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

F. Javier Cabañes[1*], Bart Theelen[2], Gemma Castellá[1] and Teun Boekhout[2].

[1]Grup de Micologia Veterinària. Departament de Sanitat i d' Anatomia Animals, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, E-08193 Spain.

[2] Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

***corresponding author**

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 –80ºC (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 (32ºC, 37ºC and 40ºC) 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 32ºC 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 µl 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 µl of 3Msodium acetate and 75 µl of 1M NaCl were added to 350 µl 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 predenaturation step at 94ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 50ºC for 1 min and extension at 72ºC for 1 min, plus a final extension of 7 min at 72ºC. 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.* (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 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 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 $MgCl_2$, 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 96ºC, followed by 35 cycles each with a denaturation step of 45 sec at 96ºC, annealing of 1 min at 54ºC, an elongation step of 2 min at 72ºC and a final elongation step of 6 min at 72ºC.

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 96ºC, followed by 35 cycles each with a denaturation step of 30 sec at 96ºC, annealing of 30 sec at 59ºC, an elongation step of 2 min at 72ºC and a final elongation step of 6 min at 72ºC. Amplicons were purified using the GFX^{Tm} 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^{Tm} 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 purifed using the MultiScreen^{Tm} Filtration System (Millipore, Etten-Leur, The Netherlands) in combination with Sephadex^{Tm} 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 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 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 growth 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 ºC 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 37º C. None of these isolates grew at 40 ºC, 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 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 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 40º 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 40º C and because they showed poor or no growth at 37º 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 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 32º C albida vel cremea, 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. 37ºC non vel paulum crescit neque 40ºC. 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 32ºC, 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 37ºC and no growth occurs at 40ºC. 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 32º 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. 37ºC paulum crescit. 40ºC 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 32ºC, 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 37ºC and no growth occurs at 40ºC. 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*.

ACKNOWLEDGMENTS

This work was supported by grants NL-TAF-864, SYNTHESYS project of the European Commission's Research Infrastructure Action (EU) and 2005SGR00684 from the DURSI, Generalitat de Catalunya, Spain.

References

Aizawa T, Kano R, Nakamura Y, Watanabe S, Hasegawa A (1999) Molecular heterogeneity in clinical isolates of *Malassezia pachydermatis* from dogs. *Vet Microbiol* **70**: 67-75.

Batra R, Boekhout T, Guého E, Cabañes FJ, Dawson TL & Gupta AK (2005) *Malassezia* Baillon, emerging clinical yeasts. *FEMS Yeast Res* **5**:1101-1113.

Bond R, Anthony RM, Dodd M & Lloyd DH (1996) Isolation of *Malassezia sympodialis* from feline skin. *J Med Vet Mycol* **34**: 145-147.

Bond R, Howell SA, Haywood PJ & Lloyd DH (1997) Isolation of *Malassezia sympodialis* and *Malassezia globosa* from healthy pet cats. *Vet Rec* **141**: 200-201.

Cabañes FJ, Hernández JJ & Castellá G (2005) Molecular analysis of *Malassezia sympodialis* related strains from domestic animals. *J Clin Microbiol* **43**: 277-283.

Crespo MJ, Abarca ML & Cabanes FJ (1999) Isolation of *Malassezia furfur* from a cat. *J Clin Microbiol* **37**: 1573-1574.

Crespo MJ, Abarca ML & Cabanes FJ (2000a) Otitis externa associated with *Malassezia sympodialis* in two cats. *J Clin Microbiol* **38**: 1263-1266.

Crespo MJ, Abarca ML & Cabanes FJ (2000b) Evaluation of different preservation and storage methods for *Malassezia* spp. *J Clin Microbiol* **38**: 3872-3875.

Crespo MJ, Abarca ML & Cabanes FJ (2002a) Occurrence of *Malassezia* spp. in the external ear canals of dogs and cats with and without otitis externa. *Med Mycol* **40**: 115-121.

Crespo MJ, Abarca ML & Cabanes FJ (2002b) Occurrence of *Malassezia* spp. in horses and domestic ruminants. *Mycoses* **45**: 333-337.

Duarte ER, Melo MM, Hahn RC & Hamdan JS (1999) Prevalence of *Malassezia* spp. in the ears of asymptomatic cattle and cattle with otitis in Brazil. *Med Mycol* **37**: 159-162.

Duarte ER, Lachance MA & Hamdan JS (2002) Identification of atypical strains of *Malassezia* spp. from cattle and dog. *Can J Microbiol* **48**: 749-752.

Guého E, Midgley G & Guillot J (1996) The genus *Malassezia* with the description of four new species. *Antonie van Leeuwenhoek* **69**: 337-355.

Guého E, Boekhout T, Ashbee HR, Guillot J, van Belkum A & Faergemann J (1998) The role of *Malassezia* species in the ecology of human skin and as pathogens. *Med. Mycol.* **36**, 220-229.

Guillot J & Bond R (1999) *Malassezia pachydermatis*: a review. *Med Mycol* **37**: 295-306.

Guillot J & Guého E (1995) The diversity of *Malassezia* yeasts confirmed by rRNA sequence and nuclear DNA comparisons. *Antonie van Leeuwenhoek* **67**: 297-314.

Guillot J, Guého E, Lesourd M, Midgley G, Chevrier G & Dupont B (1996). Identification of *Malassezia* species, a practical approach. *J Mycol Méd* **6**: 103-110.

Gupta AK, Boekhout T, Theelen B, Summerbell RC & Batra R (2004) Identification and typing of *Malassezia* species by amplified fragment length polymorphism (AFLP) and sequence analyses of the internal transcribed spacer (ITS) and large subunit (LSU) regions of ribosomal DNA. *J Clin Microbiol* **42**: 4253-4260.

Hirai A, Kano R, Makimura K *et al.* (2004) *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals. *Int J Syst Evol Microbiol* **54**: 623-627.

Matheny PB, Liu YJ, Ammirati JF & Hall BD (2002) Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). *Am J Bot* **89**: 688-698.

Mayser P, Haze P, Papavassilis C, Pickel M, Gruender K & Guého E (1997) Differentiation of *Malassezia* species: selectivity of cremophor EL, castor oil and ricinoleic acid for *M. furfur*. *Br J Dermatol* **137**: 208-213.

Midreuil F, Guillot J, Guého E, Renaud F, Mallie M & Bastide JM (1999) Genetic diversity in the yeast species *Malassezia pachydermatis* analysed by multilocus enzyme electrophoresis. *Int J Syst Bacteriol* **49**: 1287-1294.

Nell A, James SA, Bond CJ, Hunt B & Herrtage ME (2002) Identification and distribution of a novel *Malassezia* species yeast on normal equine skin. *Veter Rec* **150**: 395-398.

Scorzetti G, Fell JW, Fonseca A & Statzell-Tallman, A (2002) Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeasts Res* **2**: 495-517.

Simmons RB & Guého E (1990) A new species of *Malassezia*. *Mycol Res* **94:** 1146-1149.

Sugita T, Takashima M, Shinoda T *et al.* (2002) New yeast species *Malassezia dermatis* isolated from patients with atopic dermatitis. *J Clin Microbiol* **40**: 1363-1367.

Sugita T, Takashima M, Kodama M, Tsuboi R & Nishikawa A (2003) Description of a new yeast species, *Malassezia japonica*, and its detection in patients with atopic dermatitis and healthy subjects. *J Clin Microbiol* **41**: 4695-4699.

Sugita T, Tajima M, Takashima M, Amaya M, Saito M, Tsuboi R & Nishikawa A (2004) A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. *Microbiol Immunol* **48**: 579-583.

Swofford, D.L. PAUP*. Phylogenetic Analysis Using Parsimony (*And Other Methods). Version 4.0b10, (2002) , Sinauer Associates, Sunderland, MA. USA.

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics, *PCR protocols. A guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ & White TJ, eds), pp. 315-322. Academic Press, San Diego, CA. USA.

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

Figure 2. Cells of *M. equina* CBS 9969^{T} (MA146) cultured on mDA at 32ºC 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
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[a]	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]	Pig
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

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

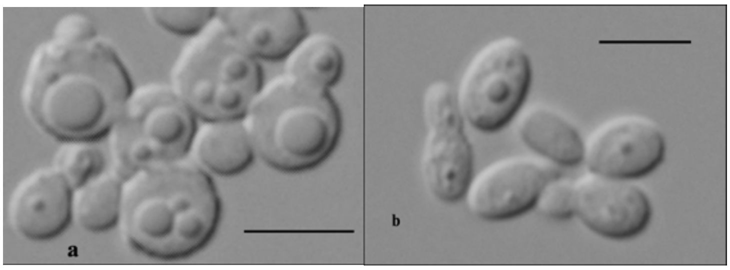

Figure 1.

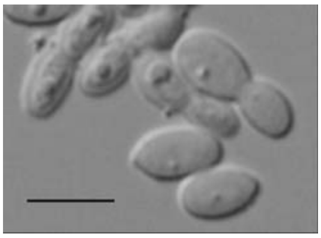

Figure 2.