Rapid and accurate identification of microorganisms contaminating cosmetic products based on DNA sequence homology

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Introduction

Because cosmetics are applied directly to human skin, contamination of such products by microorganisms should be carefully avoided. Since cosmetics are usually kept at room temperature and contain large amounts of nitrogen and carbon sources, they may easily become contaminated by a variety of microorganisms, such as bacteria, filamentous fungi, and yeasts. The rapid and accurate identification of these microorganisms is essential to prevent further expansion of such contamination and the damage it causes. However, more than 30 days and laboratory skills are usually necessary in order to identify microorganisms in cosmetic materials. These time and labor constraints may allow further damage of the cosmetic products and thereby harm the consumer.

By the recent development of molecular biological techniques, huge quantities of DNA sequence data from a wide range of organisms, including microorganisms, have been compiled in DNA databases. Since these DNA sequence data are available on the Internet, anybody can use them to search

for DNA sequence homologies and thus identify the contaminating species.

We report a procedure for the rapid and accurate identification of microorganisms contaminating cosmetic products, through the use of the nucleotide sequences of rDNA regions.

Materials and Methods

DNA extraction, PCR, and Sequencing Mycelia or colonies grown on agar media were placed in a microtube containing 400 μL of AP1 buffer of a Dneasy Plant Mini Kit (Qiagen). Several glass beads were then put into the tube and the microorganisms were homogenized using FastPrep FP120 (BIOgene). The other steps were performed according to the manufacturer's instructions.

PCR amplification The nuclear rDNA region spanning the ITS1, ITS2, and 5.8S rRNA gene was amplified twice by PCR. Primers ITS5 (White et al. 1990) and P3 (Kusaba and Tsuge 1995), and the nested primer set ITS5 and ITS4 (White et al. 1990) were used for the first and second amplification, respectively. The following thermal cycling conditions were performed in a thermal cycler TP-400 (TaKaRa): an initial denaturing step at 95℃ for 2 min; thermocycling for 30 cycles, where each cycle consisted of 30 sec at 95℃ followed by 30 sec at 52℃ for annealing, and 30 sec at 72℃ for extension; and a final extension cycle of 7 min. at 72□. The PCR product from the second amplification was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. All amplifications yielded only a single visible DNA product. The DNA product band was excised from the ethidium bromide stained gel (Fig. 1) and purified using a JETSORB kit (GENOMED) following the manufacturer's protocol.

DNA sequencing Direct sequencing of PCR products was done in a CEQ2000XL (BECKMAN COULTER) sequencer using a CEQ DTCS Quick Start kit (BECKMAN COULTER) following the manufacturer's protocol. Four primers, ITS2 (White et al. 1990), ITS4, ITS5, and T4 (Hirata and Takamatsu 1996), were used for sequencing in both directions (Fig. 2).

Database Search

Database search was using the FASTA program in the URL site of the DNA Database of Japan (DDBJ: http://www.ddbj.nig.ac.jp/Welcome-j.html).

RESULTS

Example 1.

A particular microorganism was found to grow at the inside of the cap of a bottle of lotion used for monitoring test (Fig. 3). The microorganism was cultured on a sterilized cellophane sheet on GP agar containing 50mg/L of chloramphenicol, and incubated at 28℃ for 5 days (Fig. 4). The DNA sequence of the rDNA ITS region was determined and a DNA database search was performed according to the method described in "Materials and Methods". The result showed that the DNA sequence of the microorganism was completely identical to the sequence of a mitosporic fungus *Aureobasidium pullulans* isolate BK (AY225163) (Fig. 5). The fungus regarded as a black, yeast-like fungus. Hyphae of this fungus are septate, and hyaline or brown in color. This fungus prefers humid environments, and is frequently found in bathrooms and on fermented food products. The color of the colony changes from pale-pink to dark brown during cultivation (H.Takatori et al2002).

Example. 2

A particluar microorganism was found to grow in a bottle of lotion used for monitoring tests. Themicroorganism was cultured on a SCD agar, incubated at 28℃ for 5 days, and kept at 4℃ for 6 months (Fig. 6). The stock colony of the microorganism was put into a microtube containing 400 ⌈L of AP1 buffer without subculture, and subjected to DNA extraction and sequencing. The result indicated that the DNA sequence of the microorganism was almost identical to the sequence of a *Rhodotorula mucilaginosa* strain CB (AF444541, 99.837%) (Fig. 7) . The fungus is known as a red yeast-like fungus, which has ball-shaped or elongated budding cells, and does not have pseudomycelium, chlamydospore, or a capsule. This fungus prefers humid environments, and is frequently found in bathrooms, on food products, on the skins of animals and humans, and in human intestines. Its colony is orange in color. Sometimes it becomes an allergen. Its pigment is produced at middle or low temperatures, but not at high temperatures (H.Takatori et al2002).

DISCUSSION

In this paper, we reported a rapid and accurate method of identifying microorganisms contaminating cosmetic products based on the DNA sequence of the rDNA ITS region and a DNA database search of FASTA. We were able to finish the identification within 3-7 days. In general, almost one month is necessary for the identification of microorganisms in order to check their morphological, physiological, biochemical characteristics. Expert skills are required for such identification, and misidentifications occur frequently. Some kinds of identification kits are now available for public use, but those kits do not cover a wide range of microorganisms. A huge number of nucleotide sequence data are being compiled in DNA databases. The ribosomal DNA of Eucaryotes consists of 18S rDNA, 5.8S rDNA, 28S rDNA, and the spacer regions (ITS region). It is well known that the evolution rate of nucleotide sequences differs among the rDNA regions (Takamatsu 1998). Among them, 5.8S rDNA is not suitable for the present research because of its short length and low evolution rate. The 18S rDNA region is also conservative. However, the region is available for molecular identification if the complete sequence of the region would be available. The D1, D2 regions of 28S rDNA may be more suitable for current use than the 18S rDNA, because of its faster evolution rate. Yet the amount of the sequence data compiled in the DNA databases is not sufficient for practical use. The evolution rate of the rDNA ITS region is much faster than those of the other rDNA regions, and thus a 100–200 base nucleotide sequence may be sufficient to use for species identification. A huge number of DNA sequence data are available in the DNA databases. Therefore, the rDNA ITS region is the best target DNA for the purpose of molecular identification of eucaryotic microorganisms. The result of this research work will contribute to the rapid and accurate identification of microorganisms contaminating cosmetic products.

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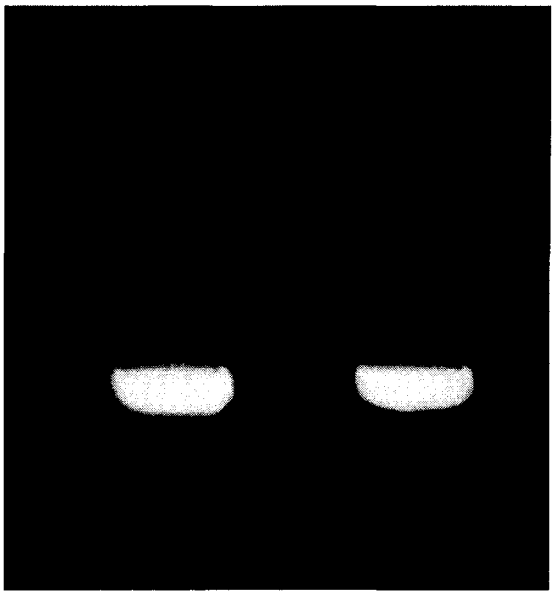

Fig. 1. PCR product detected in the ethidium bromide stained gel

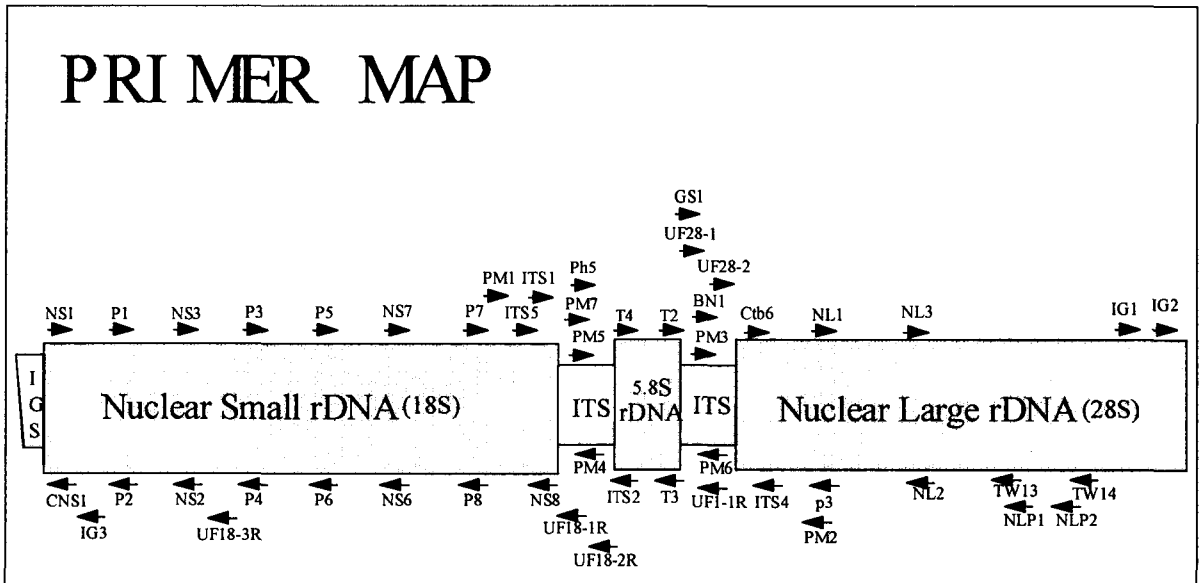

Fig. 2. Four primers, ITS2 (White et al., 1990), ITS4, ITS5, and T4 (Hirata and Takamatsu, 1996), used for sequencing in both directions .

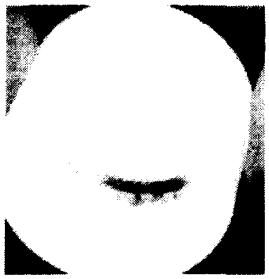

Fig. 3. Microorganism found to grow at inside of the cap of a bottle of lotion used for monitoring test .

Fig. 4. Microorganism cultured on a sterilized cellophane sheet on GP agar containing 50mg/L of chloramphenicol.

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ID
XX
SQ   Sequence  480 BP;  117 A;  116 C;  124 T;  123 G;  0 other.
     GTAAAAGTCG TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTA AAGAGTAAGG      60
     GTGCTCACGG CCCGACCTCC AACCCTTTGT TGTTAAAACT ACCTTGTTGC TTTGCCCGGA     120
     CCGCTCCGTC TCGACCCCCT GCCGATTCGT CCCACGCGAG CCCCCCCCAG AGTTAAACCA     180
     AACTCTTGTT ATTTAACCGG TCGTCTGAGT TAAAATTTTG AATAAATCAA AACTTTCAAC     240
     AACGGATCTC TTGGTTCTCG CATCGATGAA GAACGCAGCG AAATGCGATA AGTAATGTGA     300
     ATTGCAGAAT TCAGTGAATC ATCGAATCTT TGAACGCACA TTGCCCCCCT TGGTATTCCG     360
     AGGGGCATGC CTGTTCGAGC GTCATTACAC CACTCAAGCT ATGCTTGGTA TTGGGTGCCG     420
     TCCTTAGTTG GCCCCGCCTT AAAGACCTCG GCGAGGCCTC ACCGGCTTTA GGCGTAGTAG     480
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Fig. 5. AY225163|AY225163.1 Aureobasidium pullulans isolate BK (589 nt) initn: 2375 init1: 2375 opt: 2375 Z-score: 958.1 expect() 4.6e-46
100.000% identity in 475 nt overlap (6-480:1-475)

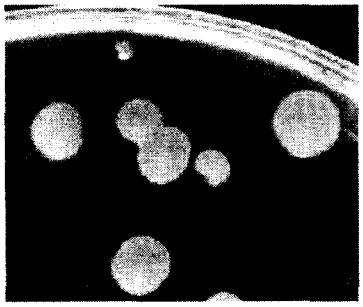

Fig. 6. Microorganism found to grow in a bottle of lotion used for monitoring test .

ID
XX
SQ Sequence 632 BP; 181 A; 129 C; 185 T; 137 G; 0 other.

GTAAAAGTCG TAACAAGGTT TCCGTAGGTG AACCTGCGGA AGGATCATTA GTGAATATAG 60
GACGTCCAAC TTAACTTGGA GTCCGAACTC TCACTTTCTA ACCCTGTCCA CTTGTTTGGG 120
ATAGTAACTC TCGCAAGAGA GCGAACTCCT ATTCACTTAT AAACACAAAG TCTATGAATG 180
TATTAAATTT TATAACAAAA TAAAACTTTC AACAACGGAT CTCTTGGCTC TCGCATCGAT 240
GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT 300
CTTTGAACGC ACCTTGCGCT CCATGGTATT CCGTGGAGCA TGCCTGTTTG AGTGTCATGA 360
ATACTTCAAC CCTCCTCTTT CTTAATGATT GAAGACGTGT TTGGTTTCTG ACGCCTCCTG 420
GCCTTTACGG TCTAGCTCGT TCGTAATGCA TTAGCATCCG CAATCGAACT TCCGATTGAC 480
TTCGCGTAAT AGACTATTCG CTGAGGAATT CTAGTCTTCG GATTAGAGCC GGGTTCGGTT 540
AAAGGAACCT TCTAATCAGA ATGTCTACAT CTTAAGATTA GATCTCAAAT CACGTACGAC 600
TACCCCCTGA ACTTAAGCAT ATCAATAAGC GG 632

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Fig. 7. AF444541|AF444541.1 Rhodotorula mucilaginosa strain CB (620 nt) initn: 3051 init1: 3051 opt: 3051 Z-score: 1207.4 expect() 5.8e-60 99.837% identity in 612 nt overlap (21-632:1-612)