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Physiological and Molecular Characterization of Atypical Isolates of *Malassezia furfur*▽

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The species constituting the genus *Malassezia* are considered to be emergent opportunistic yeasts of great importance. Characterized as lipophilic yeasts, they are found in normal human skin flora and sometimes are associated with different dermatological pathologies. We have isolated seven *Malassezia* species strains that have a different Tween assimilation pattern from the one typically used to differentiate *M. furfur*, *M. sympodialis*, and *M. slooffiae* from other *Malassezia* species. In order to characterize these isolates of *Malassezia* spp., we studied their physiological features and conducted morphological and molecular characterization by PCR-restriction fragment length polymorphism and sequencing of the 26S and 5.8S ribosomal DNA-internal transcribed spacer 2 regions in three strains from healthy individuals, four clinical strains, and eight reference strains. The sequence analysis of the ribosomal region was based on the Blastn algorithm and revealed that the sequences of our isolates were homologous to *M. furfur* sequences. To support these findings, we carried out phylogenetic analyses to establish the relationship of the isolates to *M. furfur* and other reported species. All of our results confirm that all seven strains are *M. furfur*; the atypical assimilation of Tween 80 was found to be a new physiological pattern characteristic of some strains isolated in Colombia.

The genus *Malassezia* comprises lipophilic yeasts found in the normal flora of human skin and other mammals. These yeasts were described by Eichstedt in 1848 as being associated with pityriasis versicolor (PV) lesions (13). The taxonomy and nomenclature of the genus *Malassezia* was controversial for many decades. Indeed, until 1990 only three species were recognized: *M. furfur*, *M. sympodialis*, and *M. pachydermatis*, a non-lipid-dependent species (17, 21, 38). The species *M. globosa*, *M. restricta*, *M. obtusa*, and *M. slooffiae* were described in 1995 by morphology, ultrastructure, physiology, and molecular techniques (18, 20). In the last few years, *M. dermatis*, *M. japonica*, *M. nana*, and *M. yamatoensis* (26, 40–42) have been reported as *Malassezia* species. Recently, Cabañes et al. described two new species, *M. equina* and *M. caprae*, which were isolated from domestic animals (4).

Nine of the 13 species within the genus, *M. furfur*, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. slooffiae*, *M. obtusa*, *M. dermatis*, *M. japonica*, and *M. yamatoensis*, are associated with normal human flora and pathologies. Four species, *M. pachydermatis*, *M. nana*, *M. equina*, and *M. caprae*, are associated with animals (4, 6, 11, 17, 18, 20, 21, 26, 34, 38, 40–42).

Malassezia species have been associated with diverse dermatological pathologies, including PV, seborrheic dermatitis dandruff, atopic dermatitis, folliculitis, psoriasis, onicomycosis, and blepharitis. *M. furfur* and *M. pachydermatis* have been associated with systemic infections in patients with underlying diseases and those receiving intravenous lipid emulsions (6, 7, 9–11, 16, 29, 33, 34).

Although the role of *Malassezia* species in the development of these diseases is not clear, some authors suggest that *M. globosa* is the causal agent of PV, while others have found a greater percentage of isolates of *M. sympodialis* associated with the disease. Differences in diagnosis might be due to sampling methods and differences between the culture media used, leading to controversies in clinical studies of these dermatological pathologies (1, 7, 9, 10).

New physiological patterns for identification have been described, and recently the availability of molecular biology and sequencing techniques has allowed the species to be distinguished more clearly (17–21). Despite the difficulty in isolating, maintaining, and identifying these yeasts, different characteristics of the genus, such as macroscopic and microscopic morphology and some physiological aspects (e.g., the presence/absence of catalase, selective growth on Cremophor EL, β-glucosidase activity, and growth and pigment production on a pigment-producing medium [p-agar]), allow them to be differentiated (18, 22, 30, 31). The assimilation of Tween 20, 40, 60, and 80 by *M. furfur*, *M. sympodialis*, and *M. slooffiae* yields a specific pattern that easily differentiates them from other species (18, 22).

In a previous study, we isolated a total of 154 strains of *Malassezia* spp., 7 of which were omitted and remained uncharacterized because of an atypical Tween assimilation pattern (35). The aim of the present study was to characterize these seven isolates by investigating their physiological features and conducting morphological and molecular characterization by PCR-restriction fragment length polymorphism (RFLP) and the sequencing of the 26S and 5.8S ribosomal DNA-internal transcribed spacer 2 (rDNA-ITS2) regions for all of the strains. Establishing a relationship with reported species through phylogenetic analyses will support our findings.

MATERIALS AND METHODS

Strains and growth conditions. The strains of *Malassezia* spp. with a Tween assimilation pattern different from that of the other strains were obtained as

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TABLE 1. Sources and origins of strains from *Malassezia* species used in this study

Malassezia species	Strain	Origin	Accession no.	
			D1/D2 26S rDNA	5.8S rDNA-ITS2
M. furfur ($n = 3$)	13 NL	Healthy individuals	EU815312	EU815321
	28 NL	Healthy individuals	EU815306	EU815315
	39 NL	Healthy individuals	EU815304	EU815320
M. furfur ($n = 2$)	1828 PV	PV patient	EU815309	EU815319
	45 PV	PV patient	EU815311	EU815322
M. furfur	12 DSHIV+	Seborrheic dermatitis- and human immunodeficiency virus-positive patient	EU815303	EU815313
M. furfur	4 DS	Seborrheic dermatitis patient	EU815310	EU815318
M. furfur	CBS 1878[b]	Human pityriasis capitis	EU815307	EU815316
M. furfur	CBS 7019[b]	Human PV	EU815308	EU815317
M. furfur	CBS 6094	Normal skin (of the rump)	EU815305	EU815314
M. globosa	CBS 7966[a]	Human PV	AY743604	AY387133-AY38713335
M. restricta	CBS 7877[a]	Human normal skin	AY743067	AY387143-AY38714345
M. pachydermatis	CBS 1879[b]	Dog otitis externa	DQ915500-DQ915502, AY743605	AY387139-AY38713942
M. slooffiae	CBS 7956[a]	Healthy ear of pig	AY743606, AJ249956	AY387146-AY38714649
M. sympodialis	CBS 7222[a]	Human normal skin	AY743615, AY743627, AY743628	AY743638-AY743640

[a] Type strain.
[b] Neotype strain.

uncharacterized isolates from a previous study (35). All strains were preserved in 10% skim milk at −80°C (8) and recovered in modified Dixon agar by incubation for 4 to 5 days at 32°C (18, 22).

Phenotypic characterization. The morphological characteristics of the colonies were recorded, and Gram staining was performed. The morphology of the stained cells was assessed by light microscopy. Physiological characteristics were assessed as previously described. The following tests were performed five times: assimilation (Tween 20, 40, 60, and 80 and Cremophor EL), enzyme production (β-glucosidase and catalase), pigment production on tryptophan-based medium, and growth (on Sabouraud, Sabouraud plus 10% Tween 20, and Dixon agar at 37 and 42°C) (18, 22, 30, 31).

Molecular characterization. (i) DNA extraction. Colonies grown on Dixon agar at 32°C for 4 to 5 days were transferred to 600 μl sterile distilled water and centrifuged at 10,000 rpm for 5 min. DNA was extracted as previously described (36).

(ii) PCR-RFLP of 26S and 5.8S rDNA-ITS2 regions. The 26S rDNA region was PCR amplified using forward and reverse primers as previously described (32). The 5.8S rDNA-ITS2 regions were PCR amplified using primers ITS3 and ITS4 (15). All PCRs were performed in a final volume of 25 μl (46).

The amplified 26S rDNA product was digested with 10 U of the restriction enzymes CfoI (Sigma-Aldrich) and BstF5I (SibEnzyme, Novosibirsk, Russia) by incubation for 4 h at 37 and 65°C, respectively. The amplified 5.8S rDNA-ITS2 product was digested with 10 U of AluI (Promega) by incubation at 37°C for 4 h. The products were visualized by 2% agarose gel electrophoresis using Quantity One software (Bio-Rad, Hercules, CA).

(iii) Sequencing and phylogenetic analysis. Single electrophoretic bands from the PCR products were sequenced using a 3730xl DNA analyzer (PE Applied Biosystems). Sequence assembly and editing were performed manually on the software CLC DNA Workbench.

For each region (26S and 5.8S rDNA-ITS2), a data set was formed containing sequences from reported *Malassezia* species in GenBank. Each data set was aligned using the ClustalW algorithm (45) under default settings in the BIODEDIT package (25). To assess phylogenetic relationships, maximum-parsimony analysis

TABLE 2. Physiological characteristics of *Malassezia* isolates included in this study

Species	Utilization of :					β-Glucosidase activity	Growth with 10% Tween 20	Catalase reaction	Growth in Sabouraud	Growth and pigment production in p-agar at:				Growth in Dixon agar at:	
	Tween 20	Tween 40	Tween 60	Tween 80	Cremophor EL					25°C	30°C	37°C	42°C	37°C	40°C
M. furfur 13 NL[a]	−	−	−	+	+	−	−	+	−	+	+	+	−	+	+
M. furfur 28 NL[a]	−	−	−	+	+	−	−	+	−	+	+	+	−	+	−
M. furfur 39 NL[a]	−	−	−	+	+	−	−	+	−	+	+	+	−	+	−
M. furfur 1828 PV[a]	−	−	−	+	+	−	−	+	−	+	+	+	−	+	−
M. furfur 45 PV[a]	−	−	−	+	+	−	−	+	−	+	+	+	−	+	−
M. furfur 12 DSHIV+[a]	−	−	−	+	+	−	−	+	−	+	+	+	−	+	−
M. furfur 4 DS[a]	−	−	−	+	+	−	−	+	−	+	+	+	−	+	−
M. furfur CBS 1878	+	+	+	+	+	−	+	+	−	+	+	+	−	+	+
M. furfur CBS 7019	+	+	+	+	+	−	+	+	−	+	+	+	−	+	+
M. furfur CBS 6094[a]	−	−	−	+	+	−	−	+	−	−	−	−	−	+	+
M. globosa CBS 7966	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
M. restricta CBS 7877	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. pachydermatis CBS 1879	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+
M. slooffiae CBS 7956	+	+	+	+	−	−	+	+	−	−	−	−	−	+	−
M. sympodialis CBS 7222	−	+	+	+	−	+	−	+	−	−	−	−	−	+	+

[a] Isolates with an atypical pattern of Tween assimilation.

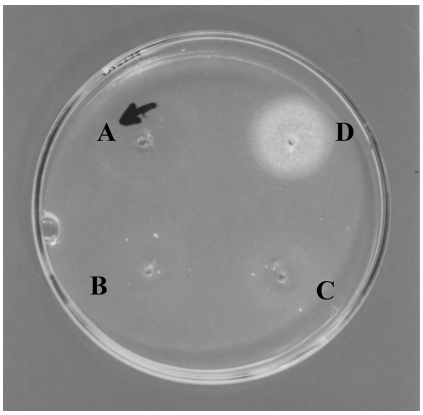

FIG. 1. Atypical Tween 80 assimilation pattern of a *Malassezia* sp. isolate. (A) Tween 20; (B) Tween 40; (C) Tween 60; (D) Tween 80.

was conducted on the aligned data sets using PAUP* 4.0b8 software under default settings (43), and *Filobasidiella neoformans* was used as the outgroup for both analyses. A bootstrap resampling was conducted for 1,000 replicates to assess relative branch support (14).

Nucleotide sequence accession number. The nucleotide sequences (26S and 5.8S rDNA-ITS2 regions) obtained in this study have been deposited in GenBank with accession numbers EU815303 to EU815322 (National Center for Biotechnology Information, Bethesda, MD).

RESULTS

To characterize the isolates of *Malassezia* spp., we studied their physiological features and conducted morphological and molecular characterization by PCR-RFLP and the sequencing of the 26S and 5.8S rDNA-ITS2 regions in three *M. furfur* strains from healthy individuals, four *M. furfur* clinical strains, and eight *Malassezia* spp. reference strains (Table 1).

Phenotypic characterization. All *M. furfur* colonies were smooth, opaque, and umbonate. Microscopically, all *M. furfur* isolates appeared as small ovoid cells (1 to 1.5 by 2 to 2.5 μm), and buds were formed on a broad base. The physiological tests other than Tween assimilation showed the results expected for *M. furfur* (Table 2). The Tween assimilation test result was different from the standard patterns so far reported for different *Malassezia* species but coincided with that of the *M. furfur* CBS 6094 strain. Tween 80 was the only Tween assimilated, as shown in Fig. 1. However, *M. furfur* CBS 6094 does not produce pigment at 25, 30, 37, or 42°C.

Molecular characterization. The 26S rDNA PCR amplified an ~580-bp product; two bands of ~180 and ~400 bp were obtained after BstF51 digestion, and three bands of ~260, ~130, and ~70 bp were obtained after CfoI digestion. The 5.8S-ITS2 PCR amplified an ~500-bp product. AluI digestion yielded two bands of ~290 and 250 bp (Table 3).

Phylogenetic analysis. The isolates with the atypical Tween assimilation pattern formed a single cluster that contained the reference *M. furfur* sequences, with high bootstrap support values of 80 and 100% for the 26S and 5.8S rDNA-ITS2 phylogeny, respectively (Fig. 2 and 3). Although the topologies differed somewhat in support values, the 5.8 rDNA-ITS2 topology showed higher bootstrap support values overall than the 26S topology, suggesting that this region provides a better resolution of the *Malassezia* spp. Taking both topologies into account, it remains unclear which is the sister group of *M. furfur*: the 26S topology places this group next to *M. yamatoensis*, while the 5.8S rDNA-ITS2 topology places it next to *M. obtusa*.

DISCUSSION

The *Malassezia* species are difficult microorganisms to identify and to maintain in culture (9). To overcome these difficulties, several physiological and molecular techniques have been implemented recently that allow the species to be differentiated and new ones to be described (2, 15, 17, 18, 20–24, 27, 38, 44). In this work, we thoroughly characterized seven isolates with an atypical Tween 80 assimilation pattern, and our results support the view that these isolates correspond to *M. furfur*.

Because these species are lipophilic, the Tween assimilation pattern allows us to identify different *Malassezia* species (18, 22). This test is based on the ability of *Malassezia* spp. to use different fatty acids. Some authors attribute this characteristic to lipase activity and propose that it is related to the adaptation of *Malassezia* spp. to host body regions rich in fatty acids under given conditions (3, 12, 37, 47). Brunke et al. described the lipophilic activity of MfLip in *M. furfur*; this extracellular lipase apparently is involved in cellular growth processes and related to pathogenicity mechanisms (3).

TABLE 3. RFLP 26S rDNA and 5.8S rDNA-ITS2 PCR products generated by BstF51, CfoI, and AluI, respectively, for *Malassezia* isolates included in this study

Malassezia isolate(s)	26SrDNA amplification product (bp)	5.8S rDNA-ITS2 amplification product (bp)	BstF51 digest (bp)	CfoI digest (bp)	AluI digest (bp)
Strains with atypical Tween pattern[a]	580	500	400, 180	260, 130, 70	290, 250
M. furfur CBS 1878	580	500	400, 180	260, 130, 70	290, 250
M. furfur CBS 7019	580	500	400, 180	260, 130, 70	290, 250
M. furfur CBS 6094	580	500	400, 180	260, 130, 70	290, 250
M. globosa CBS 7966	580	410	480, 100	480,100	240, 170
M. restricta CBS 7877	580	410	500, 70	NRS[b]	NRS
M. pachydermatis CBS 1879	580	470	500, 70	250, 230, 100	360, 100
M. slooffiae CBS 7956	580	430	NRS	250, 110, 100, 70	320, 110
M. sympodialis CBS 7222	580	400	400, 180	390, 190	NRS

[a] Isolates with the atypical pattern of Tween assimilation were 13 NL, 28 NL, 39 NL, 1828 PV, 45PV, 12 DSHIV, and 4DS.
[b] NRS, no restriction site.

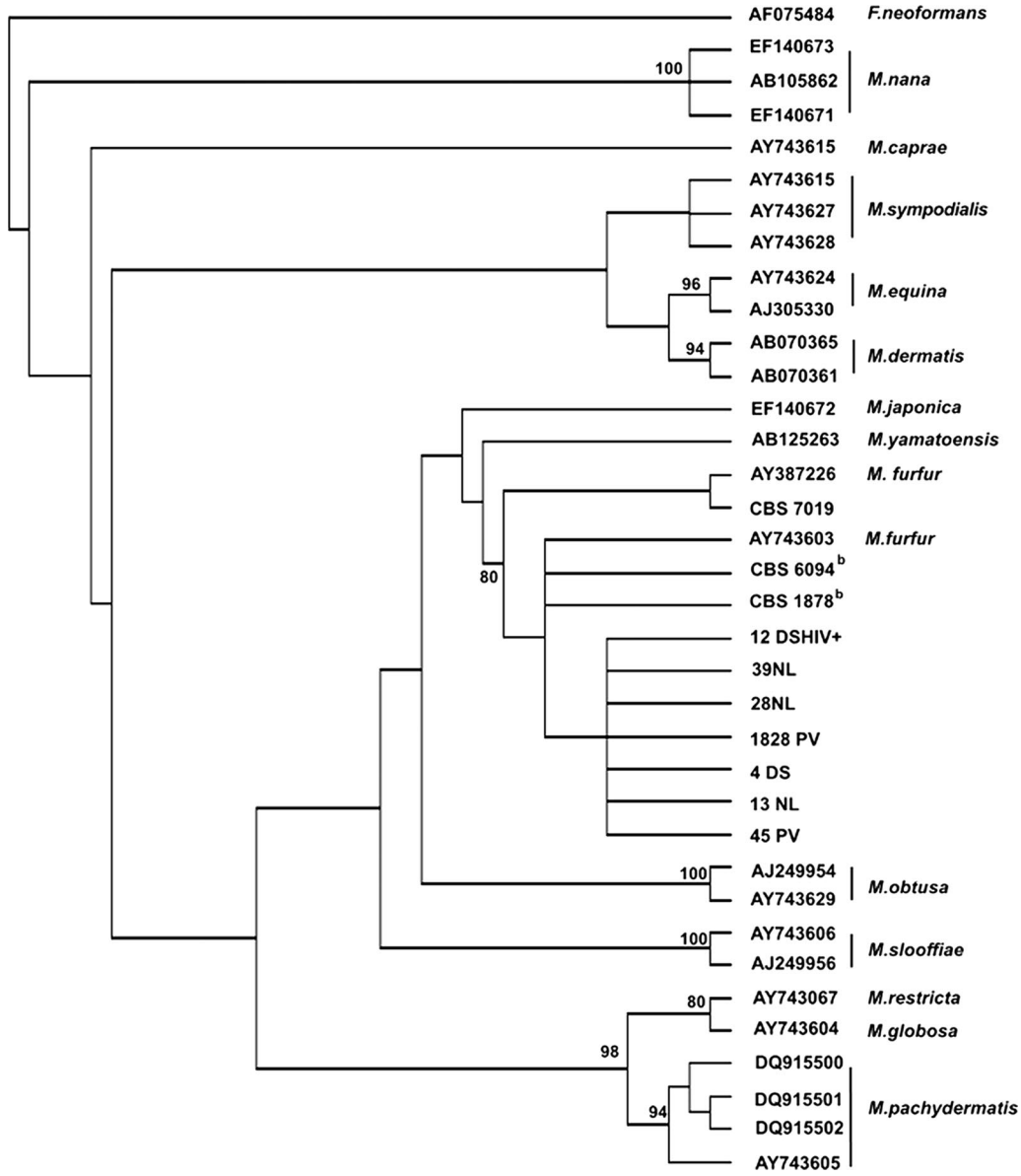

FIG. 2. Maximum-parsimony phylogeny of the genus *Malassezia* based on the D1 and D2 sequences from the 26S rDNA gene. The majority-rule consensus tree is shown. Values above branches correspond to parsimony bootstrap proportions (>80) of 1,000 replicates.

On the other hand, *M. furfur* has been described as showing high phenotypic and genotypic variability. Indeed, colonies of this species vary in size and form and show a high degree of cellular pleomorphism, including oval, cylindrical, and spherical cells (18, 22). Techniques such as randomly amplified polymorphic DNA and amplified fragment length polymorphism have demonstrated high intraspecific variability in this species (2, 5, 44). The atypical assimilation pattern of our isolates, involving an inability to utilize Tween 20, 40, or 60, reveals a metabolic variation probably resulting from alternate gene expression.

Such variability has been observed in related organisms such as *Candida* spp. Lan et al. found a relationship between phenotypic variation and metabolic flexibility in the genus *Candida*, which increases its selection according to the availability of nutrients in certain body regions (28). Phenotypic and genotypic variability such as the atypical Tween assimilation pattern in the case of *M. furfur* may, like the variability in *Candida* spp., be a determining factor in the infection strategy used by the microorganism, which in turn could be related to the expression of genes involved in pathogenic processes and colonization (39).

Shifts in metabolic processes could be related to a change of condition from commensal to pathogenic. The resulting high variability also may be influenced by host responses such as the generation of adverse microenvironments, which perturb the host-pathogen balance (7, 9, 16, 29, 33). Nonetheless, the variation in the Tween assimilation pattern observed in this research (based on the seven atypical strains) was not related to a particular dermatological condition,

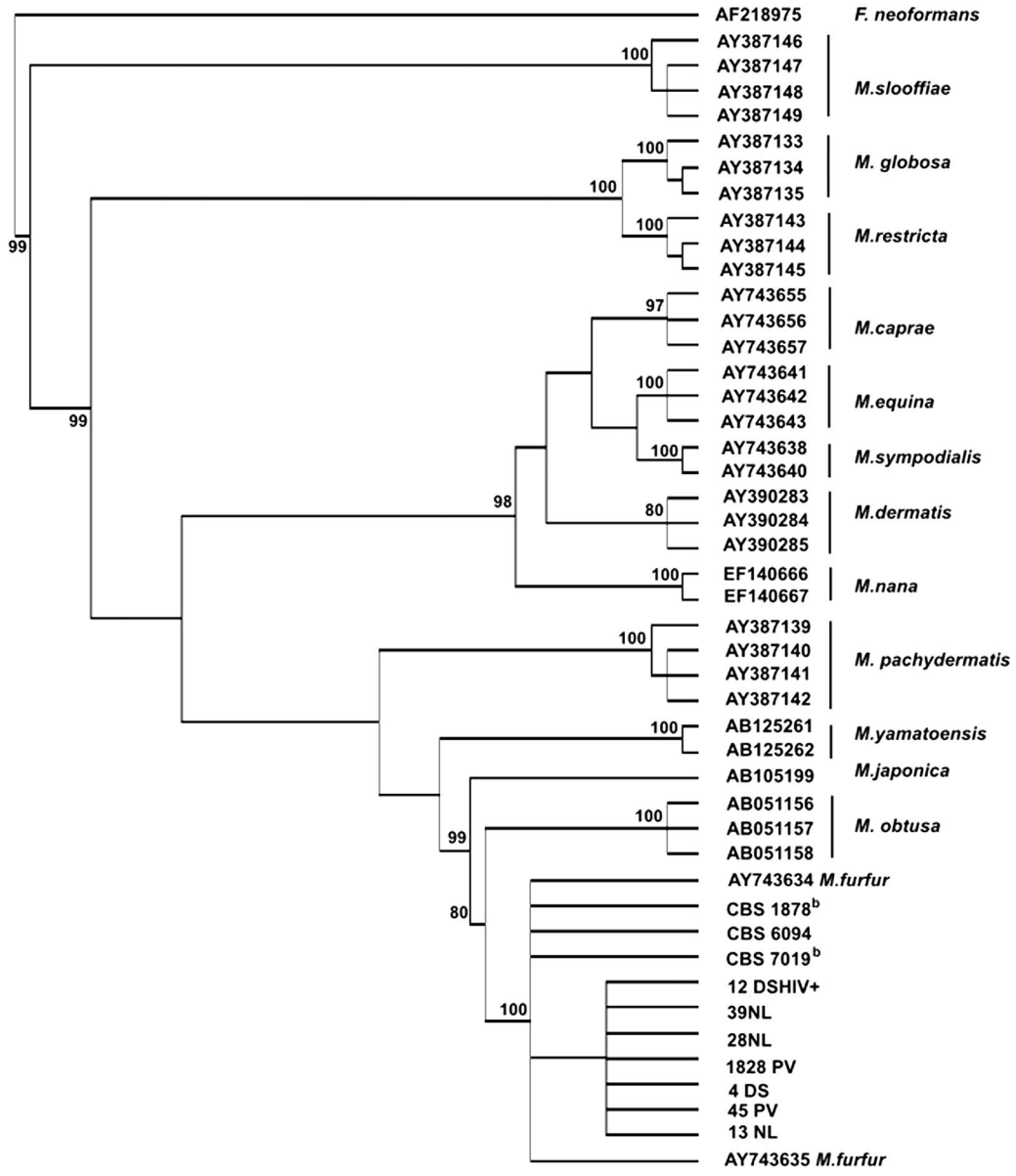

FIG. 3. Phylogenetic relationships of the genus *Malassezia* based on the sequences of the 5.8S ITS-rDNA region. The majority-rule consensus tree is shown. Values above branches correspond to parsimony bootstrap proportions (>80) of 1,000 replicates.

since it was present both in patients with a dermatological condition and those without apparent injury.

The expected results for 26S (32) and 5.8S rDNA-ITS2 (15) were obtained with all 15 isolates, including the reference ones. The PCR products and the products of digestion revealed no differences between the isolates with the atypical Tween assimilation pattern and the reference strains *M. furfur* CBS 1878 and CBS 7019. Our sequence analyses based on homology searches of the ribosomal regions identified our isolates as *M. furfur*. Furthermore, the phylogenetic analyses for 26S and 5.8S rDNA-ITS2 grouped our isolates and reference strains together in a single cluster with high bootstrap values (Fig. 2 and 3). The phylogenies obtained in this study support our findings and the identity of the isolates as *M. furfur*; the atypical assimilation of Tween 80 was found to be a new physiological pattern characteristic of certain strains isolated in Colombia.

In conclusion, we suggest further studies that will explain the genetic background of the atypical Tween assimilation pattern and its relationship to the establishment of the yeast in certain host body regions. The evaluation of the metabolic pathways and the gene products involved in the fatty acid degradation, and even the relationship between gene expression and diverse host conditions, would produce a better understanding of this unique Tween assimilation pattern, which constitutes a new parameter for the identification of *M. furfur*.

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