A Unique Isolate of *Malassezia* from a Cat

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ABSTRACT. An isolate of *Malassezia* from a cat with otitis externa was examined mycologically as well as molecularly. The isolate was similar to *M. sympodialis* in morphological and biochemical characteristics. In molecular analysis, however, it differed from the 7 species of *Malassezia* previously reported. Therefore, this clinical isolate from a cat might be a new species of *Malassezia*.

KEY WORDS: feline, *Malassezia*, molecular analysis.

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Malassezia species are recognized as members of microbiological flora of human and animal skin. *Malassezia* species are also etiological agents of pityriasis versicolor and *Malassezia* folliculitis in humans and otitis externa in animals [5]. *M. pachydermatis* and *M. sympodialis* have been isolated from otitis externa of cats [4, 8].

Malassezia species are identified by morphological, biochemical and molecular characteristics [9, 10, 16]. Molecular typings are applied to these species and sequences of the ribosomal internal transcribed spacer 1 (ITS1) region located between 18S and 5.8S ribosomal DNA and the 5' end of large-subunit (26S) ribosomal DNA (D1/D2 region) have been utilized for the identification of *Malassezia* species [6, 13]. In our previous study, we sequenced and analyzed the chitin synthase 2 (*CHS2*) gene of the 7 recognized species of *Malassezia* in order to uncover their phylogenetic relationships [1, 2, 12].

The present study describes a unique isolate of *Malassezia* isolated from otitis externa of a cat. This clinical isolate was investigated by morphological, biochemical and molecular analyses.

The clinical isolate was obtained from a 6-month-old female Japanese domestic cat which had been deserted. The cat was referred with the chief complaint of ear discharge to the Yasuda Animal Hospital, Nishinomiya, Hyogo, Japan. Physical examination revealed pruritus and discharges from both ears. Microscopic examination disclosed yeast cells in the specimens of ear discharge.

The yeast cells were cultured on modified Dixon agar [9] and Sabouraud's dextrose agar at 37°C for 1 week. After 1 week of incubation on modified Dixon agar, the colonies which developed were glistening, smooth and convex, and had an average diameter of 2 mm (Fig. 1). The texture of the colonies was soft and viscous. Microscopic examination of the isolate revealed ovoid to globose cells with nar-

row base (Fig. 2). These yeast cells showed monopolar budding. No growth was obtained on Sabouraud's dextrose agar.

The biological analysis was carried out by the method of Guého *et al.* [9]. The catalase reaction and DBB reaction were positive. No growth occurred on glucose/peptone agar with 10% Tween 20 as the sole source of lipid [9]. Growth was observed on glucose/peptone with supplements of 0.1–10% Tween 40, 60 or 80 [9]. Good growth occurred at 37°C, with a maximum temperature of 40–41°C.

These results suggested that the clinical isolate was similar to *M. sympodialis* in biochemical analysis [9].

The yeast cells were collected by scraping colonies from the surface of the agar plate. From these cells, high molecular weight DNAs were obtained as reported previously [12]. These samples were dissolved in TE buffer (10 mM

Fig. 1. Clinical isolate cultured on modified Dixon agar at 37°C for 1 week.

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Fig. 2. Microscopic examination of the clinical isolate revealed ovoid to globose cells with narrow base.

Tris-hydrochloride, pH 8.0 and 1 mM EDTA), then used for polymerase chain reaction (PCR) amplification [1, 2, 12].

The sequences of the degenerate primers for the *CHS2* gene were based on the sequences of *M. pachydermatis* (in accession no. AB016719), reported in our previous study: primer Mala S1, 5'-CAC TCG AAC CAT GCA TGG TGT-3' (nt. 39–59 in *M. pachydermatis CHS2*) and primer Mala R1, 5'-CAC CAC AGG CAC CGC CTA C-3' (nt. 517–535 in *M. pachydermatis CHS2*) [1, 2, 12]. This primer pair amplified the conserved region of the *CHS2* gene in other fungi [3]. PCR amplification of the *CHS2* gene was carried out for 35 cycles consisting of template denaturation (1 min, at 94°C), primer annealing (2 min, at 50°C) and polymerization (3 min, at 72°C).

The sequences of the degenerate primers for the *ITS1* region were the same as those used in our previous study (primer 18SF1, 5'-AGG TTT CCG TAG GTG AAC CT-3' and primer 58SR1, 5'-TTC GCT GCG TTC TTC ATC GA-3') [13]. PCR amplification of the *ITS1* region was carried out for 25 cycles consisting of template denaturation (1 min, at 94°C), primer annealing (15 sec, at 60°C) and polymerization (15 sec, at 72°C).

The sequences of the degenerate primers for 26S ribosomal DNA were based on the sequences reported by Fell *et al.* (primer F63, 5'-GCA TAT CAA TAA GCG GAG GAA AAG-3' and primer LR3, 5'-GGT CCG TGT TTC AAG ACG-3') [6]. PCR amplification of the 26S ribosomal DNA was carried out for 35 cycles consisting of template denaturation (1 min, at 94°C), primer annealing (2 min, at 55°C) and polymerization (2 min, at 72°C).

The PCR products were electrophoresed through 2 % agarose gel and then stained with ethidium bromide.

The sizes of the amplifications with the degenerate primers for *CHS2* gene, *ITS1* region and 26S ribosomal DNA were 497 bp, 182 bp and 577 bp, respectively.

The cloning and sequencing of the PCR products were performed according to the methods of previous reports [1, 2, 12]. To examine phylogenetic relationships, the nucleotide

Fig. 3. A tree showing the phylogenetic relationships of *CHS2* gene fragments of the clinical isolate and the seven recognized *Malassezia* species. The bootstrap values are generated from 1,000 bootstrap trees. The parentheses indicate the GenBank accession nos. of the species.

otide sequences were analyzed by CLUSTAL W multiple sequence alignment programs [18] and a phylogenetic tree was constructed using the TREEVIEW program for displaying phylogenesis [14]. Bootstrap analysis was performed on 1,000 random samples taken from multiple alignments as described by Felsenstein [7], and analyzed by the CLUSTAL W programs.

The nucleotide sequence analysis of the *CHS2* gene fragment of the clinical isolate showed 99.2% similarity to that of *M. pachydermatis* reported previously [12]. The result indicated that the isolate might be *M. pachydermatis*, since intraspecies sequence variation of the *CHS2* gene fragment is less than 1% in *Malassezia* species [12]. Phylogenetic analysis of the *CHS2* gene fragment of the isolate revealed that it was genetically close to *M. pachydermatis* than *M. sympodialis* (Fig. 3).

The nucleotide sequence analysis of the *ITS1* region of the clinical isolate showed 70.1% similarity to that of *M. sympodialis* reported previously [13]. Species identification and strain typing by the *ITS1* region are carried out by the method of cluster analysis. Congenous strains were shown to group into identical clusters and are classified accordingly [13]. In phylogenetic analysis of the *ITS1* region, the isolate was genetically distinct from the 7 recognized *Malassezia* species (Fig. 4).

The nucleotide sequence analysis of 26S ribosomal DNA

Fig. 4. A tree showing the phylogenetic relationships of *ITS1* region of the clinical isolate and the seven recognized *Malassezia* species. The bootstrap values are generated from 1,000 bootstrap trees. The parentheses indicate the GenBank accession nos. of the species.

of the clinical isolate showed 96.9% similarity to that of *M. sympodialis* reported previously [6]. Intraspecies sequence variation of 26S ribosomal DNA is less than 1% [15, 17]. Therefore, the difference between the isolate and *M. sympodialis* was beyond the intraspecies variation. Furthermore, in phylogenetic analysis of 26S ribosomal DNA, the isolate was genetically distinct from the 7 recognized *Malassezia* species, as in the case of phylogenetic analysis of *ITS1* region (Fig. 5).

The molecular analysis of *ITS1* region and 26S ribosomal DNA of the clinical isolate suggested that the isolate might be a new species of *Malassezia*, though biochemical study and *CHS2* gene analysis showed that the isolate might be *M. sympodialis* and *M. pachydermatis*, respectively.

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Fig. 5. A tree showing the phylogenetic relationships of 26S ribosomal DNA of the clinical isolate and the seven recognized *Malassezia* species. The bootstrap values are generated from 1,000 bootstrap trees. The parentheses indicate the GenBank accession nos. of the species.

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