Two new lipid-dependent *Malassezia* species from domestic animals

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Abstract

During a study on the occurrence of lipid-dependent *Malassezia* spp. in domestic animals, some atypical strains, phylogenetically related to *Malassezia sympodialis* Simmons et Guého, were shown to represent novel species. In this study, we describe two new taxa, *Malassezia caprae* sp. nov. (type strain MA383 = CBS 10434), isolated mainly from goats, and *Malassezia equina* sp. nov. (type strain MA146 = CBS 9969), isolated mainly from horses, including their morphological and physiological characteristics. The validation of these new taxa is further supported by analysis of the D1/D2 regions of the 26S rRNA gene, the ITS1–5.8S–ITS2 rRNA, the RNA polymerase subunit 1 and chitin synthase nucleotide sequences, and the amplified fragment length polymorphism patterns, which were all consistent in separating these new species from the other species of the genus, and those of the *M. sympodialis* species cluster, specifically.

Introduction

Since the genus *Malassezia* was created by Baillon in 1889, its taxonomy has been a matter of controversy. The genus remained limited to *Malassezia furfur* and *M. pachydermatis* for a long time (Batra *et al*., 2005). Traditionally, the lipid-dependent species *M. furfur* (*sensu lato*) was thought to occur only on human skin, whereas the lipophilic, but nonlipid-dependent, species *M. pachydermatis* was restricted to animal skin. *Malassezia sympodialis*, a lipid-dependent species described in 1990 (Simmons & Guého, 1990), was the third species accepted in the genus, a century after the description of *M. furfur*. Later, the genus *Malassezia* was revised on the basis of morphological, physiological and rRNA gene sequencing studies, and four new lipid-dependent species were described: *M. globosa*, *M. obtusa*, *M. restricta* and *M. slooffiae* (Guého *et al*., 1996). More recently, another four new lipid-dependent *Malassezia* species have been described, namely *M. dermatis* (Sugita *et al*., 2002), *M. japonica* (Sugita *et al*., 2003), *M. nana* (Hirai *et al*., 2004) and *M. yamatoensis* (Sugita *et al*., 2004).

Malassezia pachydermatis is frequently found on wild and domestic carnivores, and rarely on humans (Guillot & Bond, 1999). Lipid-dependent *Malassezia* yeasts have also been isolated from healthy dogs and cats (Bond *et al*., 1996, 1997; Crespo *et al*., 1999, 2002a) and from the healthy skin of horses and different domestic ruminants, being the major component of the lipophilic mycobiota occurring on these later animals (Crespo *et al*., 2002b). Some of these isolates from horses and ruminants could not be identified, because the different physiological tests results and their morphological characteristics precluded fitting them into any of the previously described species of the genus. A new species, tentatively named '*M. equi*', was reported from normal equine skin (Nell *et al*., 2002), but without a valid description or a type specimen. It was identified by 26S rRNA gene D1/D2 sequence analysis as a member of the genus *Malassezia*, and was found to be most closely related to *M. sympodialis*. Unfortunately, the only strain that was deposited in the NCYC yeast collection (Norwich, UK) is no longer alive (C. Bond, pers. commun.). Crespo *et al*. (2000a) reported for the first time lipid-dependent yeasts associated with otitis externa in cats with similar morphological characteristics and some shared physiological characteristics with the type strain of *M. sympodialis*. Recently, Hirai *et al*. (2004) described *M. nana*, a novel species from otic discharges of a cat and cows, which is also closely related to *M. sympodialis*.

However, difficulty in obtaining a high level of certainty in the identification of some of these lipid-dependent strains using physiological tests has been also reported (Crespo *et al.*, 2002b; Gupta *et al.*, 2004; Batra *et al.*, 2005). The speciation of lipid-dependent isolates from animals by means of physiological tests presents some difficulties, and some of them cannot even be identified (Duarte *et al.*, 1999, 2002; Crespo *et al.*, 2000a, b). Recently, some lipid-dependent strains similar to the *M. sympodialis* type strain and isolated from various domestic animal species were studied using DNA sequence analysis, and their phylogenetic relationships with the *M. sympodialis*-related species, *M. dermatis* and *M. nana*, were discussed (Cabañes *et al.*, 2005). Phylogenetic analysis of both the D1/D2 regions of the 26S rRNA gene and ITS–5.8S rRNA gene sequences showed four distinct clades. One cluster included isolates from different domestic animal species and the type culture of *M. sympodialis* that originated from humans. The remaining three clusters included isolates from cats, grouping together with the *M. nana* AB075224 sequence, and isolates from horses and goats, respectively.

On the basis of a polyphasic approach, we describe here two new lipid-dependent species in the genus *Malassezia*, *Malassezia caprae* sp. nov. and *Malassezia equina* sp. nov., isolated mainly from healthy skin of goats and horses, respectively.

Materials and methods

Strains

The strains examined that correspond to the new species are listed in Table 1. Each strain was isolated from a single animal, and mainly from healthy skin of the ears from goats, and from the healthy skin of the anus from horses. They are from a survey carried out in the Autonomous University of Barcelona (Spain) in the years 1997 and 1998 (Crespo *et al.*, 2002b). Type strains and other strains included in this study are also listed in Table 1. The strains were stored at $-80\,^{\circ}C$ (Crespo *et al.*, 2000b).

Table 1. Strains analyzed and their hosts

Strain	Host
M. caprae CBS 10434^{T} (MA383)	Goat
M. caprae CBS 9967 (MA80)	Goat
M. caprae CBS 9973 (MA400)	Goat
M. caprae MA125	Horse
M. caprae MA333	Goat
M. dermatis CBS 9169^{T}	Human
M. furfur CBS 1878*	Human
M. furfur CBS 7019^{NT}	Human
M. equina CBS 9969^{T} (MA146)	Horse
M. equina CBS 9986 (MA88)	Cow
M. equina MA250	Horse
M. equina MA461	Horse
M. equina MA470	Horse
M. globosa CBS 7966^{T}	Human
M. japonica CBS 9431^{T}	Human
M. japonica CBS 9432	Human
M. nana CBS 9557^{T}	Cat
M. nana CBS 9558	Cow
M. nana CBS 9561	Cow
M. obtusa CBS 7876^{T}	Human
M. obtusa CBS 7968	Human
M. pachydermatis CBS 1879^{NT}	Dog
M. pachydermatis CBS 1919	Dog
M. pachydermatis CBS 4165	Dog
M. restricta CBS 7877^{T}	Human
M. slooffiae CBS 7956^{T}	Dog
M. sympodialis CBS 7222^{T}	Human
M. sympodialis CBS 7978	Human
M. sympodialis CBS 7979	Human
M. sympodialis CBS 8740	Human
M. sympodialis CBS 9968 (MA73)	Sheep
M. sympodialis CBS 9970	Horse
M. sympodialis CWB1	Human
M. yamatoensis CBS 9725^{T}	Human

**M. furfur* CBS 1878 is the neotype of *Pityrosporum ovale*.

Morphological and physiological characterization

The characterization of lipid-dependent yeasts was based on the inability to grow on Sabouraud glucose agar (SGA) and on the ability to use certain polyoxyethylene sorbitanesters (Tween-20, Tween-40, Tween-60 and Tween-80), following the current identification scheme for species described by Guého *et al.* (1996) and the Tween diffusion test proposed by Guillot *et al.* (1996). The Cremophor EL assimilation test (Mayser *et al.*, 1997) and the splitting of esculin (β-glucosidase activity) (Mayser *et al.*, 1997; Guého *et al.*, 1998) were used as additional key characters. Other tests, such as the catalase reaction, growth at different temperatures (32, 37 and 40 °C) on modified Dixon agar (mDA) (36 g of malt extract, 6 g of peptone, 20 g of desiccated ox bile, 10 mL of Tween-40, 2 mL of glycerol, 2 mL of oleic acid and 12 g agar L^{-1}, pH 6.0), and the morphological characteristics after incubation at 32 °C for 7 days in the same culture medium, were also performed (Guého *et al.*, 1996).

D1/D2 26S rRNA gene and ITS–5.8S rRNA gene sequencing and analysis

Methods to isolate the DNA, and sequencing of the D1/D2 domain of the 26S rRNA gene and the ITS–5.8S rRNA gene, were similar to those described previously (Cabañes *et al.*, 2005). The strains were inoculated in 1.5-mL eppendorf tubes containing 500 mL of Sabouraud broth (2% glucose, w/v; 1% peptone w/v) supplemented with chloramphenicol

(1 mg L^{-1}), and incubated overnight in an orbital shaker at 300 r.p.m. and 30 °C. Cells were recovered after centrifugation, washed with 0.9% (w/v) NaCl, frozen in liquid nitrogen, and ground to a fine powder with a pipette tip. The powder was incubated for 1 h at 65 °C in 500 mL of extraction buffer (Tris-HCl 50 mM, EDTA 50 mM, sodium dodecyl sulfate 3%, and 2-mercaptoethanol 1%). The lysate was extracted with phenol/chloroform (1 : 1, v/v), 3 M NaOAc, and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum, and resuspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). DNA was cleaned with Geneclean kit II (BIO 101, Inc., La Jolla, CA), according to the manufacturer's instructions.

The ITS rRNA gene and the 5.8S rRNA gene were amplified using a Perkin Elmer 2400 thermal cycler and primer pairs ITS5 and ITS4 (White *et al.*, 1990). PCR consisted of a predenaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, plus a final extension of 7 min at 72 °C. The molecular masses of the amplified DNA were estimated by comparison with a 100-bp DNA ladder (Bio-Rad Laboratories S.A., Barcelona, Spain).

The PCR product was purified with the GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden), following the supplier's protocol, and purified PCR products were used as a template for sequencing. The protocol BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Nieuwerkerk aan de IJsel, The Netherlands) was used for sequencing. The primers ITS5 and ITS4 described by White *et al.* (1990) were used in the sequencing reaction, and an Applied Biosystems 3100 sequencer was used to obtain the DNA sequences. The sequences were aligned using the software program CLUSTAL X (1.81). The MEGA package, version 2.1, was used to perform a neighbor-joining analysis of a distance matrix (Kimura two-parameter model, transition to tranversion rate 2.0) with 1000 bootstrap replicates and a maximum parsimony analysis.

Chitin synthase and RNA polymerase subunit 1 (RPB1) sequence analysis

The chitin synthase gene was amplified using the primers ChiSyn2f (5′-CTG AAG CTT CAN ATG TAY AAY GAR GAY) and ChiSyn2r (5′-GTT CTC GAG YTT RTA YTC RAA RTT YTG) (Aizawa *et al.*, 1999) in 50-μl reaction volumes containing 3 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer, 1 U of Taq DNA polymerase (Bioline, Gentaur, Brussels, Belgium), and 1 μL of isolated genomic DNA. The following PCR conditions were used: initial denaturation for 5 min at 96 °C, followed by 35 cycles each with a denaturation step of 45 s at 96 °C, annealing for 1 min at 54 °C, an elongation step of 2 min at 72 °C, and a final elongation step of 6 min at 72 °C.

For the RPB1 gene, primers RPB1-Af (5′-GAR TGY CCD GGD CAY TTY GG) and RPB1-Cr (5′-CC NGC DAT NTC RTT RTC CAT RTA) (see http://faculty.washington.edu/benhall/) were used in a reaction mixture as previously described (Matheny *et al.*, 2002). The gene fragment was amplified using the following conditions: initial denaturation of 5 min at 96 °C, followed by 35 cycles each with a denaturation step of 30 s at 96 °C, annealing for 30 s at 59 °C, an elongation step of 2 min at 72 °C, and a final elongation step of 6 min at 72 °C. Amplicons were purified using the GFX PCR DNA purification kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). One to 10 ng of the purified PCR products were used in the cycle sequencing reaction in a total volume of 10 μL, containing 3 μL of 5 × sequencing buffer, 1 μL of BigDye terminator mix, v. 3.1 (both from Applied Biosystems), and 400 nM primer. The sequencing primers were the same as for the PCR reactions. Sequence amplicons were purified using the MultiScreen Filtration System (Millipore, Etten-Leur, The Netherlands) in combination with Sephadex G-50 Super fine (Amersham Pharmacia Biotech).

The sequences were size-fractionated on an ABI 3700 capillary sequencer (Applied Biosystems) and were analyzed using the LASERGENE software package (DNASTAR Inc., Madison, WI). Phylogenetic trees were generated using PAUP* version 4.0b10 for Macintosh (Swofford, 2002). Neighbor-joining analysis was performed with the uncorrected ('p') substitution model, alignment gaps were treated as missing data, and all characters were unordered and of equal weight. For parsimony analysis, gaps were treated as missing data, and all characters were unordered and of equal weight. The heuristic search was performed with 1000 random taxa additions, and tree bisection and reconstruction as the branch-swapping algorithm. Branches of zero length were collapsed, and all equally parsimonious trees were saved. The robustness of the obtained trees was evaluated by 1000 bootstrap replications. Other statistical measures included tree length, consistency index, retention index, and rescaled consistency index.

Amplified fragment length polymorphism (AFLP) analysis

AFLP analysis was performed according to the manufacturer's instructions in the AFLP microbial fingerprinting protocol (Applied Biosystems), with some modifications (Gupta *et al.*, 2004). Restriction and ligation were performed simultaneously on 10 ng of genomic DNA using 1 U of MseI, 5 U of EcoRI, and 3 U of T4 DNA ligase (Biolabs, Westburg, The Netherlands). The sequences of the primers EcoRI and

MseI were 5′-GACTGCGTACCAATTCAC-3′ and 5′-GATGAGTCCTGAGTAAC-3′, respectively. The adaptors used were EcoRI (5′-CTCGTAGACTGCGTACC-3′, forward; 3′-CATCTGACGCATGGTTAA-5′, reverse) and MseI (5′-GACGATGAGTCCTGAG-3′, forward; 3′-CTACTCAGGACTCAT-5′, reverse). The reaction took place in a total volume of 5.5 μL with the following constituents: a 0.36 μM concentration of the EcoRI adaptor and a 3.64 μM concentration of the MseI adaptor from the AFLP microbial fingerprinting kit, 0.1 M NaCl, 0.91 mM Tris-HCl (pH 7.8), 0.18 mM $MgCl_2$, 0.18 mM dithiothreitol, 18 μM ATP, and 91.36 μg of bovine serum albumin mL^{-1}. The restriction ligation mixture was incubated for 2 h at 37 °C, and later diluted by adding 25 μL of sterile double-distilled water. The first PCR was performed with two preselective primers (EcoRI core sequence and MseI core sequence) and the AFLP amplification core mix from the AFLP microbial fingerprinting kit, according to the manufacturer's manual, under the following conditions: 2 min at 72 °C, followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C each. The PCR product was diluted by adding 25 μL of sterile double-distilled water. The second PCR used more selective primers, EcoRI-A FAM and MseI-G. The conditions were 2 min at 94 °C, 10 cycles consisting of 20 s at 94 °C, 30 s at 66 °C (decreasing 1 °C every step of the cycle), and 2 min at 72 °C, and then 25 cycles consisting of 20 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C. The samples were prepared for acrylamide capillary electrophoresis with the following loading mix: 2.0 μL of selective amplification product, 24 μL of de-ionized formamide, and 1 μL of GeneScan-500 labeled with 6-carboxy-X-rhodamine (Applied Biosystems) as an internal size standard. After incubation for 5 min at 95 °C, the samples were run on an ABI 310 genetic analyser for 30 min each. Data were analyzed with the BIONUMERICS software package (version 2.5; Applied Maths, Kortrijk, Belgium), using: (1) Pearson correlation based on similarities of the densitometric curves; and (2) the unweighted pair group method with arithmetic means analysis (UPGMA).

Results and discussion

Morphology and physiology

Malassezia isolates belonging to the new species were characterized using the current morphological and physiological identification scheme (Table 2). The phenotypic characteristics of the new species, *M. caprae* and *M. equina*, and the other described *Malassezia* species, are summarized in Table 3. The isolates belonging to the new species did not grow in SGA without any lipid supplementation. In general, they grew very slowly and formed small colonies (< 0.5–2 mm in diameter; average diameter of *M. caprae* = 1 mm; average diameter of *M. equina* = 1.3 mm) on mDA at 32 °C after 7 days of incubation. After 21 days of incubation at the same temperature, colonies were 3–6 mm in diameter. All the isolates of *M. equina* and two isolates belonging to *M. caprae* grew slowly at 37 °C. None of these isolates grew at 40 °C, and they thus differed from other *M. sympodialis*-related species, such *M. dermatis*, *M. nana* and *M. sympodialis*, which can grow at this temperature.

Malassezia caprae cells were ellipsoidal to subglobose (Fig. 1, Table 2), and *M. equina* cells were mainly ovoidal (Fig. 2, Table 2). Special micromorphological characteristics have been cited for some *Malassezia* spp. In the case of *M. furfur*, the micromorphology appears to be variable in size and shape, including oval, cylindrical or spherical cells, with buds formed on a broad base (Guého *et al*., 1996). In contrast, *M. globosa* has spherical cells with buds formed on a narrow base. *Malassezia sympodialis*-related species are known to have a small cell size in comparison to other *Malassezia* spp. (Crespo *et al*., 2000a; Hirai *et al*., 2004) and buds formed on a narrow base (Simmons & Guého, 1990;

Table 2. Main phenotypical characteristics of the strains belonging to the new species described in this study

Strain	Cell morphology	T 20[a]	T 40[a]	T 60[a]	T 80[a]	Cremophor EL	Catalase	β-Glucosidase	Growth at 37 °C	Growth at 40 °C
MA80	Globose	−	−[2]	−[2]	−	−	+	+	w	−
MA125	Ellipsoidal	−[2]	+[1]	+[1]	+[1]	−	+	−	w	−
MA333	Globose	−[2]	−[2],+	−[2],+	−[2],+,+[1]	−	+	+	−	−
MA383 (CBS 10434[T])	Globose	−[2]	+,+[1]	+,+[1]	+,−[2]	−	+	+	−	−
MA400	Ellipsoidal	−	+[1]	+,+[1]	−	−	+	+	−	−
M. caprae sp.nov.	Globose, ellipsoidal	−[2]	+[1]	+[1]	+[1], (−)	−	+	+, (−)	−, (w)	−
MA88	Ellipsoidal	w[2]	w[2]	w[2]	w[2]	−	+	+	w	−
MA146 (CBS 9969[T])	Ellipsoidal	w[2]	+	+[1]	+[1]	−	+	−	w	−
MA250	Ellipsoidal	w[2]	+	+[1]	+[1]	−	+	−	w	−
MA461	Ellipsoidal	w[2]	+	+[1]	+[1]	−	+	−	w	−
MA470	Ellipsoidal	w[2]	+	+[1]	+	−	+	−	w	−
M. equina sp. nov.	Ellipsoidal	w[2]	+	+[1]	+[1]	−	+	−, (+)	w	−

w, weak; [1], growth may be inhibited near the well where the substrate is placed; [2], growth may occur at some distance from the well where the substrate is placed; ( ) indicate rare deviations from main pattern; [a]Tween diffusion test proposed by Guillot *et al*. (1996).

Table 3. Main phenotypical characteristics of *Malassezia* species*

Species	Cell morphology	Lipid dependency	T 20[a] 10%	T 40[a] 0.5%	T 60[a] 0.5%	T 80[a] 0.1%	T 20[b]	T 40[b]	T 60[b]	T 80[b]	Cremophor EL	Catalase	β-Glucosidase	Growth at 37 °C	Growth at 40 °C
M. dermatis	Ellipsoidal, globose	+	+	+	+	+	+	+	+	+	W,(+)	+	?	+	+
M. furfur	Globose, ellipsoidal, cylindrical	+	+	+	+	+	+,(−)	+,(−)	+,(−)	+,(−)	+,(−)	+,(−)	−,(w)	+	+
M. globosa	Globose	+	−	−	−	−	−	−[2]	−[2]	−	−	+	−	−,(w)	−
M. japonica	Globose, ellipsoidal	+	−	w	+	−	−	w	+	−	?	+	?	+	−
M. nana	Ellipsoidal	+	+,(−)	+	+	w	v	+	+	w	−	+	−	+	v
M. obtusa	Ellipsoidal, cylindrical	+	−	−	−	−	−	−	−	−	−	+	+	−,(w)	−
M. pachydermatis	Ellipsoidal	−,(w)	−	+	+	+	+[1]	+	+	+	+[1]	+,w	+,(−)	+	+
M. restricta	Globose, ellipsoidal	+	−	−	−	−	−	−[3]	−[3]	−	−	−	−	v	−
M. slooffiae	Ellipsoidal, cylindrical	+	+,(w)	+	+	−	+,w,(−)	+	+	−,(w)	−	+	−	+	+
M. sympodialis	Ellipsoidal	+	−	+	+	+	−,w[2]	+	+	+	−,(w)	+	+	+	+
M. yamatoensis	Ellipsoidal	+	+	+	+	+	+	+	+	+	?	+	?	+	−
M. caprae sp. nov.	Globose, ellipsoidal	+	−	w(−)	w	w	−[2]	+[1]	+[1]	+[1],(−)	−	+	+,(−)	−,(w)	−
M. equina sp. nov.	Ellipsoidal	+	−	w	w	w	w[2]	+	+[1]	+[1]	−	+	−,(+)	w	−

*With the exception of the new proposed species, data are from Guého *et al*. (1996), Guillot *et al*. (1996), Guého *et al*. (1998), Sugita *et al*. (2002), Sugita *et al*. (2003), Sugita *et al*. (2004), Hirai *et al*. (2004) and Batra *et al*. (2005) (+, positive; −, negative; v, variable; ?, not included in the description of the species;w, weak; v, variable; ( ) indicate rare deviations from main pattern; [1], growth may be inhibited near the well where the substrate is placed; [2], growth may occur at some distance from the well where the substrate is placed; [3], opaque zone may occur); [a] Tween dilution test proposed by Guého *et al*. (1996); [b] Tween diffusion test proposed by Guillot *et al*. (1996).

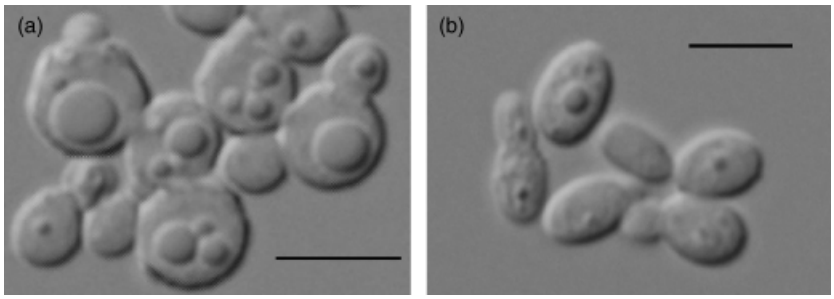

Fig. 1. Cells of *Malassezia caprae*, (a) CBS 10434[T] (MA383) and (b) CBS 9973 (MA400), cultured on mDA at 32 °C for 7 days. Bar, 4 μm.

Guého *et al*., 1996; Crespo *et al*., 2000a; Hirai *et al*., 2004). Occasionally, sympodial budding (Simmons & Guého, 1990; Guého *et al*., 1996; Crespo *et al*., 2000a) has been reported. However, the separation of *Malassezia* species on the basis of morphological characteristics may be considered to be subjective (Guého *et al*., 1996) or unreliable (Guillot & Guého, 1995).

Although the two new species had similar Tween assimilation profiles to *M. sympodialis* and *M. nana*, the isolates analyzed in this study did not completely fit the assimilation profiles of any described species, and hence could not be identified (Table 2). Following the Tween dilution test proposed by Guého *et al*. (1996), the isolates grew poorly on glucose – peptone agar with 0.5% Tween-40%, 0.5% Tween-60, or 0.1% Tween-80, and they did not grow on 10% Tween-20. In the Tween diffusion test proposed by Guillot *et al*. (1996), most of the isolates showed inhibition areas around Tween-40, Tween-60 and Tween-80. These inhibition areas were wider around the Tween-20 wells, and in most cases the isolates did not grow around this compound. These inhibition areas are related to the toxic effects of these compounds at higher concentrations. In fact, the initial poor growth that these isolates showed on culture media for lipid-dependent species, containing different Tweens or other lipidic sources, such as mDA, may be related to their fungistatic properties. None of the isolates grew around Cremophor EL. All, except one (MA 125), of the *M. caprae* isolates showed strong β-glucosidase activity, which was revealed by the splitting of esculin. In contrast, most of the *M. equina* isolates were β-glucosidase-negative.

Among other differences, the isolates belonging to the new species can be distinguished from *M. pachydermatis* by

their inability to grow in SGA, from *M. dermatis*, *M. furfur*, *M. slooffiae* and *M. sympodialis* by their inability to grow at 40 °C, from *M. japonica* by their ability to assimilate Tween-80, from *M. yamatoensis* by their inability to assimilate Tween-20, from *M. obtusa* and *M. globosa* by their ability to assimilate Tween-40 and Tween-60, from *M. restricta* by their catalase activity, and from *M. nana* by their inability to assimilate Tween-20, by their inability to grow at 40 °C, and by poor or no growth at 37 °C.

Molecular analysis

On the basis of the sequence divergence observed in the D1/D2 domains of the 26S rRNA gene (Fig. 3), the ITS regions and the 5.8S rRNA gene (Fig. 4), as well as the chitin synthase (Fig. 5) and RPB1 genes (Fig. 6), we concluded that *M. sympodialis* represents a species complex. Full concordance was observed with clustering of the isolates using the above-mentioned partial genome sequences as well as the AFLP analysis (Fig. 7). Here, we formally describe two of these species. Molecular sequences and AFLP data for species included in Figs 3–7 were compared to confirm that the isolates studied were distinct from the other species of the genus and represent undescribed species.

Figure 3 shows the molecular phylogenetic tree based on the D1/D2 regions of the 26S rRNA gene sequences constructed by the neighbor-joining method. Figure 4 shows the molecular phylogenetic tree based on the ITS1–5.8S–ITS2 rRNA sequences. The isolates belonging to the new proposed species formed a cluster with *M. sympodialis*, *M. dermatis* and *M. nana*.

The isolates belonging to the novel proposed species *M. caprae* had identical D1/D2 sequences. Dissimilarities between *M. caprae* strains and *M. sympodialis* CBS 7222^T, *M. dermatis* CBS 9169^T and *M. nana* CBS 9557^T in their D1/D2 sequences were 1.5%, 1.8% and 2.8%, respectively. Their ITS1–5.8S–ITS2 rRNA gene sequences were also identical, with dissimilarities between *M. caprae* strains and *M. sympodialis* CBS 7222^T, *M. dermatis* CBS 9169^T and *M. nana* CBS 9557^T of 6.5%, 3.4% and 9.9%, respectively.

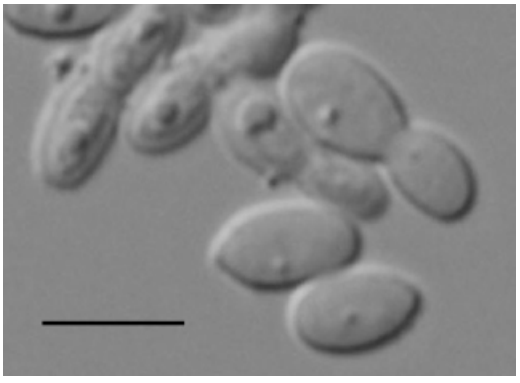

Fig. 2. Cells of *Malassezia equina* CBS 9969^T (MA146) cultured on mDA at 32 °C for 7 days. Bar, 4 μm.

Isolates from the novel proposed species *M. equina* showed nearly identical D1/D2 and ITS sequences, thus indicating that these are conspecific strains. Dissimilarities between *M. equina* CBS 9969^T and the *M. sympodialis*, *M. dermatis* and *M. nana* type strains in the D1/D2 regions of the 26S rRNA gene and ITS1–5.8S–ITS2 rRNA were 1.3% and 9.1%, 1.3% and 6.7%, and 3.5% and 12.2%, respectively. The sequences of '*M. equi*' AJ305330 (Nell *et al*., 2002) and *M. equina* CBS 9969^T were identical, but unfortunately, we were not able to analyze '*M. equi*' ITS1–5.8S–ITS2 rRNA sequences, because there is no such sequence deposited in the GenBank and, furthermore, no '*M. equi*' type strain is preserved in culture collections.

In each novel species, the strains were found to be closely related to each other. Phylogenetic analysis of sequences from these novel species showed that they were clearly distinct from the other 11 described *Malassezia* species, exceeding the variation generally observed to occur between species (Scorzetti *et al*., 2002).

The clades obtained with the analyzed strains of *M. caprae* and *M. equina* using chitin synthase (Fig. 5) and RPB1 (Fig. 6) sequences are also close to those of *M. sympodialis*. These sequences showed the following dissimilarities: between *M. sympodialis* and *M. caprae*, 1.6% and 9.4% respectively; between *M. sympodialis* and *M. equina*, 19.7% and 12.9%, respectively. The same sequences showed that *M. dermatis* differs from *M. caprae* by 7.5% and 18% respectively, and from *M. equina* by 12.2% and 4.5%, respectively. Those from *M. nana* differ from those from *M. caprae* by 12.2% and 14.2%, respectively, and from those from *M. equina* by 17% and 14%, respectively. Therefore, these data clearly support the distinction of our new species from the remaining species of the genus *Malassezia*.

The UPGMA dendrogram (Fig. 7) calculated from the AFLP fingerprints obtained from the different *Malassezia* strains clearly differentiated the strains belonging to the proposed novel species *M. caprae* and *M. equina* from the rest of the species belonging to the genus *Malassezia*. The similarities in the AFLP profile among the analyzed *M. caprae* and *M. equina* strains were 89.8% and 95.7%, respectively. On the other hand, the similarities of these two novel species in comparison with other species in the genus *Malassezia* were 62.7% for *M. caprae* with its closest relative *M. sympodialis*, and 13.0% between *M. equina* and all other *Malassezia* species.

Mechanisms of divergence

All four targeted genome regions supported the *sympodialis* lineage within *Malassezia* with high statistical support, thus indicating the reliability of our analysis. The five species,

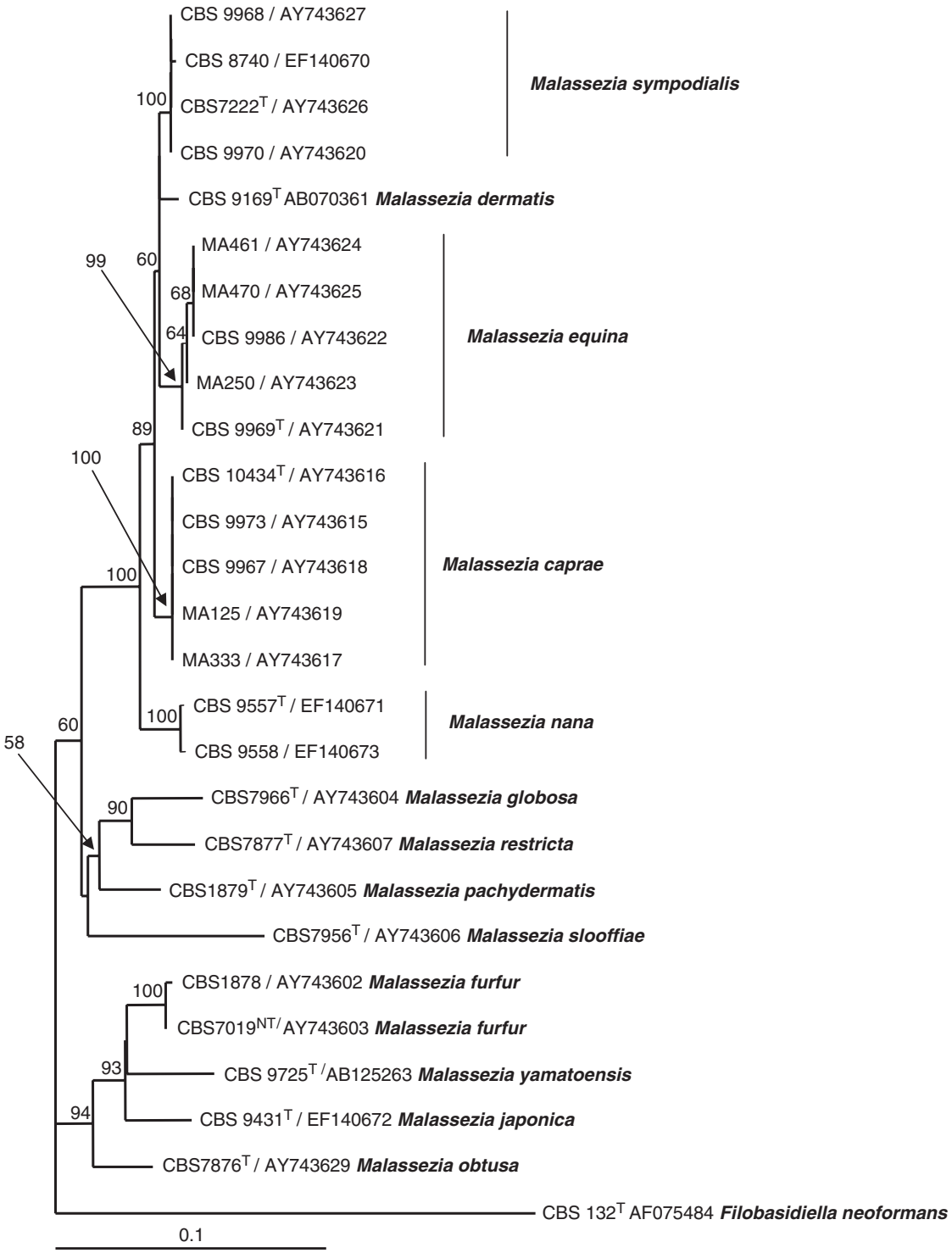

Fig. 3. Molecular phylogenetic tree constructed using the D1/D2 sequences of the 26S rRNA gene of members of the genus *Malassezia*. The numbers at branch points are the percentages of 1000 bootstrapped datasets that supported the specific internal branches. Outgroup: *Filobasidiella neoformans* CBS 132^{T}. Species with GenBank numbers represent sequences obtained from GenBank.

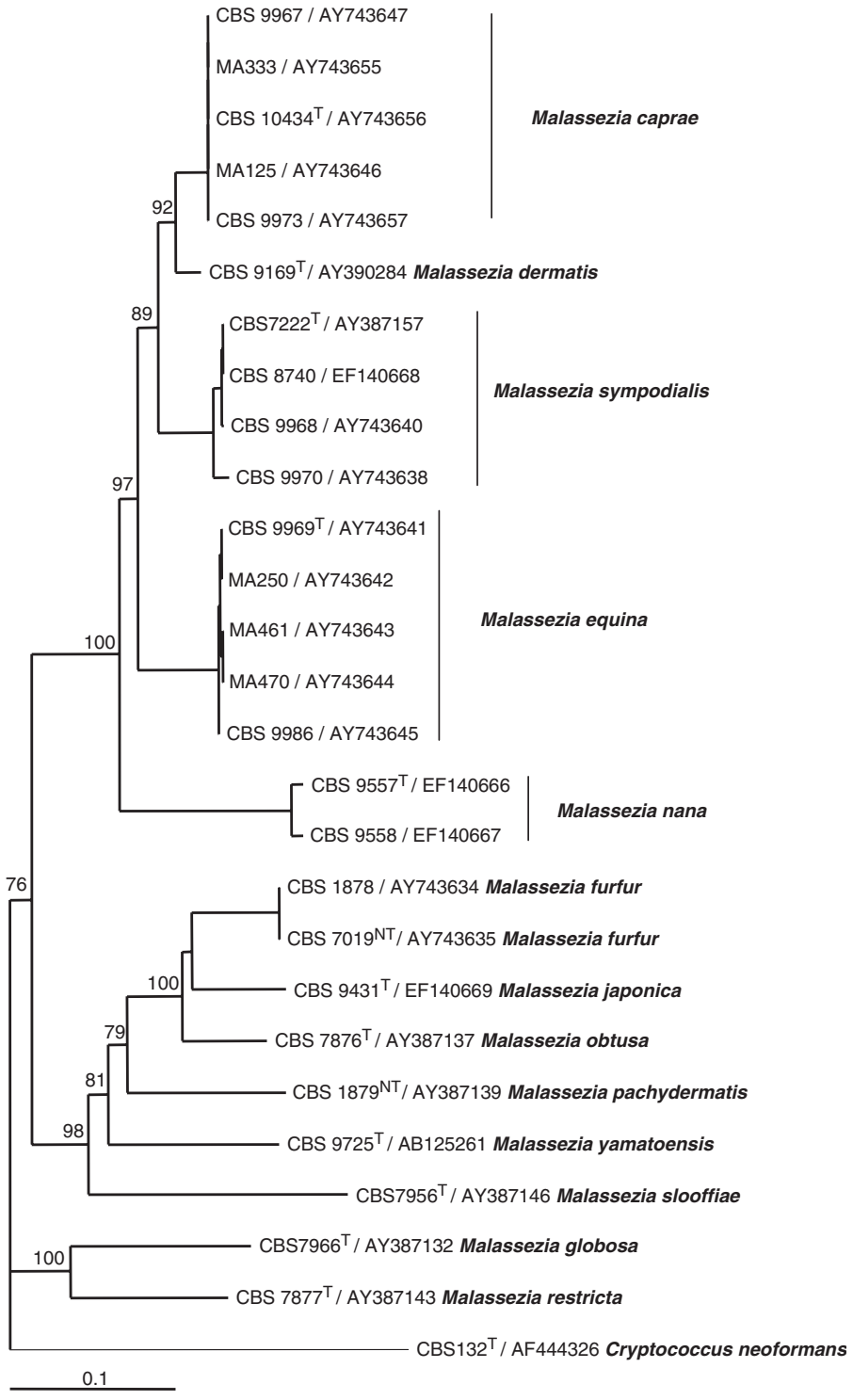

Fig. 4. Molecular phylogenetic tree constructed using the ITS–5.8S rRNA gene sequences of members of the genus *Malassezia*. The numbers at branch points are the percentages of 1000 bootstrapped datasets that supported the specific internal branches. Outgroup: *Cryptococcus neoformans* CBS 132^{T}. Species with GenBank numbers represent sequences obtained from GenBank.

M. nana, *M. dermatis*, *M. sympodialis*, *M. equina* and *M. caprae*, all formed separate and well-supported clades in the analysis of each molecular marker, as well as in the AFLP analysis. However, within the *sympodialis* lineage, the topology of the species was not concordant between the four markers investigated. Three main topologies were observed, with ITS–5.8S rRNA gene and RPB1 supporting the same topology, and both D1/D2 and chitin synthase supporting alternative topologies. Interestingly, all these topologies received high nodal support. In the case of speciation through

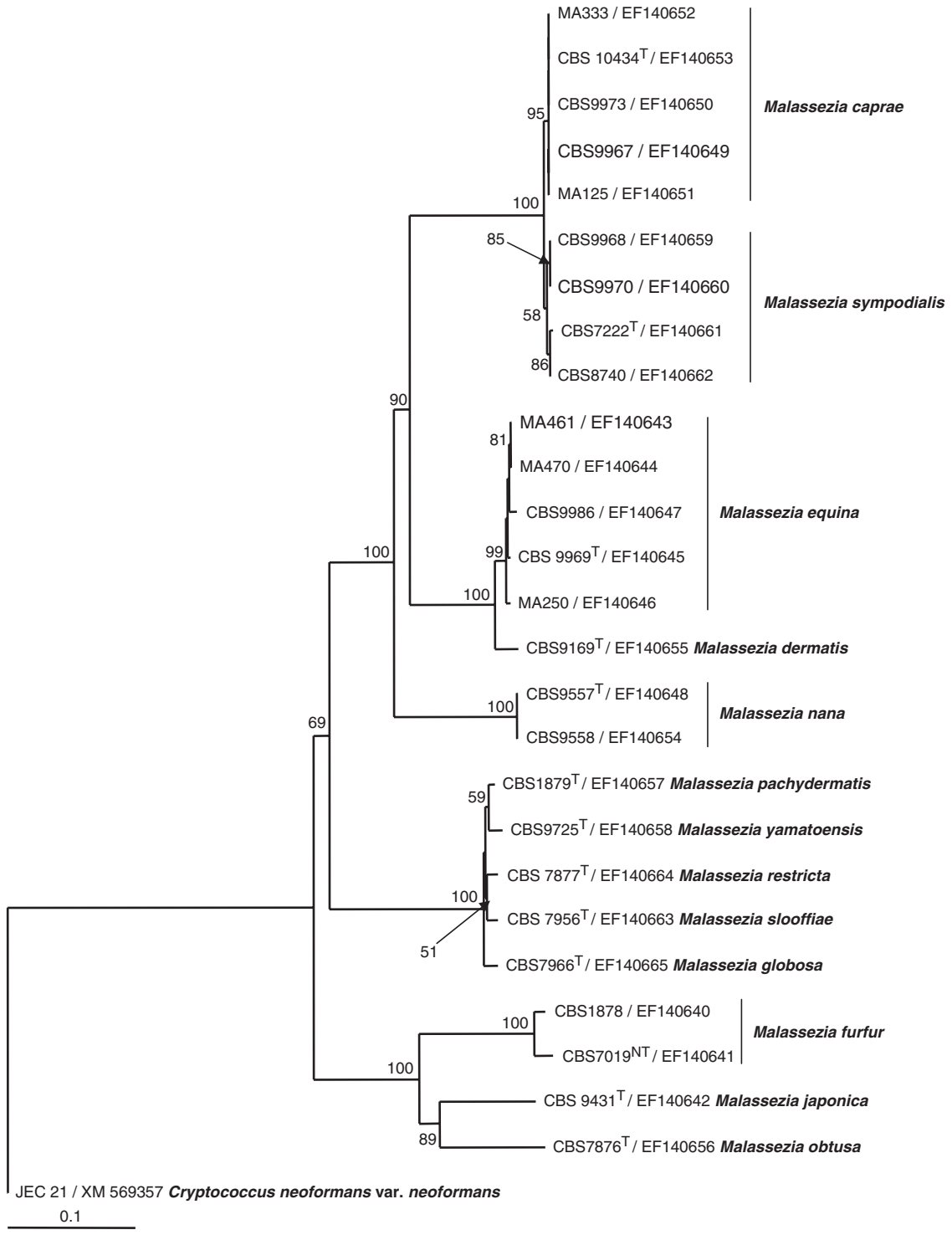

Fig. 5. Molecular phylogenetic tree constructed using the sequences of chitin synthase gene sequences of members of the genus *Malassezia*. The numbers at branch points are the percentages of 1000 bootstrapped datasets that supported the specific internal branches. Outgroup: *Cryptococcus neoformans* JEC 21. Species with GenBank numbers represent sequences obtained from GenBank.

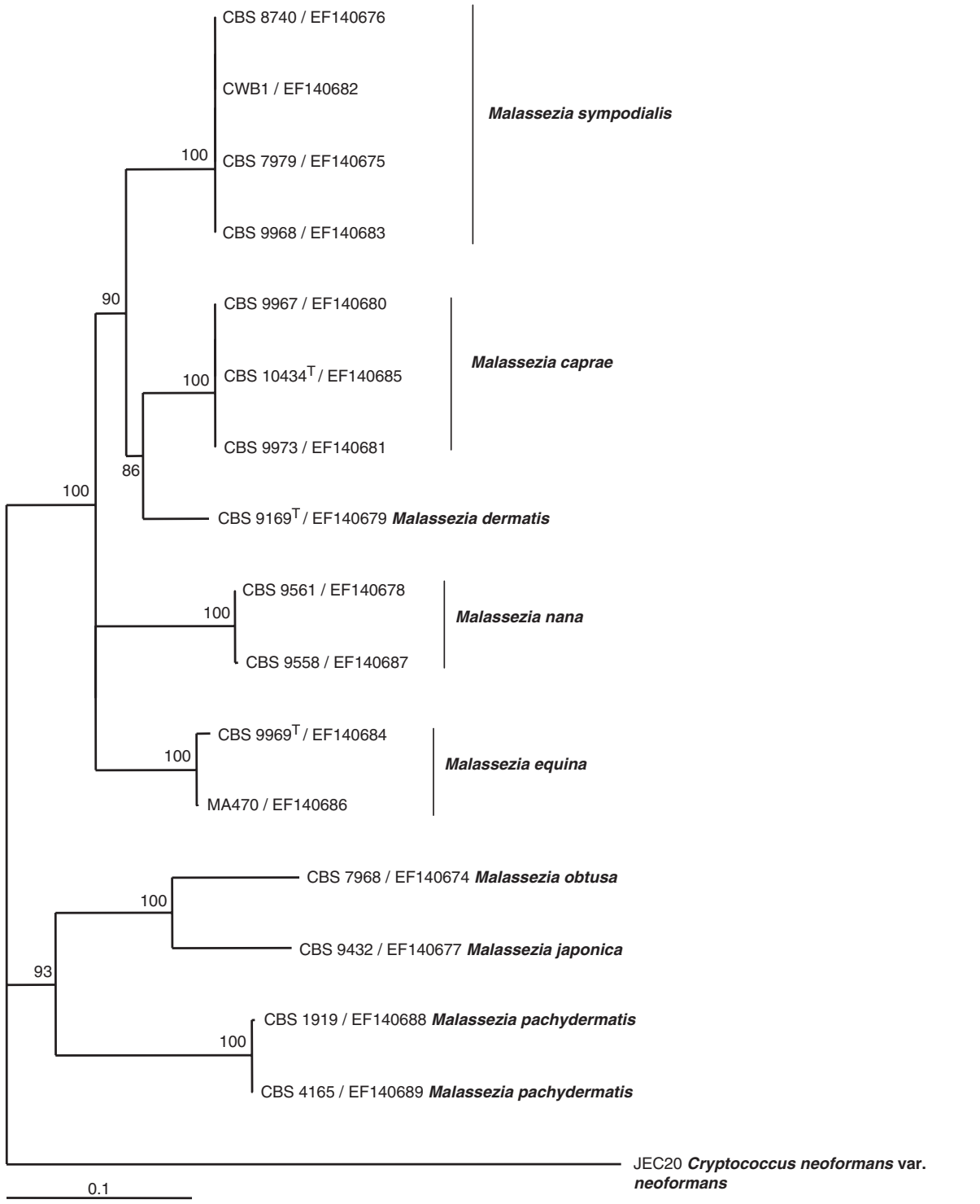

Fig. 6. Molecular phylogenetic tree constructed using the sequences of RNA polymerase subunit 1 gene sequences of members of the genus *Malassezia*. The numbers at branch points are the percentages of 1000 bootstrapped datasets that supported the specific internal branches. Outgroup: *Cryptococcus neoformans* JEC 20. Species with GenBank numbers represent sequences obtained from GenBank.

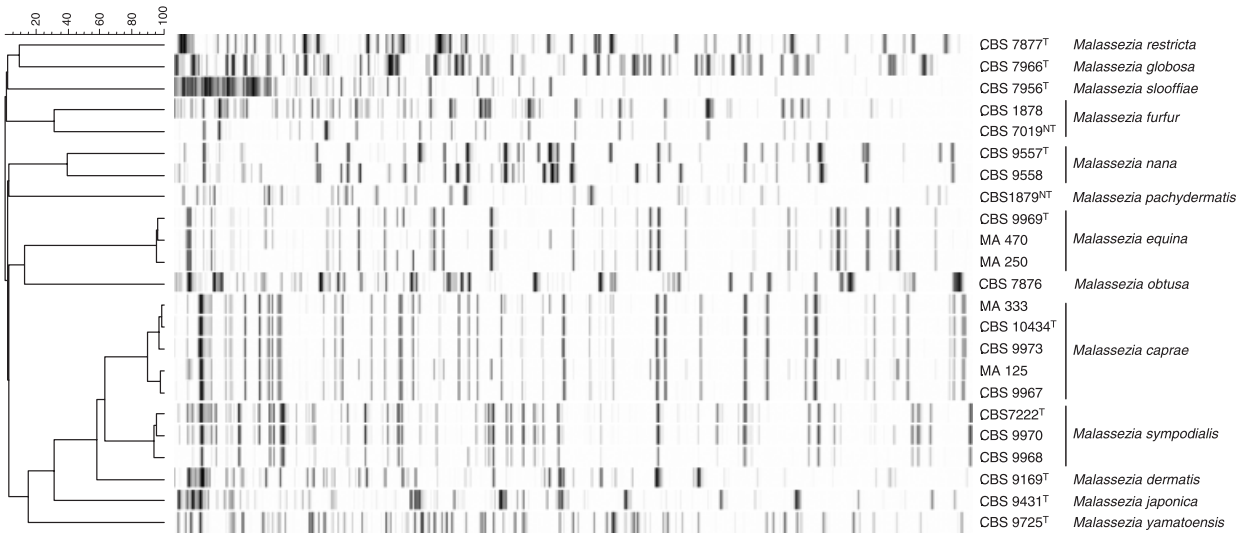

Fig. 7. UPGMA dendrogram assessed from the comparison of AFLP fingerprints of *Malassezia* species.

clonal divergence and genetic drift, probably followed by some host adaptation, one would expect concordance between the phylogenetic patterns of each individual gene. This clearly is not the case, and the lack of concordance may indicate that recombination has played a role in the divergence of these species. This is particularly interesting, as sexual reproduction is unknown in *Malassezia*. However, recombination has been suggested to occur in *M. pachydermatis*, on the basis of isozyme analyses (Midreuil *et al.*, 1999), and in the *M. furfur* complex, a putative hybrid genotype has been observed (R. Batra and T. Boekhout, unpubl. observ.), thus suggesting that cell fusion, karyogamy and meiosis may be possible within the genus.

Interestingly, within the *sympodialis* lineage, the cat- and cow-associated *M. nana* was found to be a basal species in all cases. The subsequent ingroup lineage was found to be *M. equina* (ITS–5.8S rRNA gene) or *M. caprae* (D1/D2), and these analyses placed the two species from human hosts, *M. sympodialis* and *M. dermatis*, together with isolates from goats (*M. caprae*). The D1/D2 analysis, in contrast, placed the two human-associated species with *M. equina*, a horse-associated species, whereas the chitin synthase analysis placed *M. sympodialis* as a sister group to *M. caprae*, and *M. dermatis* as a sister group to *M. equina*. Therefore, all our data support the proposition that a cat- and cow-associated species (i.e. *M. nana*) formed a basal lineage to the other species. Moreover, the data also support that host shifts from animals to humans may have occurred more than once. In order to better understand the mechanism of speciation in relation to host jumps in this interesting asexual and clinically important group of yeasts, considerable effort is needed to sequence more loci across the known biodiversity of the genus. This is even more true given that we have to include the other lineages known to exist within *Malassezia* (e.g. the *furfur* lineage, the *globosa* lineage, and *M. slooffiae*), as the exact infrageneric relationships among these lineages are not yet clear from our data.

To summarize this molecular analysis, we point out that members of these new species form two well-supported clades on the basis of comparative analysis of five molecular markers. However, the differences found in the different genes analyzed among the strains under study support the recognition of two distinct species, for which the names *M. caprae* sp. nov. and *M. equina* sp. nov. are proposed.

Latin diagnosis of *M. caprae* Cabañes et Boekhout, sp. nov.

Cultura in agaro Dixonii post 7 dies ad 32 °C albida vel cremea, glabra, lucida aut hebetata, butyracea, moderate convexa, margine expresso (1 mm). Cellulae ovoidae aut globosae, 2.7–4.5 × 1.7–4.5 μm, e base angusta gemmantes. In agaro glucoso-peptonico Tween-40 (0.5%), Tween-60 (0.5%) et Tween-80 (0.1%) addito paulum crescit. In agaro glucoso-peptonico Tween-20 (10%) addito non crescit. 37 °C non vel paulum crescit neque 40 °C. Teleomorphis ignota. Typus CBS 10434 (MA383 = JCM 14561); isolatus ex cute caprina; depositus in collectione zymotica CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.

Description of *M. caprae* Cabañes & Boekhout, sp. nov.

Malassezia caprae [*caprae*: this Latin-derived species epithet refers to the the host animal from which the yeast was first

isolated (ca'prae. L. fem. n. *capra* goat; L. fem. gen. n. *caprae* of a goat)].

On mDA, after 7 days at 32 °C, colonies are small (average diameter 1 mm, < 0.5–1.8 mm), whitish to cream-colored, smooth, glistening or dull, butyrous and moderately convex with entire margins. Cells are ovoidal to spherical, 2.7–4.5 × 1.7–4.5 μm, with buds formed monopolarly on a narrow base. No growth is obtained on SGA. Catalase reaction is positive, and β-glucosidase activity is usually positive, except for isolate MA 125. No growth occurs on glucose–peptone agar with 10% Tween-20. Poor growth is observed on glucose–peptone agar with Tween-40 (0.5%), Tween-60 (0.5%) and Tween-80 (0.1%). No growth is observed on glucose–peptone agar with Cremophor EL. No or weak growth appears at 37 °C, and no growth occurs at 40 °C. The teleomorph is unknown.

The type strain CBS 10434 (= JCM 14561; originally strain MA383) was isolated from healthy skin of the ear of a goat in Barcelona, Spain. The strains were deposited in the CBS Fungal Biodiversity centre, Utrecht, The Netherlands, and in the Japan Collection of Microorganisms, Saitama, Japan, as CBS 10434 and JCM 14561, respectively.

Latin diagnosis of *M. equina* Cabañes et Boekhout, sp. nov.

Cultura in agaro Dixonii post 7 dies ad 32 °C albida vel cremea, glabra, lucida aut hebetata, butyracea, moderate convexa, margine expresso (1.3 mm). Cellulae ovoidae, 2.9–4.7 × 1.2–3.1 μm, e base angusta gemmantes. In agaro glucoso-peptonico Tween-40 (0.5%), Tween-60 (0.5%) et Tween-80 (0.1%) addito paulum crescit. In agaro glucoso-peptonico Tween-20 (10%) addito non crescit. 37 °C paulum crescit. 40 °C non crescit. Teleomorphis ignota. Typus CBS 9969 (MA146 = JCM 14562); isolatus ex cute equina; depositus in collectione zymotica CBS Fungal Biodiversity Centre, Utrecht, The Netherlands.

Description of *M. equina* Cabañes & Boekhout, sp. nov.

Malassezia equina [*equina*: this Latin-derived species epithet refers to the the host animal from which the yeast was first isolated (e.quin'a. L. adj. *equina* of horses)].

On mDA, after 7 days at 32 °C, colonies are small (average diameter 1.3 mm, range < 0.5–2 mm), whitish to cream-colored, smooth, glistening to dull, butyrous and moderately convex with an entire margin. Cells are ovoidal, 2.9–4.7 × 1.2–3.1 μm, with buds formed monopolarly on a narrow base. No growth is obtained on SGA. Catalase reaction is positive, and the β-glucosidase activity is usually negative. No growth occurs on glucose–peptone agar with 10% Tween-20. Poor growth is observed on glucose–peptone agar with Tween-40 (0.5%), Tween-60 (0.5%) and Tween-80 (0.1%). No growth is observed on glucose–peptone agar with Cremophor EL. Poor growth appears at 37 °C, and no growth occurs at 40 °C. The teleomorph is unknown. The type strain CBS 9969 (= JCM 14562; originally strain MA146) was isolated from healthy skin of the anus of a horse in Barcelona, Spain. The strains were deposited in the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands, and in the Japan Collection of Microorganisms, Saitama, Japan, as CBS 9969 and JCM 14562, respectively.

Nell *et al*. (2002) reported the presence of a novel *Malassezia* species from normal equine skin, which they tentatively named '*Malassezia equi*', but without including a valid description. It was identified by 26S rRNA gene D1/D2 sequence analysis as a member of the genus *Malassezia*, and was found to be most closely related to *M. sympodialis*. The D1/D2 sequences of '*M. equi*' (AJ305330) (Nell *et al*., 2002) and of the type species of *M. equina* (CBS 9969^T = MA146) are identical (Cabañes *et al*., 2005), so they are very related organisms, and probably conspecific. We were not able to analyze the '*M. equi*' ITS sequences or any other gene sequences, because there is no other sequence deposited in GenBank. Moreover, '*M. equi*' has not formally been described (e.g. no Latin diagnosis and no type strain indicated), and data on morphological and physiological characteristics, such as growth on the various Tweens, esculin and Cremophor EL, were not provided in the description. Furthermore, no strain has been preserved for this taxon. For these reasons, this species (Nell *et al*., 2002) is an invalidly described species. Therefore, the name '*M. equi*' Nell *et al*. can be considered to be nonexistent. To avoid any future confusion, we decided to provide our species the epithet *equina*.

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