

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 revealed to represent novel species. In the present 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 26S rDNA, the ITS1-5.8S-ITS2 rDNA, the RNA polymerase subunit 1 (RPB1) and chitin synthase nucleotide sequences and by analysis of the amplified fragment length polymorphism (AFLP) patterns, which were all consistent in separating these new species from the other species of the genus, and the *M. sympodialis* species cluster specifically.

Keywords: *Malassezia caprae*, *Malassezia equina*, taxonomy, yeast, domestic animals, skin, asexual speciation

Introduction

Since the genus *Malassezia* was created by Baillon in 1889, its taxonomy has been a matter of controversy. The genus remained limited to *M. 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, while the lipophilic, but non lipid-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 of the description of *M. furfur*. Afterwards, the genus *Malassezia* was revised on the basis of morphological, physiological and rRNA 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; Bond *et al.*, 1997; Crespo *et al.*, 1999; Crespo *et al.*, 2002a) and from the healthy skin of horses and different domestic ruminants, being the major component of the lipophilic mycobiota in 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 including a valid description, nor a type specimen. It was identified by 26S rDNA 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 not alive anymore (C. Bond, personal communication). Crespo *et al.* (Crespo *et al.*, 2000a) reported for the first time lipid-dependent yeasts associated with otitis externa in cats having similar morphological characteristics and some shared physiological characteristics with the type strain of *M. sympodialis*. Recently, Hirai *et al.* (Hirai *et al.*, 2004) described *M. nana*, a novel species from otic discharges of a cat and cows, which are also closely related to *M. sympodialis*.

However, the difficulty to obtain 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 can not even be identified (Duarte *et al.*, 1999; Crespo *et al.*, 2000a; Crespo *et al.*, 2002b Duarte *et al.*, 2002). 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 26S rDNA and ITS-5.8S rDNA sequences showed 4 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.

Based on 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.

Material and methods

Strains

The strains examined corresponding 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 listed also in this table. The strains were stored at -80°C (Crespo *et al.*, 2000b).

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 (Tweens 20, 40, 60 and 80), following the current identification scheme of species described by Guého *et al.* (Guého *et al.*, 1996) and the Tween diffusion test proposed by Guillot *et al.* (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°C, 37°C and 40°C) on modified Dixon agar (mDA) (36 g malt extract; 6 g peptone, 20 g desiccated ox-bile; 10 ml Tween 40; 2 ml glycerol; 2 ml oleic acid and 12 g agar per litre, 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 rDNA and ITS-5.8S rDNA sequencing and analysis

Methods to isolate the DNA and sequencing of the D1/D2 domain of the 26S rDNA and the ITS regions and the 5.8S rDNA were similar to those described previously (Cabañes *et al.*, 2005). Cells were harvested from 4- to 5-day-old cultures in modified Dixon's medium. The cells were incubated for 1h at 65°C in 500 μ l of extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, and 1% 2-mercaptoethanol). The lysate was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v). Then 65 μ l of 3M sodium acetate and 75 μ l of 1M NaCl were added to 350 μ l of the supernatant, and the resulting volume was incubated at 4°C for 30min. DNA was recovered by isopropanol precipitation and washed with 70% (v/v) ethanol, dried under a vacuum, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). DNA was cleaned with the GeneClean kit II (BIO 101, Inc., La Jolla, Calif.) according to the manufacturer's instructions.

ITS rDNA and 5.8S rDNA were amplified using a Perkin Elmer 2400 thermal cycler and primer pairs ITS5 and ITS4 (White *et al.*, 1990). PCR consisted of a pre-

denaturation 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 IJssel, The Netherlands) was used for sequencing. The primers ITS5 and ITS4 described by White *et al.* (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 by 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 2-parameter model, transition to transversion 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 ACN 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 MgCl₂, 200 µM of each dNTP, 1 µM of each primer and 1U DNA Taq polymerase (Bioline, Gentaur, Brussels, Belgium) and 1 µl of isolated genomic DNA. The

following PCR conditions were used: initial denaturation of 5 min at 96°C, followed by 35 cycles each with a denaturation step of 45 sec at 96°C, annealing of 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 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 sec at 96°C, annealing of 30 sec 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 ten ng of the purified PCR products were used in the cycle sequencing reaction in a total volume of 10 µl, containing 3 µl 5x sequencing buffer and 1 µl BigDye[™] terminator mix, v3.1 (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 analysed using the Lasergene software package (DNASTAR Inc., Madison, Wisconsin, U.S.A.). Phylogenetic trees were generated using PAUP* version 4.0b10 for Macintosh (Swofford, 2002). Neighbour 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 (TBR) 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 statistic measures included tree length, consistency index, retention index and rescaled consistence index (TL, CI, RI and RC).

Amplified fragment length polymorphism 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 by using 1 U of MseI, 5 U of EcoRI, and 3 U of T4 DNAligase (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 μ l with the following constituents: a 0.36 μ M concentration of the EcoRI adaptor and a 3.64 μ M 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 MgCl₂, 0.18 mM dithiothreitol, 18 μ M ATP, and 91.36 μ g 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 deionised formamide, and 1 µl of GeneScan-500 labelled 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 analysed with the Bionumerics software package (version 2.5; Applied Maths, Kortrijk, Belgium), by using (i) Pearson correlation based on similarities of the densitometric curves and (ii) 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 by using the current morphological and physiological identification scheme (Table 2). The

phenotypical 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 reached 3-6 mm of 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, thus differing from other *M. sympodialis* related species, such *M. dermatis*, *M. nana* or *M. sympodialis*, which can grow at this temperature.

Cells were ellipsoidal to subglobose in *M. caprae* (Fig. 1, Table 2) and mainly ovoidal in *M. equina* (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). On the contrary, *M. globosa* has spherical cells with buds formed on a narrow base. *M. 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; 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 based on 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 analysed in the present 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.* (Guého *et al.*, 1996) the isolates grew poorly on glucose-peptone agar with 0.5% Tween 40, 0.5% Tween 60, 0.1% Tween 80, and they did not grow on 10% Tween 20. In the Tween diffusion test proposed by Guillot *et al.* (Guillot *et al.*, 1996) most of the isolates showed inhibition areas around the Tweens 40, 60 and 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 lipodic 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 a strong β -glucosidase activity, which was revealed by the splitting of esculin. On the contrary, 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 because they showed poor or no growth at 37° C.

Molecular analysis

Based on the sequence divergence observed in the D1/D2 domains of the 26S rDNA (Fig. 3), the ITS regions and the 5.8S rDNA (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 Figures 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 rDNA sequences constructed by the neighbor-joining method. Figure 4 shows the molecular phylogenetic tree based on the ITS1-5.8S-ITS2 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 *Malassezia 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 rDNA sequences were also identical having 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.

Isolates from the novel proposed species *Malassezia 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 26S and ITS1-5.8S-ITS2 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 analyse “*M. equi*” ITS1-5.8S-ITS2 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 eleven described *Malassezia* species, exceeding the variation generally observed to occur between species (Scorzetti *et al.*, 2002).

The clades obtained with the analysed 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 dissimilarity between *M. sympodialis* and those from *M. caprae*: 1.6% and 9.4% respectively and from *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* differs from *M. caprae* by 12.2% and 14.2% respectively and 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 similarity of these two novel species in comparison with other species in the genus *Malassezia* was 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, *M. nana*, *M. dermatis*, *M. sympodialis*, *M. equina* and *M. caprae*, formed all 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 rDNA 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 case of speciation through 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 probably 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* based on 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 rDNA) 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, placed *M. sympodialis* as a sister group to *M. caprae*, and *M. dermatis* to *M. equina*. Therefore, all our data support 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, a considerable effort is needed to sequence more loci across the known biodiversity of the genus. This is even more true as one has 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 is 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 using comparative analysis of five molecular markers. However, the differences found in the different genes analysed among the strains under

study support the recognition of two distinct species, for which the names *Malassezia caprae* sp. nov and *Malassezia equina* sp. nov., are proposed.

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

Cultura in agaro Dixonii post 7 dies ad 32° C albida vel crenea, glabra, lucida aut hebetata, butyracea, moderate convexa, margine expresso (1 mm). Cellulae ovoidae aut globosae, 2.7-4.5 x 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 Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Description of *Malassezia 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-coloured, smooth, glistening or dull, butyrous and moderately convex with entire margins. Cells are ovoidal to spherical, 2.7-4.5 x 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 Collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and in the Japan Collection of Microorganisms, Saitama, Japan, as CBS 10434 and JCM 14561, respectively.

Latin diagnosis of *Malassezia 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 x 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 Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Description of *Malassezia 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-coloured, smooth, glistening to dull, butyrous and moderately convex with an entire margin. Cells are ovoidal, 2.9-4.7 x 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 Collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and in the Japan Collection of Microorganisms, Saitama, Japan, as CBS 9969 and JCM 14562, respectively.

Nell *et al.* (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 rDNA 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*” was not formally been described (e.g. no latin diagnosis and no type strain indicated) and data on morphological and physiological description, 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 that, consequently does not exist. Therefore the name “*M. equi*” Nell et al. can be considered as non-existent. To avoid any future confusion we decided to provide our species the epithet *equina*.

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

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

Figure 3. Molecular phylogenetic tree constructed using the sequences of D1/D2 26S rDNA of members of the genus *Malassezia* species. The numbers at branch point are the percentages of 1,000 bootstrapped data sets that supported the specific internal branches. Outgroup: *Filobasidiella neoformans* CBS 132^T. Species with GenBank numbers represent sequences obtained from GenBank.

Figure 4. Molecular phylogenetic tree constructed using the sequences of ITS-5.8S rDNA gene sequences of members of the genus *Malassezia*. The numbers at branch point are the percentages of 1,000 bootstrapped data sets that supported the specific internal branches. Outgroup: *Cryptococcus neoformans* CBS 132^T. Species with GenBank numbers represent sequences obtained from GenBank.

Figure 5. Molecular phylogenetic tree constructed using the sequences of chitin synthase gene sequences of members of the genus *Malassezia*. The numbers at branch point are the percentages of 1,000 bootstrapped data sets that supported the specific internal branches. Outgroup: *Cryptococcus neoformans* JEC 21. Species with GenBank numbers represent sequences obtained from GenBank.

Figure 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 point are the percentages of 1,000 bootstrapped data sets that supported the specific internal branches. Outgroup: *Cryptococcus neoformans* JEC 20. Species with GenBank numbers represent sequences obtained from GenBank.

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

Table 1. Strains analyzed and their hosts.

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

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

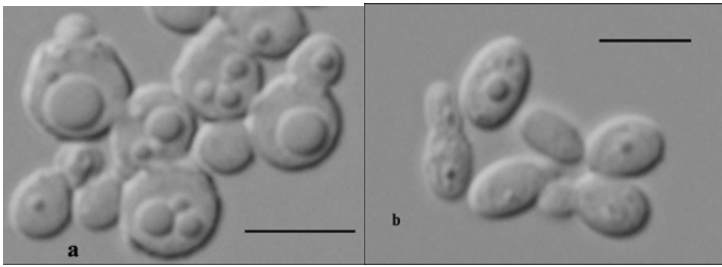


Figure 1.

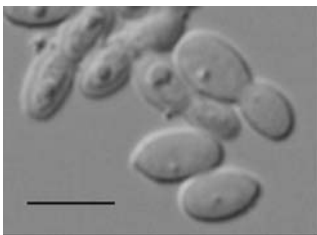


Figure 2.