoensis*, and 12 and 13 negative control

tested with each of the multiplex PCR tubes for the specificity of technique. No PCR products were obtained with template DNAs used with each of the M-PCR tubes (Fig. 2).

### Comparison of Conventional Methods and Multiplex PCR for Identification and Differentiation of *Malassezia* Strains

Of 62 skin scrapings, 55 were microscopically positive for *Malassezia* elements. Fifty-five samples were tested with the M-PCR. M-PCR diagnosed and differentiated *Malassezia* strains in all 55 samples (Table 2). Fifty (90 %) of the microscopically positive specimens yielded *Malassezia* colony in the culture. Grown colonies were tested with conventional methods described in “Materials and Methods” section. DNAs were extracted from colonies grown in culture and used in multiplex PCR. Identical multiplex PCR results were obtained with DNA templates extracted from the colonies or skin samples obtained from the patients (Table 2). The conventional methods, however, led to different results for eight yeasts grown in culture. While three colonies were identified as *M. obtusa* with the conventional methods, multiplex PCR





**Fig. 2** Evaluation of the specificity of the M-PCRs. M. molecular size marker, 1, 2, 3, and 4 are positive controls, for M-PCR tube 1: 1 *M. restricta*, 2 *M. sympodialis*, 3 *M. japonica*, and 4 *M. globosa*; for M-PCR tube 2: 1 *M. pachydermaditis*, 2 *M. obtusa*, 3 *M. furfur*, and 4 *M. dermatis*; for M-PCR tube 3: 1 *M. slooffiae*, 2 *M. nana*, 3 *M. yamatoensis*, 5 *S. aureus*, 6 *E. coli*, 7 *C. albicans*, 8 *T. rubrum*, 9 *M. canis*, 10 *A. fumigatus*, 11 human DNA, and 12 negative control

identified them as *M. globosa*. In addition to this, the conventional methods were unable to differentiate four *Malassezia* strains completely (Table 2). The PCR products of each of yeasts were sequenced. Sequences obtained were subjected to a BLAST search with registered sequences in the GenBank database. Results of sequence analysis with the BLAST program revealed that out of the examined yeasts, three were *M. globosa* and the remaining five were *M. slooffiae*, which is the same result obtained with multiplex PCR (Fig. 3; Table 2).

## Discussion

A multiplex PCR-based diagnostic assay can use different primers specific to different species in a single test, which means that it can combine different diagnostic tests for different microorganisms into a single, comprehensive, all-encompassing test. However, using a large number of primers in a single PCR may increase the nonspecific background. An approach to decrease nonspecific background is using

consensus forward and reverse primer pair [15]. In this study, a new multiplex PCR technique that can diagnose and differentiate 11 *Malassezia* species is developed. In this new technique, we divided the multiplex-PCR primer content into three parts to circumvent the problem of nonspecific background resulting from the use of a large number of primers in a single PCR. In addition to this, we used consensus forward primer, which could recognize all of *Malassezia* strains in the multiplex PCR, to decrease the primer numbers in each multiplex PCR tube. It is assumed that these approaches decrease nonspecific background and increase the sensitivity and specificity.

As culture-based methods do not always allow the species-specific identification, a range of molecular methods have been developed as tools to diagnose and differentiate *Malassezia* strains [5, 9]. Fingerprinting molecular methods such as DGGE, PFGE, RAPD, and AFLP have been employed to screen genomes for the identification and differentiation of *Malassezia* spp. In spite of their utility, each technique has its own limitation. Most of them are time-consuming to conduct and are thus less suited as a routine diagnostic tool. For example, although useful, DGGE and PFGE are time-consuming to perform and technically difficult, and PFGE requires large amounts of high molecular weight genomic DNA for analysis. RAPD has limited reproducibility of results. AFLP is relatively time-consuming to conduct (because of the digestion and ligation steps involved) [5]. Importantly, most of the methods mentioned above require cultivation to perform the test. Five studies have been reported to differentiate complex *Malassezia* strains from skin without cultivation [16, 17]. Four of them require either separate amplification with specific primer sets for each species or restriction digestion [16–18]. The other study used nested PCR which needed more than one PCR. In addition to time and technical requirements, the method was used for the detection of only six *Malassezia* species [17].

Therefore, in this study, the focus is placed on testing the multiplex PCR with the samples taken directly from the skin. Initially, the effectiveness of multiplex PCR is validated with DNA templates obtained from the skin and standard *Malassezia* strains using extraction method described by Harju et al. [14]. Since relatively low-yielding PCR products or no products were obtained from both samples, DNA

**Table 2** Results of M-PCRs with template DNAs obtained from the skin of patients and from the yeast grown in culture and the results of conventional methods

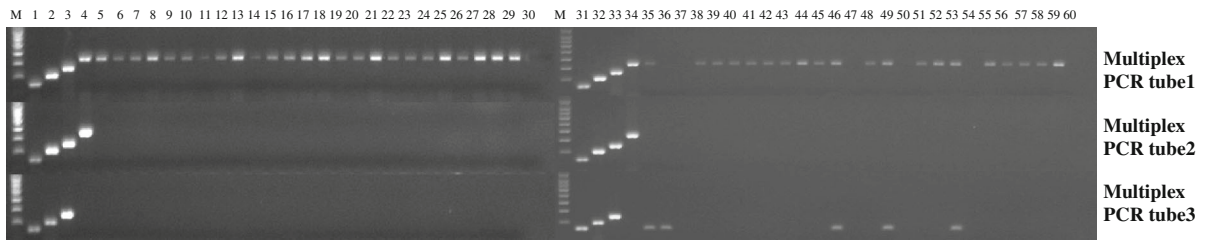
Sample no	Result of M-PCR using DNAs obtained from skin	Result of M-PCR using DNAs obtained from the yeasts grown in culture	Result of conventional methods
1	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. obtusa</i>
2	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
3	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
4	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
5	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
6	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
7	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
8	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
9	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
10	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
11	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
12	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
13	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. obtusa</i>
14	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
15	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
16	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
17	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
18	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
19	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
20	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. obtusa</i>
21	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
22	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
23	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
24	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
25	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
26	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
27	<i>M. sloffiae</i>	<i>M. sloffiae</i>	<i>M. furfur–M. sloffiae?</i>
28	<i>M. sloffiae</i>	<i>M. sloffiae</i>	<i>M. furfur–M. sloffiae?</i>
29	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
30	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
31	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
32	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
33	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
34	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
35	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
36	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
37	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>

**Table 2** continued

Sample no	Result of M-PCR using DNAs obtained from skin	Result of M-PCR using DNAs obtained from the yeasts grown in culture	Result of conventional methods
38	<i>M. sloffiae</i>	<i>M. sloffiae</i>	<i>M. furfur–M. sloffiae?</i>
39	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
40	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
41	<i>M. sloffiae</i>	<i>M. sloffiae</i>	<i>M. furfur–M. sloffiae?</i>
42	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
43	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
44	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
45	<i>M. sloffiae</i>	<i>M. sloffiae</i>	<i>M. furfur–M. sloffiae?</i>
46	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
47	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
48	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
49	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
50	<i>M. globosa</i>	<i>M. globosa</i>	<i>M. globosa</i>
51	<i>M. globosa</i>	No growth	
52	<i>M. globosa</i>	No growth	
53	<i>M. globosa</i>	No growth	
54	<i>M. globosa</i>	No growth	
55	<i>M. globosa</i>	No growth	

extraction method is found to be ineffective. Hence, liquid nitrogen was used instead of the  $-80^{\circ}\text{C}$  freezer to break down the yeast membrane. This modification in DNA extraction increased sensitivity of the multiplex PCR dramatically in both samples obtained from the skin and the yeast grown in culture.

The samples from the 55 patients diagnosed with PV were tested with the multiplex PCR. The multiplex PCR identified and differentiated *Malassezia* species in all the 55 samples obtained directly from the skin. The isolation rate of *Malassezia* spp. was found to be 90 % similar to some other studies [19, 20]. In addition to low isolation rate of conventional method, eight samples were misdiagnosed or not completely differentiated with the conventional method. DNA sequence analysis of the seven strains confirmed that results of the multiplex PCR were correct. Similar to our results, most studies have shown some



**Fig. 3** Results of multiplex PCRs using DNAs obtained from the skin of the patients. M, 1-kb-molecular-size marker: For the multiplex PCR tube 1, lanes 1 and 31 are positive control of *M. restricta*; lanes 2 and 32 are positive control of *M. sympodialis*; lanes 3 and 33 are positive control of *M. japonica*; and lanes 4 and 34 are positive control of *M. globosa*. For the multiplex PCR tube 2, lanes 1 and 31 are positive control of *M. pachydermatitis*; lanes 2 and 32 are positive control of *M. obtusa*; lanes 3 and 33 are positive control of *M. furfur*; and lanes 4 and 34 are

positive control of *M. dermatis*. For the multiplex PCR tube 3, lanes 1 and 31 are positive control of *M. slooffiae*; lanes 2 and 32 are positive control of *M. nana*; and lanes 3 and 33 are positive control of *M. yamatoensis*. For the multiplex PCR tube 1 and tube 2, lanes 5–29, 35–59 are the results of the multiplex PCR performed for DNA templates obtained directly from the patients. For the multiplex PCR tube 3, lanes 4–28, 34–58 are the results of the multiplex PCR performed for DNA templates obtained directly from the patients

discrepancies between phenotypic and molecular-based identifications of *Malassezia*. In a study of 46 clinical isolates, identified using a conventional phenotypic approach as *M. furfur*, 22 were genetically identified as *M. sympodialis* and five as *M. slooffiae* by sequencing of the ITS-1 region [21]. In another study, an apparent misidentification rate of 13.8 % was recorded by comparing the results from AFLP as well as ITS sequencing with those based on the physiological identification of *Malassezia* spp. For example, one isolate identified previously as *M. furfur* was classified as *M. sympodialis*, and three isolates identified as *M. globosa* were categorized as *M. sympodialis* [22].

The multiplex PCR tests performed with templates obtained from the yeast–serum mixture showed that M-PCR can effectively identify *Malassezia* extracted from the serum. Therefore, one may conclude that the multiplex PCR can be used for the identification and differentiation of *Malassezia* that causes systemic infections [2].

In conclusion, a new multiplex PCR method is developed that can be applied for the identification of 11 *Malassezia* strains. This new method requires only PCR, therefore technically less demanding than most other molecular biological approaches and conventional methods. The method is capable of identifying species with high accuracy and reliability and would be simple, fast, and cost-effective for application. More importantly, the method works efficiently for the diagnosis of *Malassezia* species obtained directly from patient samples.

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**Conflict of interest** None.

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