Molecular analysis of *Malassezia pachydermatis* isolated from canine skin and ear in Korea

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We investigated *Malassezia* species and genotypes colonizing dogs, comparing those recovered from the ear canal with those from other anatomical body sites, as well as from diseased and healthy skin. The *Malassezia* isolates were obtained from four types of skin samples, i.e., diseased ear, diseased skin, healthy ear, and healthy skin. Sequences of the 26S ribosomal DNA region, the intergenic spacer 1 (IGS-1) and the internal transcribed spacer 1 (ITS-1) DNA region were analyzed. These confirmed the presence of *Malassezia pachydermatis*, which could be separated into three main sequence genotype groups (A, B, C). Genotype A was the most common, only two genotype B isolates were recovered from diseased skin lesion and genotype C was more likely to be isolated from ear samples than from other healthy or diseased-skin sites. The present findings provide the basis for further studies of genotypic diversity in *M. pachydermatis*, as well as their pathogenic potential.

**Keywords** *Malassezia pachydermatis*, intergenic spacer 1 (IGS-1), internal transcribed spacer 1 (ITS-1)

**Introduction**

The lipophilic, non-mycelial, unipolar budding yeasts of the genus *Malassezia* populate the skin of warm-blooded vertebrates and occasionally are associated with skin disease [1,2]. *Malassezia* spp. are primarily categorized into either lipid-dependent or non-lipid-dependent [3]. Lipid-dependent *Malassezia* species originally were thought to occur only on human skin [4], but such species as *M. furfur*, *M. globosa*, and *M. sympodialis* have since been isolated from healthy and diseased skin, the ear canals of dogs and cats [5–9], as well as humans. The non-lipid dependent *M. pachydermatis* is a major pathogen of animal skin and is detected particularly on the skin of dogs with seborrhic dermatitis, atopic dermatitis, or otitis externa [10,11]. Although zoophilic, *M. pachydermatis* also is a causative agent of nosocomial infections in high-risk infants [12,13] and can be detected as a commensal microorganism on the skin of dog owners [14].

Several genetic variants of *M. pachydermatis*, isolated from dog skin, have been found by sequence analysis of the large subunit (LSU) ribosomal DNA (rDNA), the internal transcribed spacer 1 (ITS-1), the intergenic spacer 1 (IGS-1) of rDNA, and the chitin synthase 2 gene (chs-2) [15–19]. DNA sequencing of ITS-1 is a rapid and useful method of genotyping *Malassezia* species to facilitate phylogenetic analysis of closely related isolates [20]. However, the IGS-1 region is thought to exhibit more intraspecific diversity than the ITS-1 region [19] and has been used to differentiate isolates of *Cryptococcus neoformans* and *C. gattii* [21], *Trichosporon* spp. [22], and *M. globosa* [23], *M. restricta* [24], *M. pachydermatis* [19].

Many predisposing factors have been proposed to explain the change of *Malassezia* species from commensals to pathogens, with increased humidity and sebum-rich condition reported as among the most important factors [9,25]. The frequencies and population sizes of *M. pachydermatis* vary across anatomical sites in dogs. Cafarchia *et al.* [26] described that certain *M. pachydermatis*
subgenotypes are localized on particular body regions. The external ear canal is regarded as more sebum-rich and humid than other parts of the body, which may influence its colonization by *Malassezia* species. We hypothesized that the sebum-rich and humid ear canal may be associated with a different distribution of *Malassezia* isolates compared to other body-sites.

In this study, *Malassezia* isolates recovered from the ear and other skin sites (e.g., nasal folds, axillary, inguinal, interdigital, and perianal regions) of dogs in Korea were investigated to evaluate the prevalence of lipid-dependent and non-lipid-dependent species. Furthermore, we investigated genotypes and subgenotypes by ITS-1 and IGS-1 DNA sequences, and the distribution of *M. pachydermatis* genotypes and subgenotypes in the sebum-rich and humid ear versus other parts of the skin.

**Materials and methods**

**Strains and animals**

We analyzed 134 of a total of 165 *Malassezia* isolates obtained from dogs referred to the Seoul National University Hospital for Animals (Korea) in 2010 and 2011. Sixty-one swab samples were obtained from ear canals and 73 from other skin sites. The study population included 21 intact male dogs, 28 neutered males, 27 intact females, and 22 spayed females (total of 98 dogs). The ages of the animals varied from 3 months to 20 years (mean age: 8.7 years). Shih Tzu (*n* = 25) and Cocker spaniel (*n* = 24) were the most common breeds but other breeds that were included were Beagle (*n* = 10), Maltese (*n* = 9), Yorkshire terrier (*n* = 7), Poodle (*n* = 4), and Miniature Schnauzer (*n* = 3). The remaining 16 dogs were of several other breeds. Animals with diseased skin or ear lesions were put on antifungal therapy for 4 weeks to verify that *Malassezia* yeasts were the causative agent. Antifungal agents used were oral ketoconazole at 5 mg per kg of body weight given twice a day or itraconazole at 10 mg per kg of body weight for 51, 396–404.

**Fungal DNA extraction and polymerase chain reaction (PCR)**

Freshly scraped yeast colonies were homogenized using TissueLyser (Qiagen, Hilden, Germany) with 5 mm-diameter stainless steel beads. Genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer’s instructions and aliquoted into 50 μl samples to be stored at −20°C until PCR amplification.

To identify the strains of *Malassezia*, 26S ribosomal DNA was amplified using the following primers: forward 5′-TAA CAA GGA TTC CCC TAG TA-3′, reverse 5′-ATT ACG CCA GCA TCC TAA G-3′ [30]. The PCR protocol used included denaturation at 94°C for 7 min; 30 cycles of template denaturation at 94°C for 45 sec, primer annealing at 55°C for 45 sec, polymerization at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were electrophoresed through a 1.5% agarose gel and stained with ethidium bromide. Fragments identified by a size of approximately 580 bp were purified using the MEGAquick-spin PCR & Agarose Gel DNA Extraction System (Intron Biotechnology, Seoul, Korea) for direct sequencing.

ITS-1 and IGS-1 regions were amplified with primers 18SF1 (5′-AGG TTT CCG TAG GTG AAC CT-3′ and 5′-TTC GGT CCT GCG TTT TCC ATC ATC GA-3′) [20] and with primers 26S-F (5′-ATC TTT TGC AGA CGA CTG GA-3′) and Mala-R (5′-TGC TTA ACT TCG CAG ATC GG-3′) [31]. PCR mixtures were pre-denatured at 94°C for 5 min followed by 30 to 35 cycles of 94°C for 1 min, 60°C for 30 sec, and 72°C for 30 sec for ITS-1, and by 30–35 cycles of 94°C for 45 sec, 57°C for 45 sec, and 72°C for 1 min for IGS-1. Both protocols included a final extension.
at 72°C for 7 min. Amplicons were purified using MEGAquick-spin PCR & Agarose Gel DNA Extraction System (Intron Biotechnology).

Cloning and direct sequencing

Purified amplicons were directly sequenced using Abi Prism BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) and analyzed on a 3730XL automatic sequencer (Applied Biosystems). For confirmation, a randomly chosen sample from each group was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) using the cloning system supplied by the manufacturer. Identification of M. pachydermatis was done by direct sequencing of the D1/D2 domains of the rDNA and comparing with sequences available in GenBank using the BLAST program (www.ncbi.nlm.nih.gov/blast). Nucleotide sequences for ITS-1 and IGS-1 were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). GenBank accession numbers for the ITS-1 region were: genotype A1, JQ619042; A2, JQ619044; A3, JQ619043; B1, JQ619045; C1, JQ619047; C2, JQ619046; C3, JQ619048; Gen Bank accession numbers for the IGS-1 region were: genotype 1a, JQ619029; 1b, JQ619030; 1c, JQ619031; 1d, JQ619032; 1e, JQ619033; 2a, JQ619034; 3a, JQ619035; 3b, JQ619036; 3c, JQ619037; 3d, JQ619038; 3e, JQ619039; 3f, JQ619040; 3g, JQ619041.

Phylogenetic analysis

Sequences were aligned using ClustalW software [32] and phylogenetic analyses were conducted using the MEGA v.4.0 program [33]. A bootstrap analysis was performed with 1,000 replications. Neighbor-joining [34] then was carried out according to the Kimura two-parameter model [35].

Statistical analysis

A two-proportion test was used to evaluate the difference between positive rates from diseased and healthy sites, the frequency of genotypes A and C, and the recovery rate of genotype C between Groups 1, 3 and Groups 2, 4. Statistical analyses were done using the Minitab® 16 program. $P < 0.05$ was considered statistically significant.

Results

Prevalence of Malassezia species

A total of 141 isolates of Malassezia spp. were recovered from 228 samples of diseased skin and ear sites of 98 dogs (61.8% rate of positivity). In contrast, 24 Malassezia positive cultures were recovered from 55 samples of healthy skin and ear (43.6% rate of positivity). Among this total of 165 isolates, only 134 were genotyped using the ITS-1 and IGS-1 regions. Fifty-two of these isolates were from the ear canal (Group 1) and 58 were derived from samples of other skin regions (Group 2). All 24 isolates from healthy sites were genotyped which included nine from ear canals (Group 3) and 15 from other skin sites (Group 4).

Confirmation of M. pachydermatis

BLAST analysis of the 26S ribosomal DNA gene sequences of the isolates revealed they were identical to several M. pachydermatis 26S ribosomal DNA sequences, including strain IFM52753 (AB118938), IFM52755 (AB118937), and CBS 1879 (AY743605, AB118941, AJ249952), showing 100% query coverage and 100% maximum identity.

ITS-1 and IGS-1 sequence analysis

The length of the ITS-1 region was approximately 250 bp (range 246–253 bp). The alignment for this region (Fig. 1) and its phylogenetic analysis identified the following seven subgenotypes; A1, A2, A3, B1, C1, C2, and C3 (Fig. 3). The IGS-1 region ranged from 546 – 939 bp in length and the alignment of each group are shown in Figure 2. The IGS-1 region was more variable than the ITS-1 region with
its alignment and phylogenetic analysis revealing 13 subgenotypes; 1a, 1b, 1c, 1d, 1e, 2a, 3a, 3b, 3c, 3d, 3e, 3f, and 3g (Fig. 3). Sequences from Group 3 isolates exhibited a conserved region characterized by a short repeat sequence (CAGCA)$_n$. Groups were classified based on their CAGCA repeat number. Sequenced amplicons could all be categorized into 1e of three major sequence types based on their levels of sequence similarity of the ITS-1 (93.7–99.6%) and IGS-1 (41.6–99.0%). Genotypic grouping results (ITS-1 = IGS-1; A = 1, B = 2, C = 3) were concordant among each locus and were considered to be identical to previously described genotypes A, B, and C [18].

Identification of M. pachydermatis genotypes and subgenotypes

Based on analyses of the ITS-1 and IGS-1 regions, the frequency of the distribution of individual genotypes was determined according to diseased and non-disease skin sites (Table 1). Six ITS-1 genotypes were identified among isolates recovered from diseased skin groups (Groups 1 and 2), three ITS-1 genotypes in isolates from healthy ear (Group 3), and four identified in healthy skin (Group 4). Twelve IGS-1 genotypes were defined in Groups 1 and 2, whereas four each were associated with Groups 3 and 4. The M. pachydermatis genotypes identified in ear canal samples were genetically more variable than those detected at other sites. Overall, subgenotype A1 of ITS-1 and subgenotype 1a of IGS-1 were detected most frequently (A1 70%, 1a 58%). ITS-1 genotype A was most prevalent in Groups 1, 2, and 4 ($P < 0.05$), but there was no statistical difference between genotypes A and C in Group 3 (healthy ear). Subgenotype B1 (IGS-1 2a) was the only genotype B that was present in both the diseased ear and the interdigital area. Genotype C occurred at other skin sites, but was more frequently detected from ear canal samples (36.5–55.5% vs. 5.1–13.3% at other sites; $P < 0.05$), regardless of their disease status.

Discussion

In total, 165 Malassezia isolates were recovered, more frequently from diseased than healthy sites ($P < 0.05$), which is in accord with previous studies [10,11,36]. These results may have been affected by various factors. For example, studies using sterile swabs, as in this investigation, resulted in a higher prevalence of Malassezia from specimens of diseased skin [11,37] when compared to direct microscopic examination which indicated no difference between samples from health ears and those with otitis [37]. The breed and age of the dogs may have influenced the prevalence of Malassezia isolates [37,38]. In addition, the population of Malassezia cells seems to play an important role in causing skin disease because the application of a large inoculum of M. pachydermatis to healthy dog ears resulted in otitis externa [39].

Since phenotypic identification methods are not very accurate for typing Malassezia isolates, the characterization of Malassezia species and isolates is best presently accomplished by genotyping, [18]. Furthermore, the genetic distinction among M. pachydermatis isolates recovered from dogs as assessed by diverse molecular methodologies has been applied as an epidemiological tool. In addition, high genotypic diversity of M. pachydermatis has been reported to be related to their host [19], body site distribution [26], healthy skin condition [40], and geographic origin [36]. The regions of ITS-1 and IGS-1 were chosen for use in this study because they are associated with more substantial intraspecies diversity than are the chs-2 or 26S LSU rDNA regions. The intraspecific variation of ITS-1 ranged from 0.4–6.4%, which suggests that this genomic region is more variable when compared to yeasts in general (<1% variability) [18,41]. IGS-1 sequences of M. pachydermatis exhibited 41.6–99.0% similarity, whereas in other Malassezia species similarity exceeds 85% [19]. This would indicate that the IGS-1 region of M. pachydermatis may be more diverse than in other Malassezia species, thus supporting its utility for strain characterization of this species.

The loci of ITS-1 and IGS-1 of M. pachydermatis isolates recovered from dogs grouped into three distinct genotypes (i.e., A, B, and C), which is in agreement with a previous study [18]. This was supported further by phylogenetic analysis. The numbers and frequencies of each genotype relative to various skin sites are summarized in Table 2. The M. pachydermatis isolates were categorized further into subgenotypes based on the ITS-1 and IGS-1 sequences. Four of the ITS-1 sequences were identical to those deposited previously (i.e., GenBank accession numbers: genotype A1, DQ915503; genotype A2, EU158826; genotype B1, DQ915504; genotype C2, DQ915505), and three novel sequences were identified. One IGS-1 sequence matched one already deposited in GenBank (subgenotype 3d; GenBank accession AB118602), and 12 IGS-1 sequences were novel. However, both loci were too variable to allow for estimation of relationships between subgenotypes and anatomical sites of isolation.

Genotype A was detected on most of the body sites sampled, indicating a common distribution which is in agreement with the results of a previous report that examined healthy and diseased skin sites of dogs [36]. In the present study, two isolates obtained from an ear exhibiting otitis externa and from an interdigital area with dermatitis, respectively, were classified as genotype B, indicating that this genotype is present in Korea and occurs on diseased
Fig. 2 Alignment of IGS-1 region sequence groups representing all *Malassezia pachydermatis* genotypes.
Malassezia pachydermatis isolated from canine skin and ear

Fig. 3  Phylogenetic analysis of ITS-1 (a) and IGS-1 (b) sequence data for Malassezia pachydermatis isolates using the Neighbor-Joining method (Kimura-2 parameter). Sequences of M. pachydermatis strains (a, b) and other Malassezia species (i.e., M. furfur, M. obtusa, M. yamatoensis, M. japonica) (a) taken from GenBank™ (http://www.ncbi.nlm.nih.gov/Genbank/index.html) were used as an out-group.
genotype B was localized exclusively on healthy skin [26], and that it was present in Japan and Europe [18,42], but not Brazil [36]. Genotype C occurred on various skin samples, but was more frequently recovered from ear samples than on other skin sites ($P < 0.05$, see Table 2) in the diseased groups (groups 1, 2). The number of isolates categorized from healthy groups (i.e., groups 3 and 4) was smaller, but genotype C was found predominantly in the ears compared to other healthy body sites.

The external ear canal is associated with unique physiological features, including lipid-rich cerumen (earwax) and high humidity due to its unique anatomical structure [43]. The cerumen of canine external ears may facilitate the growth of $M. \text{pachydermatis}$ [44], whereas human cerumen exhibits antifungal properties [45]. Additional factors of the skin microenvironment, including the presence of bacterial microbiota, pH, salts, immune response, as well as other biochemical and physiological features are considered to play an important role in yeast colonization [46]. Pathogenicity of the $\text{Malassezia}$ yeasts might depend on their adaptation to the skin with its different microenvironments, including lipid content and humidity. The unique properties of the external ear canal may explain why $M. \text{pachydermatis}$ genotype C was more frequently represented at this site. However, we could not find an

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*The recovery rate of Genotype C is higher in either healthy or diseased ear compared to other skin sites. **Statistically significant differences by two-proportions test ($P < 0.05$).
explanation for this in the present study and more work is needed. We assume that *M. pachydermatis* genotype C has an affinity to colonize sebum-rich and humid environments. The heterogeneity of *M. pachydermatis* and different characteristics of each group were addressed in a previous study. Kiss et al. [47] reported that *M. pachydermatis* is a heterogeneous species, which can be divided into two groups by its morphological, cultural, and biochemical features. In this study, we categorized three genotypes in *M. pachydermatis* using genetic features. Genotype C may have unique pathologic and physiologic features not detected in this earlier study. Based on these results, we hypothesize that different genotypes have different characteristics, and that there is a possibility *M. pachydermatis* can be further divided into additional genotypes.

This study clearly shows that *M. pachydermatis* has a variety of genotypes, and that pathogenicity may be linked to this genotypic diversity. In particular, genotype C of *M. pachydermatis* was predominantly found in the external ear canal of dogs. The reasons for the frequent colonization and pathogenesis of genotype C in the ear canal still needs explanation, Machado et al. reported that the distribution of particular *M. pachydermatis* genotypes over skin surfaces may depend on individual predispositions [36]. Also, different genotypes are associated with varying virulence [18,48]. The immune responses of the host may play an important role in the colonization of skin by yeasts [36]. The increased diversity found in this study could be due not only to geographic differences, but it may well be that *Malassezia* yeasts have adapted through genetic polymorphisms to various environments, such as differences in lipid contents. However, further study is needed to reveal the physiologic and pathologic features of *M. pachydermatis* genotype C. The present findings provide a basis for further discovery of diversity in *M. pachydermatis*, along with its pathogenic nature.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

**References**

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