Malassezia nana sp. nov., a novel lipid-dependent yeast species isolated from animals

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Five isolates of a novel species of the yeast genus *Malassezia* were isolated from animals in Japan and Brazil. Phylogenetic trees based on the D1/D2 domains of the large-subunit (26S) rDNA sequences and nucleotide sequences of the internal transcribed spacer 1 region showed that the isolates were conspecific and belonged to the genus *Malassezia*. They were related closely to *Malassezia dermatis* and *Malassezia sympodialis*, but were clearly distinct from these two species and the other six species of *Malassezia* that have been reported, indicating that they should be classified as a novel species, *Malassezia nana* sp. nov. Morphologically and physiologically, *M. nana* resembles *M. dermatis* and *M. sympodialis*, but can be distinguished from these species by its inability to use Cremophor EL (Sigma) as the sole lipid source and to hydrolyse aesculin. The type strain of *M. nana* is NUSV 1003^T (= CBS 9557^T = JCM 12085^T).

Malassezia species have been recognized as members of the microbiological flora of human and animal skin; they are also considered to be aetiological agents of pityriasis versicolor and *Malassezia* folliculitis in humans (Ashbee & Evans, 2002) and otitis externa in dogs. *Malassezia pachydermatis* and *Malassezia sympodialis* have been isolated from cats with otitis externa (Greene, 1998; Crespo *et al.*, 2000), whilst *Malassezia globosa*, *Malassezia furfur*, *Malassezia slooffiae* and *M. sympodialis* have been isolated from cattle with otitis externa (Duarte *et al.*, 1999, 2001).

The genus *Malassezia* includes lipophilic and globose to subglobose or ellipsoidal yeasts with monopolar budding (Boekhout, 1998). These species can be identified by morphological, biochemical and molecular characteristics (Guillot & Guého, 1995; Guého *et al.*, 1996). Molecular typing methods have also been applied to these species (Boekhout *et al.*, 1998; Senczek *et al.*, 1999; Gupta *et al.*,

2000; Theelen *et al.*, 2001; Gaitanis *et al.*, 2002; Gemmer *et al.*, 2002). Sequences of the D1/D2 domains of the large-subunit (26S) rDNA and nucleotide sequences of the internal transcribed spacer 1 (ITS1) region, which is located between 18S and 5.8S rDNA, have been utilized for the examination of these species (Fell *et al.*, 2000; Makimura *et al.*, 2000). Kano *et al.* (1999) and Aizawa *et al.* (1999, 2001) also sequenced and analysed the chitin synthase 2 gene of seven species of *Malassezia*, in order to understand their phylogenetic relationships.

Recently, an eighth species of *Malassezia*, newly isolated from the skin of a human with atopic dermatitis, was described as *Malassezia dermatis* by using molecular analysis (Sugita *et al.*, 2002). As a result of molecular analysis, we suggested that an isolate from a cat might belong to a novel species of the genus *Malassezia* (Hirai *et al.*, 2002). Moreover, four other isolates from cows were revealed to be conspecific with that isolate. The present study describes these five isolates as a novel species of *Malassezia*, for which the name *Malassezia nana* sp. nov. is proposed.

The origins of the novel isolates discussed in this study are given in Table 1. NUSV 1003^T was isolated from a cat with otitis externa in Hyogo, Japan (Hirai *et al.*, 2002). Modified Dixon (mDixon) agar was inoculated with discharge from the ear (Guého *et al.*, 1996) and incubated at 32 °C for

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Abbreviation: ITS1, internal transcribed spacer 1.

The GenBank/EMBL/DDBJ accession numbers for the 26S rDNA sequences reported in this paper are AB075224, AB105860–AB105862 and AY166596, and the accession numbers for the ITS1 region sequences are AB075223 and AB105863.

Table 1. Origins of strains of *M. nana*

Strain	Origin	Place of isolation
NUSV 1003 ^T	Cat (otitis externa)	Hyogo, Japan
VG Luz 776	Cow (otitis externa)	Minas Gerais, Brazil
VG Luz 979	Cow (otitis externa)	Minas Gerais, Brazil
VSF Geleia	Cow (normal ear canal)	Minas Gerais, Brazil
VM Pom Passarela	Cow (normal ear canal)	Minas Gerais, Brazil

1 week. The other four isolates were isolated from cerumen or ear discharges from cows, two of which had otitis externa and two of which were healthy, in farms at Minas Gerais, Brazil. These specimens were inoculated onto Mycosal agar medium (Difco) that was modified by addition of glucose (final concentration, 4%) and chloramphenicol (final concentration, 150 mg l⁻¹), olive oil having been added to the surface of the medium; the medium was then incubated at 32 °C for 1 week.

Isolates were cultured on mDixon agar and Sabouraud's dextrose agar at 32 °C for 1 week and were then analysed morphologically and biologically as described by Guého *et al.* (1996) and Hagler & Ahearn (1981). Furthermore, utilization of the polyoxyethylated castor oil Cremophor EL (Sigma), hydrolysis of aesculin (Mayser *et al.*, 1997) and precipitate production on mDixon agar (Hammer & Riley, 2000) were also examined.

Morphological and physiological characteristics of the five isolates were similar in many ways to those of *M. sympodialis* and *M. dermatis* (Guého *et al.*, 1996; Sugita *et al.*, 2002); however, the isolates can be distinguished from these two species by their inability to use Cremophor EL as the sole lipid source and to hydrolyse aesculin (Table 2). In terms of morphology, cells were comparatively small (1.5–2.0 × 2.5–3.0 µm) (Guého *et al.*, 1996). Because of this

morphological characteristic, we propose the name *M. nana* for the isolates.

Yeast cells were collected by scraping colonies from the surface of the agar plate. High-molecular-mass DNA was obtained from these cells by phenol/chloroform extraction. Samples were dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and used for PCR amplification (Kano *et al.*, 1999; Aizawa *et al.*, 1999, 2001). Some D1/D2 rDNA sequences were also obtained by direct amplification from whole cells (Lachance *et al.*, 1999).

Sequences of degenerate primers for 26S rDNA amplification were based on the sequences reported by Fell *et al.* (2000) (forward primer, F63: 5'-GCATATCAATAAGCGG-AGGAAAAG-3'; reverse primer, LR3: 5'-GGTCCGTGT-TTCAAGACG-3'). PCR amplification of 26S rDNA was carried out for 35 cycles, each of which consisted of template denaturation (1 min at 94 °C), primer annealing (2 min at 55 °C) and polymerization (2 min at 72 °C).

Sequences of degenerate primers for the ITS1 region were the same as described previously (forward primer, 18SF1: 5'-AGGTTTCCGTAGGTGAACCT-3'; reverse primer, 58SR1: 5'-TTCGCTGCGTTCTTCATCGA-3'; Makimura *et al.*, 2000). PCR amplification of the ITS1 region was carried out for 25 cycles, each of which consisted of template

Table 2. Characteristics of *M. nana* and other *Malassezia* species

Species: 1, *M. nana* sp. nov.; 2, *M. furfur*; 3, *M. pachydermatis*; 4, *M. sympodialis*; 5, *M. globosa*; 6, *M. obtusa*; 7, *M. restricta*; 8, *M. slooffiae*; 9, *M. dermatis*. Data are from Guého *et al.* (1996), Mayser *et al.* (1997), Hammer & Riley (2000) and Midgley (2000). +, Positive; +/–, weakly positive; –, negative; v, variable; NT, not tested.

Characteristic	1	2	3	4	5	6	7	8	9
Growth on Sab.* at 32 °C	–	–	+	–	–	–	–	–	–
Growth on mDixon at 40 °C	+ or –	+	+	+	–	–	–	+	+
Catalase reaction	+	+	+/- or +	+	+	+	–	+	+
Use of lipid sources:									
Tween 20 (10%)	+ or –	+	–	–	–	–	–	+/- or +	+
Tween 40 or 60 (0.5%)	+	+	+	+	–	–	–	+	+
Tween 80 (0.1%)	+/-	+	+	+	–	–	–	–	+
Cremophor EL	–	+	v	–	–	–	–	–	+
Hydrolysis of aesculin	–	–	v	+	–	+	–	–	–
Precipitate production on mDixon agar	+	–	NT	+	+	–	NT	–	+

*Sabouraud's agar without any lipid supplementation.

denaturation (1 min at 94 °C), primer annealing (15 s at 60 °C) and polymerization (15 s at 72 °C). PCR products were electrophoresed through 2% agarose gel and then stained with ethidium bromide.

Each PCR product was cloned into a plasmid vector and more than three clones from each sample were sequenced according to methods described previously (Kano *et al.*, 1999; Aizawa *et al.*, 1999, 2001). To examine phylogenetic relationships, we used the neighbour-joining method with the CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994); a phylogenetic tree was constructed by using TREEVIEW for displaying phylogenies (Page, 1996). Bootstrap analysis was performed on 1000 random samples taken from multiple alignments, as described by Felsenstein (1985).

Nucleotide sequence analysis of the 26S rDNA (D1/D2 regions) of these isolates showed fewer than five substitutions over 577 bp among them; the isolates differed by >23 substitutions over 577 bp from the eight *Malassezia* species that have been reported previously (Fell *et al.*, 2000; Sugita *et al.*, 2002). The difference between these isolates and the eight *Malassezia* species exceeded that generally observed between species (Scorzetti *et al.*, 2002). Phylogenetic analysis of 26S rDNA sequences from these isolates showed that they were conspecific and were clearly distinct from the other eight *Malassezia* species, although they were related closely to *M. dermatitis* and *M. sympodialis* (Fig. 1).

Fig. 1. Phylogenetic relationships of *M. nana* and eight recognized *Malassezia* species, based on the D1/D2 region of 26S rDNA sequences. The tree was constructed as described in the text. Numbers at nodes are bootstrap frequencies derived from 1000 replicates. GenBank accession numbers are shown in parentheses. Bar, 0.01 nucleotide substitutions per site.

Nucleotide sequence analysis of the ITS1 region of these isolates showed fewer than four substitutions over 182 bp between them, and >27 substitutions over 158 bp with respect to the sequences of the eight *Malassezia* species reported previously (Makimura *et al.*, 2000; Sugita *et al.*, 2002). Our paper on phylogenetic analyses of *Malassezia* species based on ITS1 DNA revealed that the sequences of members of this genus are exceptionally diverse (Makimura *et al.*, 2000). Species identification and strain typing by using the ITS1 region were carried out by means of phylogenetic analysis; congeneric strains were shown to be grouped into identical clusters and are classified accordingly (Makimura *et al.*, 2000). In phylogenetic analysis of the ITS1 region, these five isolates were shown to be conspecific and did not belong to any of the eight recognized *Malassezia* species; this is also the case for the phylogenetic analysis of 26S rDNA sequences (Fig. 2) (Makimura *et al.*, 2000; Sugita *et al.*, 2002). These results indicate that these isolates belong to a novel species of *Malassezia*.

The two phylogenetic trees show that there are slight differences between NUSV 1003^T, the Japanese isolate, and the four Brazilian isolates. Furthermore, the phylogenetic tree of 26S rDNA showed that there was some divergence among these four Brazilian isolates. Thus, in *M. nana*, 26S rDNA is more variable than the ITS1 region.

The five isolates examined are clearly conspecific, although the Japanese isolate was slightly different from the four Brazilian isolates in the phylogenetic trees. Therefore, the Japanese isolate was designated as the type strain, as it was the first to be suggested as a novel species of *Malassezia* (Hirai *et al.*, 2002).

Fig. 2. Phylogenetic relationships of *M. nana* and eight recognized *Malassezia* species, based on ITS1 region sequences. The tree was constructed as described in the text. Numbers at nodes are bootstrap frequencies derived from 1000 replicates. GenBank accession numbers are shown in parentheses. Bar, 0.05 nucleotide substitutions per site.

Fig. 3. *M. nana* NUSV 1003^T cultured on mDixon agar at 32 °C for 1 week. Bar, 2 mm.

As *M. nana* was isolated from a cat and cows with or without otitis externa, it was suggested as a member of the microbiological flora of animals, as for the other *Malassezia* species. Further studies are required to investigate whether *M. nana* plays a pathogenic role in otitis externa or other diseases of humans and animals.

Latin diagnosis of *Malassezia nana* Hirai, Kano, Makimura, Yamaguchi et Hasegawa sp. nov.

Coloniae in agar Dixonii post 7 dies 32 °C nitentes aut hebetatae, leves, convexae (2 mm). *Textura molis. Cellulae ovoideae aut globosae* (1.5–2.0 × 2.5–3.0 µm), *e base angusta gemmantes. H₂O₂ hydrolysatur. Commutatio coloris per diazonium B positiva. In agar glucosi–peptonico Tween 40, Tween 60 (0.5 %) addito crescit, Tween 80 (0.1 %) addito hebdomadale crescit, Tween 20 (10 %) addito plerumque crescit, et Cremophor EL addito non crescit. Sectura aesculini negativa. In agar Dixonii praecipitationis productio positiva. 37 °C plerumque crescit. Teleomorphis ignotus. Typus NUSV 1003^T, ex felis otite externa, Hyogo, Japan, Martius 2001, K. Yasuda isolatus est. In collectione zymotica Centraalbureau voor Schimmelcultures, Delphi Batavorum, CBS 9557^T (=JCM 12085^T) deposita est.*

Description of *Malassezia nana* Hirai, Kano, Makimura, Yamaguchi & Hasegawa sp. nov.

Malassezia nana (na'na. L. fem. n. *nana* a female dwarf; so named due to the organism's comparatively small cells).

After 1 week incubation on mDixon agar, colonies are cream to yellow, glistening to dull, smooth, convex and have a mean diameter of 2 mm (Fig. 3). Texture of colonies is soft and viscous. Microscopic examination reveals small, ovoid to globose (1.5–2.0 × 2.5–3.0 µm) cells with monopolar budding at the narrow end (Fig. 4). Catalase and

Fig. 4. Cells of *M. nana* NUSV 1003^T are small and ovoid to globose with a narrow end. Arrows indicate monopolar budding. Bar, 3 µm.

diazonium blue B reactions are positive. No growth is obtained on Sabouraud's dextrose agar without lipid supplementation. Colonies develop on glucose–peptone agar supplemented with Tweens 40 or 60 (0.5 %), grow weakly on glucose–peptone agar supplemented with Tween 80 (0.1 %) and usually grow on glucose–peptone agar supplemented with 10 % Tween 20. No growth is observed on glucose–peptone agar with Cremophor EL. Aesculin hydrolysis is negative. Precipitate production on mDixon agar is positive. Good growth usually occurs at 37 °C (Table 2). Teleomorphs are unknown.

The type strain, NUSV 1003^T (=CBS 9557^T=JCM 12085^T), was isolated from a cat with otitis externa in Hyogo, Japan, by K. Yasuda in March 2001 (Hirai *et al.*, 2002). Reference strains are VG Luz 776 (=CBS 9558=JCM 12086), VG Luz 979 (=CBS 9559=JCM 12087), VSF Geleia (=CBS 9560=JCM 12088) and VM Pom Passarella (=CBS 9561=JCM 12089).

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