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Description of a New Yeast Species, *Malassezia japonica*, and Its Detection in Patients with Atopic Dermatitis and Healthy Subjects

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Lipophilic yeasts of the genus *Malassezia* are part of the normal cutaneous microflora and are considered one of the factors that trigger atopic dermatitis (AD). We isolated two strains of *Malassezia* from a healthy Japanese female. Analysis of the D1/D2 26S ribosomal DNA and internal transcribed spacer region sequences of the isolates suggested that they are new members of the genus *Malassezia*. We propose the name *Malassezia japonica* sp. nov. for the isolates. *M. japonica* is easily distinguished from the seven known lipophilic species by its ability to assimilate Tween 40 and Tween 60 and its inability to assimilate Tween 20 and Tween 80 and to grow at 40°C. Furthermore, by applying transparent dressings to the skin lesions of 36 patients with AD and the skin of 22 healthy subjects, *M. japonica* DNA was detected by a non-culture-based method consisting of nested PCR with *M. japonica* species-specific primers. *M. japonica* DNA was detected from 12 of the 36 patients (33.3%) and 3 of the 22 healthy subjects (13.6%). Although it is not known whether *M. japonica* plays a role in AD, this species was part of the microflora in both patients with AD and healthy subjects.

Lipophilic yeasts of the genus *Malassezia* are members of the normal human cutaneous microflora and are also associated with several skin diseases. It is strongly suspected that *Malassezia* species are responsible for pityriasis versicolor, seborrheic dermatitis, *Malassezia* folliculitis, and atopic dermatitis (AD) (2, 4, 5, 19). The genus *Malassezia* includes eight species: *Malassezia globosa*, *M. restricta*, *M. obtusa*, *M. slooffiae*, *M. furfur*, *M. sympodialis*, *M. dermatis*, and *M. pachydermatis* (7, 22). *M. pachydermatis* is not a lipophilic species and is associated several animal skin diseases (1, 8). Recently, our research group found *M. dermatis* on Japanese patients with AD (22). Much research has examined the relationships between these eight species and their roles as causative agents of disease or factors that trigger disease. Most studies indicate that pityriasis versicolor and seborrheic dermatitis are likely affected by *M. globosa* and *M. sympodialis* (3, 9, 10, 15). The distribution of *Malassezia* species in the skin lesions of AD patients and healthy subjects was previously compared by a non-culture-based method (nested PCR) that is not affected by the isolation medium (21). Of the members of the genus *Malassezia*, *M. globosa* and *M. restricta* were associated with disease in more than 90% of AD patients, while the other species were detected in less than 50% of the patients. In our survey of the cutaneous *Malassezia* microflora, we isolated a new *Malassezia* species from a healthy subject. In the present study, we propose a new species, *M. japonica*, for the isolates from this subject and investigated the skin surfaces of patients with AD and healthy subjects for the presence of this species.

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MATERIALS AND METHODS

Yeast isolate. *Malassezia* strains were isolated from the left forearm of a healthy 22-year-old Japanese female. OpSite transparent dressings (3 by 7 cm; Smith and Nephew Medical Ltd., Hull, United Kingdom) were applied to the scalp, back, arm, and nape of the neck of the subject. The samples were then transferred onto modified Leeming and Notman agar (LNA; 20 g of glucose, 50 g of malt extract, 1 g of polypeptone, 20 g of bile salts [Oxoid, Basingstoke, United Kingdom], 1% Tween 40, 0.2% glycerol, and 50 μg of chloramphenicol [Sankyo, Tokyo, Japan]) and incubated at 32°C.

Direct DNA sequencing of rRNA genes. Nuclear DNA was extracted by the method of Makimura et al. (14). The D1/D2 26S rRNA and internal transcribed spacer (ITS) regions of the rRNA gene were sequenced directly from the PCR products by using primer pair NL-1 (5′-GCATATCAATAAGCGGAGGAA AAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) (13) and primer pair pITS-F (5′-GTCGTAACAAGGTTAACCTGCGG) and pITS-R (5′-TCCTCC GCTTATTGATATGC) (20), respectively. The PCR products were sequenced with an ABI 310 DNA sequencer and a BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems, Foster, Calif.), according to the instructions of the manufacturer.

Molecular phylogenetic analysis. The sequences were aligned by using Clustal W software (23). For the neighbor-joining analysis (18), the distances between sequences were calculated by using the two-parameter model of Kimura (12). A bootstrap analysis was conducted with 100 replications (6).

Morphological, physiological, and chemotaxonomic characteristics. Morphology was examined on LNA after incubation at 32°C for 7 days. Tween 20, 40, 60, and 80 utilization, catalase reactions, and diazonium blue B reactions were performed as described by Guého et al. (7). Ubiquinone molecules were identified by the method of Nakase and Suzuki (16).

Direct detection of DNA in samples from patients with AD and healthy subjects. (i) Subjects. The microfloras of 36 AD outpatients (24 men and 12 women; age range, 20 to 64 years; mean age, 33.3 ± 10.5 years) at Tokyo Medical University Hospital and 22 healthy students (7 men and 15 women; age range, 19 to 25 years; mean age, 20.7 ± 1.6 years old) at Meiji Pharmaceutical University were analyzed. AD was diagnosed according to the criteria of Hanifin and Rajka

TABLE 1. Specificities of the primers for *M. japonica* and related species

Species	Strain[a]	Specificity of primer pair:	
		26SBF and Mala-R	JP-IGSIF and JP-IGSIR
M. japonica sp. nov.	M 9966	+	+
M. japonica sp. nov.	M 9967	+	+
M. dermatis	M 9927	−	−
M. dermatis	M 9929	−	−
M. furfur	CBS 4162	+	−
M. furfur	CBS 6000	+	−
M. furfur	CBS 7982	+	−
M. globosa	CBS 7966	+	−
M. globosa	M 9972	+	−
M. obtusa	CBS 7876	+	−
M. obtusa	Clinical isolate 2-17	+	−
M. pachydermatis	CBS 1879	+	−
M. restricta	CBS 7991	+	−
M. restricta	M 9976	+	−
M. slooffiae	CBS 7956	+	−
M. slooffiae	M 9980	+	−
M. sympodialis	CBS 7222	−	−
M. sympodialis	M 9978	−	−
Candida albicans	CBS 562	−	−
Candida guilliermondii	CBS566	−	−
Candida parapsilosis	CBS 604	−	−
Rhodotorula mucilaginosa	CBS 17	−	−
Staphylococcus aureus	Clinical isolate 4-2	−	−

[a] CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands; M, Meiji Pharmaceutical University, Tokyo, Japan.

(11). Routine skin care, including intermittent applications of mild steroid ointment or petrolatum, was administered before sampling. Written informed consent was obtained from each subject.

(ii) Design of *M. japonica* species-specific primers for PCR. The sequences of the intergenic spacer (IGS) 1 region, which is located between the 18S and 5.8S rRNA genes of *M. japonica* and the phylogenetically closely related species *M. furfur* and *M. obtusa*, were determined. The IGS 1 region was amplified with primer 26SBF (5′-AGCT GCTGCCAATGCTAGCTC), which hybridizes to a sequence located at the end of the 26S rRNA gene, and primer Mala-R (5′-T ACTGCTGTGAATGCTCCAGC), which hybridizes to a sequence located in the 5.8S rRNA gene, and by use of the following program: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The amplified PCR products were directly sequenced with the primer pair 26SBF and Mala-R. A species-specific primer pair was designed on the basis of IGS 1 sequence analysis: primer JP-IGS1F (5′-G ACTGCTGATAATGCTCCAGT) and primer JP-IGS1R (5′-GTCTGCTG AT AAGTCTCACTG). To investigate the specificities of these primers, we used the other *Malassezia* and relevant species listed in Table 1.

(iii) Analysis of *M. japonica* microflora. Samples of *Malassezia* were collected by applying OpSite transparent dressings (3 by 7 cm) to erythematous lesions on the faces and necks of patients with AD and the faces and necks of healthy subjects. *Malassezia* DNA was extracted from the OpSite dressing by a previously described method (21). The DNA extracted (3 μl) from each sample was added to 47 μl of the PCR master mixture, which consisted of 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$; Takara Inc., Shiga, Japan), 4 μl of 200 μM deoxynucleoside triphosphates (an equimolar mixture of dATP, dCTP, dGTP, and dTTP; Takara), 10 pmol of each primer, and 2.5 U of Ex *Taq*DNA polymerase (Takara). PCR was performed with primers 26SBF and Mala-R, with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 57°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. In the nested PCR step, 1 μl of the first amplification product was added to a new reaction mixture with the same composition as the first one. The PCR with primers JP-IGS1F and JP-IGS1R consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 44°C, and 30 s at 72°C, with a final extension at 72°C for 10 min.

Nucleotide sequence accession numbers. The nucleotide sequences of the D1/D2 26S rRNA, ITS, and IGS regions determined in this study have been deposited with DDBJ (DNA Data Bank of Japan) and are listed in Table 2.

RESULTS AND DISCUSSION

Molecular phylogenetic analysis and taxonomic characteristics. The isolates formed a cluster with *M. furfur* and *M. obtusa*, with 100 and 99% bootstrap support on trees constructed by using the D1/D2 26S rRNA gene and ITS 1 sequences, respectively (Fig. 1A and B). The dissimilarities between the D1/D2 regions of the 26S rRNA genes of the isolates and those of the *M. furfur* and *M. obtusa* strains were 4.6% (27 of 582 bp) and 6.9% (40 of 580 bp), respectively. The ITS 1 regions of the isolates had 12.5 to 24.2% and 15.3 to 20.3% dissimilarities to those of the *M. furfur* and *M. obtusa* strains, respectively; and the ITS 2 regions of the isolates had 15.6 to 17.9% and 20.2 to 21.3% dissimilarities to those of the the *M. furfur* and *M. obtusa* strains, respectively. Since the divergence

TABLE 2. Accession numbers of the IGS, ITS, and D1/D2 26S rRNA sequences

Species	Strain	Accession no.		
		D1/D2 26S rRNA gene	ITS region	IGS 1 region
M. japonica sp. nov.	M 9966[Ta]	AB100599	AB100599	AB105063
	M 9967	AB105199	AB105199	AB105064
M. furfura	CBS 4162			AB111460
	CBS 6000			AB111459
	CBS 7982[T]	AB105198	AB105150	
	Clinical isolate 2–9		AB105151	
	Clinical isolate 2–4		AB105152	
	Clinical isolate 2–521		AB105153	
	Clinical isolate 2–52		AB105154	
M. obtusa	CBS 7876[T]	AB105197	AB105158	AB111461
	Clinical isolate 2–17		AB105155	
	Clinical isolate 2–20		AB105156	
	Clinical isolate 2–35		AB105157	

[a] T, type strain.

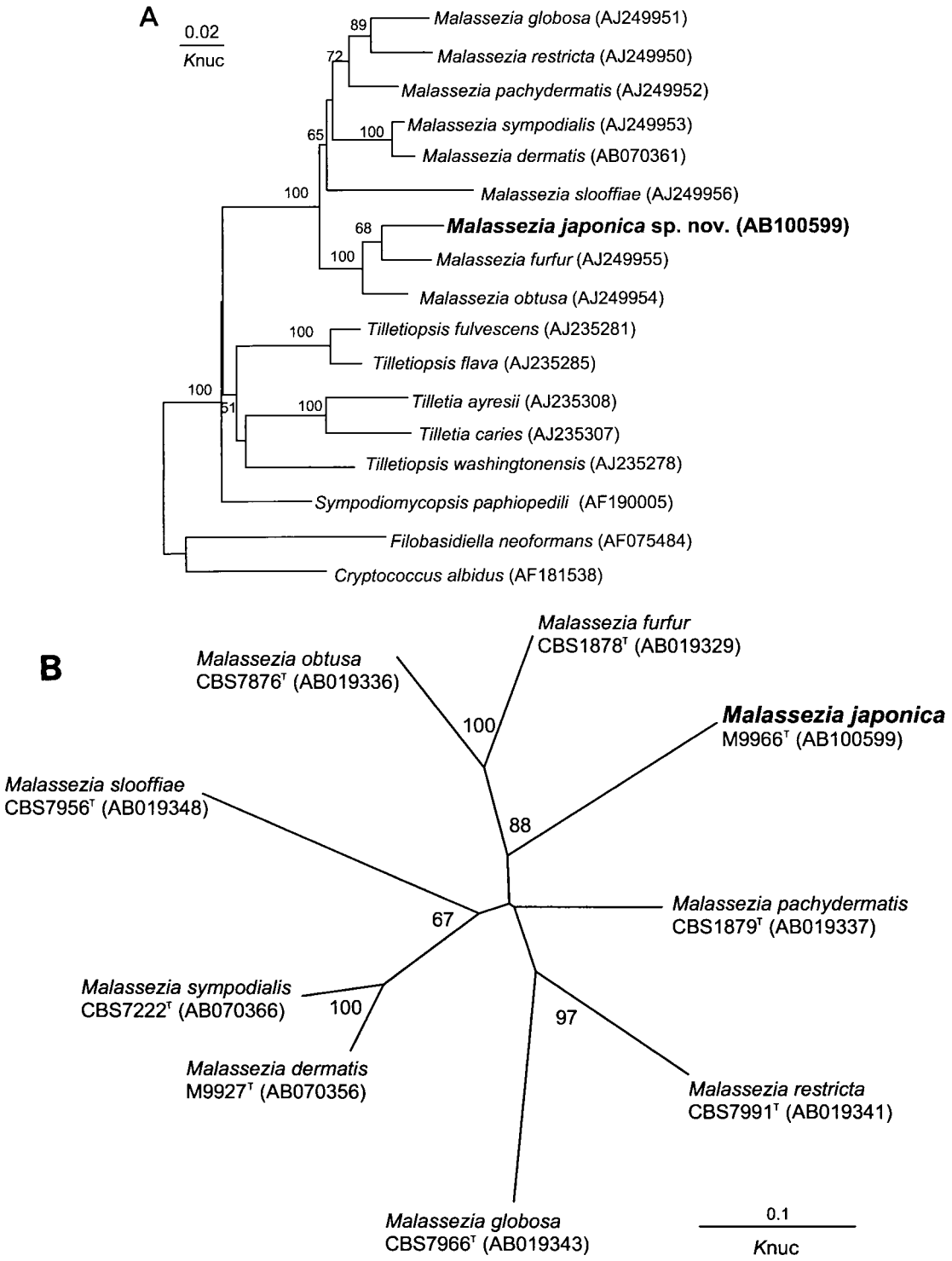

FIG. 1. Phylogenetic trees constructed by using the D1/D2 26S rRNA sequences of *M. japonica* sp. nov. and related species of the class *Ustilaginomycetes* (A) and the ITS1 region of *M. japonica* sp. nov. and other members of the genus *Malassezia* (B). The DDBJ and GenBank accession numbers are indicated in parentheses. The numerals represent the confidence levels from 100 replicate bootstrap samplings (frequencies less than 50% are not indicated). *K*nuc, Kimura's parameter (12).

between the *M. furfur* and *M. obtusa* strains and our isolates is sufficient to resolve them as individual species, we propose the name *M. japonica* for the isolates (17, 20). The species epithet used here refers to the country where the species was discovered. The characteristics differentiating the new species, *M. japonica*, and the known *Malassezia* species are summarized in Table 3. *M. japonica* is easily distinguished from the other species by its ability to assimilate Tween 40 and Tween 60 and

TABLE 3. Physiological characteristics of *M. japonica sp. nov.* and other *Malassezia* species[a]

Species	Growth on SA[b] at 32°C	Growth on mDixon[c] at			Catalase reaction	Utilization of:			
		32°C	37°C	40°C		10% Tween 20	0.5% Tween 40	0.5% Tween 60	0.1% Tween 80
M. japonica sp. nov.	−	+	+	−	+	−	±	+	−
M. slooffiae[d]	−	+	+	+	+	± or +	+	+	−
M. sympodialis[d]	−	+	+	+	+	−	+	+	+
M. furfur[d]	−	+	+	+	+	+	+	+	+
M. dermatis	−	+	+	+	+	+	+	+	+
M. globosa[d]	−	+	± or −	−	+	−	−	−	−
M. obtusa[d]	−	+	± or +	−	+	−	−	−	−
M. restricta[d]	−	+	+	−	−	−	−	−	−
M. pachydermatis[d]	+	+	+	+	± or +	−	+	+	+

[a] +, positive; −, negative; ±, weakly positive.
[b] SA, Sabouraud dextrose agar.
[c] mDixon, modified Dixon agar.
[d] Data are from Guého et al. (7).

its inability to assimilate Tween 20 and Tween 80 and to grow at 40°C.

Direct detection of *M. japonica* DNA by a non-culture-based method. The sensitivity of the nested PCR assay was examined by using *M. japonica* DNA purified from the culture. The limit of detection for purified DNA was approximately 10 fg by the nested PCR assay. The specificities of the *M. japonica* species-specific primers are shown in Table 1. Our PCR primers amplified only the targeted *M. japonica* DNA and did not amplify the DNA of any other *Malassezia* species. We confirmed the absence of false-positive reactions by determining the DNA sequences of the PCR products after they were cloned in the pCR2.1 vector (Invitrogen), since various species, including bacteria and filamentous fungi, colonize the skin surface. We collected and analyzed 142 samples from 36 patients with AD and 66 samples from 22 healthy subjects. *M. japonica* DNA was detected in 12 patients (33.3%) and 3 healthy subjects (13.6%). A non-culture-based method (PCR) was previously developed to analyze the *Malassezia* microflora on the skin surface, since the isolation media and technique influence the growth of *Malassezia* species and the growth rates of each species differ. As a result, the detection rate is higher by the PCR-based method than by traditional culture methods (21). The previous study suggests that *M. globosa* and *M. restricta* make up the major part of the microflora in patients with AD. The frequency of detection of the new species, *M. japonica*, is the same as that of *M. sympodialis* and *M. furfur*.

In conclusion, we have described a novel species, *M. japonica*, isolated from the skin surface of a healthy Japanese subject. It is not known whether this microorganism plays a significant role in AD or other skin diseases. *M. japonica* was part of the microfloras in both patients with AD and healthy subjects.

Latin description of *Malassezia japonica* Sugita, Takashima, Kodama, Tsuboi, et Nishikawa. In LNA, post dies 6 ad 32°C, cellulae vegetativae sphaericae, ovoideae vel ellipsoideae 2–5 × 2–7 μm; sympodiales gemmantes proferentes. Cultura xanthoalba, semi-nitida aut hebetata, rugosa, et butyracea et margo glabra aut lobulata. In agaro glucoso-peptonico Tween 40 et Tween 60 (0.5%) addito crescit. H_2O_2 hydrolysatur. Commutatio colori per diazonium caeruleum B positiva. GTC acid: deoxyribonucleati 60.4 mol%. Ubiquinonum majus Q-9 est. Teleomorphis ignota. Typus: JCM 11963^T, ex cute, feminae sani, Tokyo, Japonia, 2002, T. Kodama (originaliter ut M 9966), conservatur in collectionibus culturarum quas "Japan Collection of Microorganisms," Saitama, Japan, sustentat.

Description of *Malassezia japonica* Sugita, Takashima, Kodama, Tsuboi, et Nishikawa sp. nov. On LNA, after 6 days at 32°C, the vegetative cells are spherical, oval, or ellipsoidal and 2 to 5 by 2 to 7 μm, and sympodial budding is observed (Fig. 2). The colony is pale yellowish, semishining to dull, wrinkled, and butyrous and has an entire to lobed margin. The organism grows on glucose-peptone agar with 0.5% Tween 40 and Tween 60 as the sole source of lipid. The catalase reaction is positive. The diazonium blue B reaction is positive. The G+C content of nuclear DNA is 60.4 mol%, and the major ubiquinone is Q-9. The teleomorph is unknown.

JCM 11963^T (CBS 9431^T; originally strain M 9966) was isolated from the skin of a healthy Japanese subject in Tokyo, Japan, by M. Kodama in November 2002 and is maintained in the Japan Collection of Microorganisms (JCM), Saitama, Japan. The other strain, M 9967, has also been deposited in the JCM and CBS collections as strain 11964 and strain 9432, respectively.

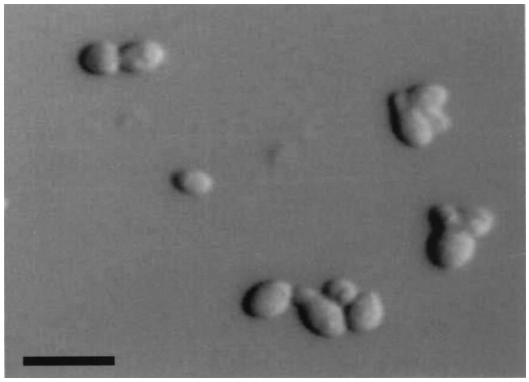

FIG. 2. Vegetative cells of *M. japonica* M 9966 grown in LNA for 7 days at 32°C. Bar, 10 μm.

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