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DIAGNOSTICS OF *MALASSEZIA* SPECIES: A REVIEW

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ABSTRACT

Yeasts from the genus *Malassezia* belongs to normal commensal skin flora of warm-blooded vertebrates. These yeasts may act as opportunistic pathogens and cause skin diseases in humans and animals under certain conditions. The identification of *Malassezia* species is based on the phenotypic or genotypic diagnostics. The methods used for the phenotypic identification is determined by: the growth on Sabouraud agar, growth on selective media (Leeming-Notman agar, Dixon agar, Chrom *Malassezia* agar), the ability to utilise different concentrations of Tween, monitoring of the growth on CEL agar (soil enriched with castor oil) and TE agar (Tween-esculine agar), and the catalase test. The genotypic identification uses molecular methods like: the pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment lenght polymorphism (AFLP), denaturing gradient gel electrophoresis (DGGE), and the DNA sequence analysis.

Key words: genotypic; identification; phenotypic; yeasts

INTRODUCTION

Malassezia yeasts are lipophilic organisms known for more than a century as a part of the natural human skin microflora, as well as the agents of some skin diseases. Yeasts have also been considered as the agents of occasional systemic infections since the 1980s [5].

After the first isolation from an Indian rhinoceros, *Malassezia* yeasts were subsequently found in wild mammals (bears, wolves, coyotes, foxes, seals, sea lions, llamas, porcupines, elephants, armadillo, monkeys, ferrets, leopards), as well as in domestic animals (dogs, cats, horses, cattle, sheep, goats, pigs). *Malassezia* yeasts were also isolated from various species of birds, therefore it is assumed that these yeasts may be found in all warm-blooded vertebrates [20] and their distribution in nature is currently being examined by molecular techniques [5].

Until the late 1980s, only two species were known within the genus, *Malassezia furfur* and *Malassezia pachydermatis* [9].

It took a long time to understand the importance of lipids for the growth of *Malassezia* yeasts, so it was difficult to maintain cultures *in vitro* [5]. *M. furfur* was considered

as lipid-dependent yeast, occurring in humans, whereas the lipophilic but not lipid-dependent species *M. pachydermatis*, was found in domestic carnivores, particularly in dogs [9, 28]. Later, other species have been found and described and the range of animal hosts greatly extended [20]. *M. sympodialis*, the third accepted species isolated from human skin, was discovered a century after *M. furfur*. The genus *Malassezia* was revised in 1996 on the basis of morphological, physiological and rRNA studies and four new lipid-dependent species were described, *M. globosa*, *M. obtusa, M. restricta* and *M. slooffiae* [26]. Many reports have shown that the skin of healthy animals, in addition to *M. pachydermatis*, may have also been colonized by the lipid-dependent species [9] as *M. obtusa, M. globosa, M. slooffiae, M. furfur and M. sympodialis* were found in cattle and goats. A new species *M. caprae* was also isolated from goats. *M. equina* was found in horses [20] and *M. nana* was isolated from cats and cattle [34].

Nowadays, *Malassezia* can be isolated from almost all domestic and wild animals [9]. Molecular methods helped reveal new types of *Malassezia* also in humans, such as *M. dermatis*, *M. yamatoensis* and *M. japonica* [5]. Currently, *Malassezia genus* includes 16 species (Table 1) which colonize human or animal skin [10].

Malassezia yeasts are primarily located in the sebaceous glands, which provide lipids necessary for their growth [19]. The sebaceous glands are mostly found on the face, head, chest and back which made these places the most affected by yeast infections [61]. The *Malassezia genus* is typical in its morphology and affinity to lipids in the medium [5]. All members of this genus, except for *M. pachydermatis*, need for their growth medium supplementation with long chain fatty acids (C12—C24). *M. pachydermatis* is able to grow without the addition of lipids in the medium, but more vigorous growth can be seen in the presence of lipids. Species, requiring the addition of lipids in the medium are called lipid-dependent [61].

The most frequently isolated zoophilic species is *M. pachydermatis*, which is often associated with the inflammation of the external ear canal and various types of

Table 1. Currently described *Malassezia* species, authors, year of the description and their main hosts

Malassezia species	Discovery	Main host/others
M. furfur (Robin, Baillon)	1889	man/cow, elephant, pig, monkey, pelican
M. pachydermatis (Weidman, Dodge)	1925	dog, cat/carnivores, birds
M. sympodialis (Simmons & Guého)	1990	man/horse, pig, sheep
M. globosa (Midgley et al.)	1996	man/cheetah, cow
M. obtusa (Midgley et al.)	1996	man
M. restricta (Guého et al.)	1996	man
M. slooffiae (Guillot et al.)	1996	man, pig/goat, sheep
M. dermatis (Sugita et al.)	2002	man
M. japonica (Sugita et al.)	2003	man
M. nana (Hirai et al.)	2004	cat, cow/dog
M. yamatoensis (Sugita et al.)	2004	man
M. caprae (Cabañes and Boekhout)	2007	goat/horse
M. equina (Cabañes and Boekhout)	2007	horse/cow
M. cuniculi (Cabañes and Castellá)	2011	rabbit
M. brasiliensis (Cabañes et al.)	2016	parrot
M. psittaci (Cabañes et al.)	2016	parrot

dermatitis in animals [13]. It usually occurs in dogs, cats and less frequently it can be isolated from other animals. This yeast is opportunistic and may become pathogenic when environmental changes of the skin surface occur or in the immunosuppressed hosts. In some breeds of dogs the growth of yeasts can be supported by different types of hypersensitivity (such as flea bite allergy, food hypersensitivity and atopy) and long term antimicrobial therapy or therapy with corticosteroids [9].

IDENTIFICATION OF *MALASSEZIA* SPECIES

Malassezia yeasts can be identified either by culture methods based on their morphological characteristics and biochemical properties or by non-culture, molecular methods [1, 30]. When using conventional culture methods, the samples are collected from healthy or affected skin, scalp, hair, nails or any other human or animal sources. The samples, cultured on special selective media in Petri dishes, are incubated in a moist environment. *Malassezia* yeasts as cutaneous mycobiota are ecologically adapted to a lower temperature and optimum cultivation temperature for them is around 32—34 °C. The vitality of yeasts on culture media decreases rapidly so it is necessary to transfer samples regularly on fresh medium every one or two months [5, 42].

Culture media supplemented with different sources of lipids are often used because of the lipophilic properties of this genus [11]. Sabouraud dextrose agar, the culture medium without the addition of lipids, is used to differentiate the lipid-dependency of the species [38]. *M. pachydermatis*, a less demanding species, is able to grow on this medium (Fig. 1) [5, 6].

Other media were developed and successfully used as a selective media for *Malassezia* species, e. g. Dixon agar, Leeming-Notman agar (Fig. 2) and Ushijima agar [1]. All of these complex media contain ox bile which is a sufficient compound for good growth of *Malassezia* yeasts. Some of the media use Tween 40 (Leeming-Notman agar) or Tween 60 (Dixon agar) but Tween 60 seems to be more efficiently utilized, thus it is better for the growth of most species [5].

Fig. 1. The growth of *Malassezia pachydermatis* on Sabourad dextrose agar

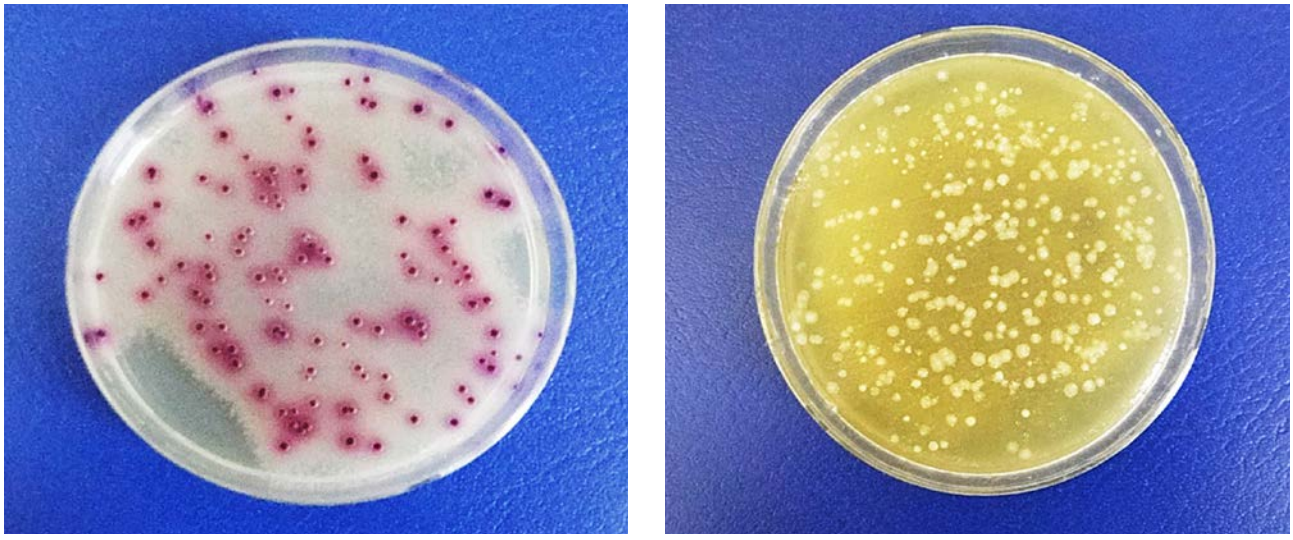
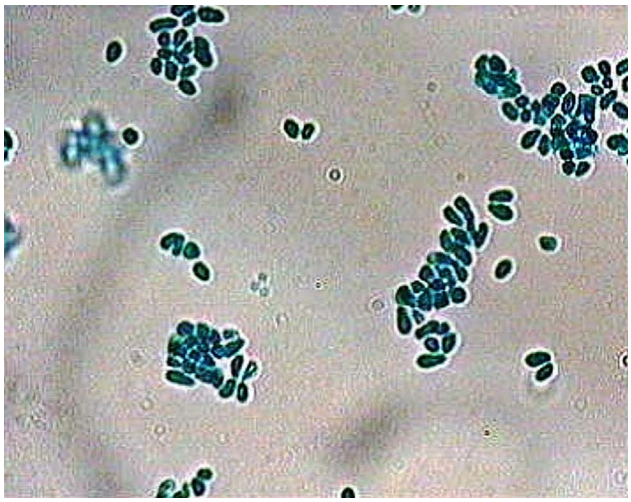


Fig. 2. The growth of *M. pachydermatis* on Leeming-Notman agar and modified CHROMagar *Candida*

By using chromogenic *Candida* agar (CHROMagar Candida) (Fig. 2), enriched with lipid components, it was possible to culture and differentiate 9 *Malassezia* species on the basis of the colony morphology [1, 37]. CHROMagar is also used for the differentiation of the genus *Malassezia* and *Candida*.

The specific media used for *Malassezia* cultivation are Cremophor EL agar and Tween 60-esculin agar. Cremophor EL agar is used to determine the ability of species to utilize the polyethoxylated castor oil and Tween-esculin agar for determining the ability of isolates to hydrolyse esculin and utilize Tween 60 [38].

The culture methods may not be objective because of the difference in growth rates and the cultivation requirements of individual species, therefore, the focus is currently on molecular techniques [32].



Fig, 3. Microscopic view of *Malassezia pachydermatis* **cells Magn. ×400**

PHENOTYPIC IDENTIFICATION

Individual species from the genus *Malassezia* can be identified by macroscopic and microscopic morphology and also by certain physiological properties [24]. Macroscopic diagnosis includes an identification of the shape, texture and colour of the colonies; microscopically they are examined by cell morphology and budding [52].

The size of colonies is determined by measuring well isolated single colonies and the isolates are divided into three groups: small (1 mm: *M. globosa, M. slooffiae* and *M. restricta*), intermediate (1—2 mm: *M. obtusa*) and large (2—5 mm: *M. pachydermatis, M. sympodialis, M. dermatis, M. furfur and M. japonica*) [38]. Unipolar cells with round to ovoid shape can be observed by microscopic examination [34, 54]. The *M. globosa* cells are typically spherical [14] (Fig. 3).

Although there are morphological differences between *Malassezia* species, their identification is usually determined also upon the basis of their physiological characteristics [14, 52]. Physiological tests used for the identification of *Malassezia* species are based on: the monitoring of catalase reaction (decomposition of 3 % hydrogen peroxide); on the ability of yeasts to utilize different concentrations of Tween (i.e. T20, T40, T60 and T80) and Cremophor EL (castor oil) as a source of lipids [38, 14]; the β-glucosidase activity [38, 44]; the ability to produce pigments [24] and fluorochromes in the presence of tryptophan as a main source of nitrogen [45]; and production of the urease enzyme (ability to hydrolyze urea to ammonia and carbon dioxide) [38]. The urease activity test is not used to distinguish *Malassezia* species but to eliminate cultures that are contaminated by bacteria or ascomycetous yeasts, such as *Candida* spp. which are quite common on the skin [5].

Malassezia furfur

M. furfur forms smooth or slightly wrinkled single colonies about 4—5 mm in diameter. The colonies are creamy coloured, dull, with straight or lobate margins. The texture of the colonies is soft. Colonies of *M. furfur* are easily distinguishable from other *Malassezia* species on CHROMagar where they form characteristic large pale pink and wrinkled colonies [38]. The cells are morphologically variable, with ovoid, globose or cylindrical shape and budding is on a broad base. Some strains are able to occasionally produce filaments [2, 5, 29, 61].

Malassezia furfur can be identified: by its ability to grow up to 41 °C; by a strong catalase reaction; the absence or a very weak β-glucosidase activity; and equal growth in the presence of Tweens 20, 40, 60, 80 and CremophorEL as a lipid sources [4, 27]. The optimum temperature for the growth is near 34 °C, but good growth occurs at 37 °C, and the maximum temperature for growth is 41 °C. In contrast to *M. globosa, M. obtusa, M. restricta, M. slooffiae* and *M. sympodialis*, this species is able to utilize glycine as a nitrogen source [50].

Malassezia pachydermatis

M. pachydermatis forms convex single colonies 1—5 mm in diameter, pale yellowish, dull, smooth, lightly wrinkled, with an entire straight margin. Cells are ovoid, ellipsoidal to cylindrical with monopolar budding on a broad base. This species does not produce filaments.

In all rDNA genotypes there occurs differences in catalase and β-glucosidase expression and Tweens 20, 40, 60, 80 and Cremophor EL (CrEL) growth reactions. All isolates grow well at 37 °C with the maximum at 40—41 °C and some primary cultures show a certain lipid-dependence [4, 5, 20, 53, 59, 61].

Malassezia sympodialis

M. sympodialis forms flat or elevated single colonies, approximately 5—8 mm in diameter, pale cream, glistening, smooth, with an entire or folded margin. Cells are small, with ovoid to globose shape and monopolar budding on a narrow base. This strain, able to grow at 40 °C, is characterized by the presence of a catalase reaction and a strong β-glucosidase activity, and good growth in the presence of Tweens 40, 60 and 80. With CrEL, growth is usually absent but fresh isolates can develop a ring of tiny colonies [2, 5, 26].

Malassezia globosa

M. globosa forms raised, wrinkled single colonies, 3—4 mm in diameter, pale yellowish, rough, shiny or dull, with a slightly lobate margin. Yeast cells are spherical, so this species is easily recognized morphologically with monopolar budding on a narrow base. This species is able to produce short filaments [2, 23, 26]. The yeast has a strong catalase activity but does not split esculin. Growth is limited at 37 °C, and no growth occurs on the five lipid substrates [2, 5, 39].

Malassezia obtusa

M. obtusa forms flat to convex, smooth single colonies, on average 1.5—2 mm in diameter, glistening or dull, butyrous, and with entire or slightly lobate margins. The cells are cylindrical with monopolar budding on a broad base. Filaments may be present. This species has a maximum temperature at 38 °C and cannot utilize any of the five lipids used in the tests as the only lipid supplement in the medium. *M. obtusa* combines the positive reactions of catalase and β-glucosidase [4, 5, 26].

Malassezia restricta

M. restricta forms small, flat or raised single colonies, 1—2 mm in diameter on average, pale yellowish-brown, dull, smooth, with a lobate margins. The shape of the cells is globose or ovoid, and budding is monopolar on a relatively narrow base. *M. restricta* does not produce any filaments. This species lacks catalase and β-glucosidase activity, does not grow at 37 °C or with any of the Tween lipid supplements. Growth with CrEL is always absent. Growth of the colonies is very restricted [2, 5, 26, 31].

Malassezia slooffiae

M. slooffiae forms flat or raised single colonies with a roughened surface, about 3—4 mm in diameter, pale yellowish-brown, shiny, butyrous and with finely folded margins. The cells are short and cylindrical with monopolar budding on a broad base. The species is not known to produce any filaments. This species is able to grow at 40 °C, and shows a catalase reaction, but absence of β-glucosidase activity. *M. slooffiae* may be misidentified as *M. furfur*, but the main difference is that growth of *M. sloofiae* with CrEL is absent. Growth with Tween 80 is always very weak in comparison with the other three Tweens [4, 5, 26, 35].

Malassezia dermatis

M. dermatis forms flat or convex single colonies, 5—6 mm in diameter, pale yellowish, glistering or dull, butyrous and with an entire or finely folded margin. The shape of the cells is globose, ovoid or ellipsoidal. Budding is monopolar on a moderately broad base. The production of filaments has not been observed. *M. dermatis* does not grow at 40 °C and can be identified by its lack of catalase and β-glucosidase activity. Growth occurs with all four Tweens but with Tween 80 it may be weaker, similar to that of *M. caprae*. CrEL is not assimilated [5, 54].

Malassezia japonica

M. japonica forms flat slightly wrinkled single colonies about 2—3 mm in diameter, pale yellowish-cream, dull and butyrous with a straight or folded margin. Cells are ovoid to cylindrical, with budding which is monopolar on a broad base. The production of hyphae has not been observed. *M. japonica* grows at 37 °C, with strong catalase and β-glucosidase reactions. From all four Tweens, only 60 and 80 are well assimilated. Tweens 20, 40 and CrEL are assimilated weakly [5, 56].

Malassezia nana

M. nana forms convex single colonies, 1.5—2 mm in diameter, yellowish, dull, smooth, butyrous, with entire to narrowly folded margins. The shape of the cells is globose to ovoid with monopolar budding on a relatively narrow base. *M. nana* does not produce any filaments.

This species grows at 37 °C, Tweens 40, 60 and 80 are well assimilated and CrEL is not utilized [2, 5, 34].

Malassezia yamatoensis

M. yamatoensis forms flat to convex single colonies about 3—4 mm in diameter, pale yellowish, shiny, smooth or wrinkled, with an entire margin. The shape of cells is ovale or cylindrical with monopolar budding on a broad base. This strain can be identified by its ability to grow at 37 °C, a strong catalase reaction and lack of β-glucosidase activity. These characteristics distinguish the species from *M. sympodialis*. Growth appears in the presence of all four Tweens and CrEL [5].

Malassezia caprae

M. caprae forms small moderately convex single colonies about 1—2 mm in diameter, whitish or cream-colored, smooth, butyrous with an entire or lobate margin. Cells are globose or ovoid with budding on a broad base. The species does not produce hyphae. *M. caprae* can be identified by: its weak growth at 37 °C, the presence of a catalase reaction, β-glucosidase activity, and good growth in the presence of all four Tweens. Growth may be weaker with Tween 80 [5, 8].

Malassezia equina

M. equina forms single colonies, about 1—3 mm in diameter, cream-colored, glistening to dull, wrinkled, butyrous, with a folded to fringed margins. The cells are ovoid or ellipsoidal with monopolar budding on a narrow base. Filaments have not been observed. *M. equina* grows at maximum temperature of 37 °C. The catalase reaction is strong, but this species lacks the β-glucosidase expression. Tweens 40, 60 and 80 are well assimilated. CrEL is not assimilated, but sometimes a weak precipitate can occur [5].

Malassezia brasiliensis

M. brasiliensis forms large convex colonies elevated in the centre with an average diameter of 2.5 mm, whitish to cream-colored, smooth, dull and butyrous with entire margins. The cells are ovoid or ellipsoidal with buds formed monopolarly on a broad base [10].

Malassezia psittaci

M. psittaci forms large moderately convex colonies, about 2.5 mm in diameter. They look similar to the colonies of *M. brasiliensis* with the whitish to cream colour and smooth, shiny, butyrous appearance. The yeast cells are globose to ovoid [10].

Phenotypic methods are usually time-consuming, multistep processes, requiring a number of cultural techniques and usually are unable to clearly differentiate newly identified species. Also, there are significant differences in the evaluation and description of *Malassezia* biochemical properties by various authors [2, 26, 38] and this complicates the phenotypic identification. The occurence of strains with atypical physiological and biochemical properties is increasing and for these reasons it is essential to support the phenotypic identification by molecular analysis [14].

GENOTYPIC IDENTIFICATION

The number of molecular methods have been developed and successfully used as tools to diagnose and differentiate *Malassezia* species, to better understand the epidemiology of *Malassezia* and their connection with diseases [14].

To overcome the limitations of culture-based techniques which do not always allow identification and typing of each *Malassezia* species, a range of molecular biology methods are used, such as: nested polymerase chain reaction (PCR) [49], real-time PCR [58], pulsed-field gel electrophoresis (PFGE) [53], random amplified polymorphic DNA analysis (RAPD — random amplification of polymorphic DNA (RAPD) [21], amplified fragment length polymorphism (AFLP) [32], denaturing gradient gel electrophoresis (DGGE) [60], single strand conformation polymorphism (SSCP) [17], terminal fragment length polymorphism (tFLP) [23], restriction fragment length polymorphism (RFLP) [16, 41, 48] and sequencing analysis [40] (Tab. 2).

Table 2. Molecular methods used for detection and identification of Malassezia species [7]

Fingerprinting methods:
PFGE-Pulsed field gel electrophoresis
RAPD-Random amplified polymorphic DNA
AFLP-Amplified fragment lenght polymorphism
DGGE-Denaturing gradient gel electrophoresis
DNA sequence analysis:
Ribosomal DNA analysis (D1/D2 region LSU- Large subunit —rDNA)
Analysis ITS (Internal transcribed spacer regions) rDNA
Analysis IGS (Intergenic spacer regions) rDNA
Restriction analysis of PCR amplicons:
RFLP-Restriction fragment length polymorphism
tFLP-Terminal fragment lenght polymorphism
Chitin synthase gene sequence analysis

FINGERPRINTING METHODS

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis is a highly discriminative molecular method based on the variable migration/separation of large genomic fragments in an agarose gel [46]. This technique characterized seven *Malassezia* spp. (i. e. *M. furfur*, *M. obtusa*, *M. globosa*, *M. slooffiae*, *M. sympodialis*, *M. pachydermatis* and *M. restricta*) and allows them to be differentiated into species on the basis of distinct karyotypes displayed by PFGE analysis [53]. PFGE is a useful diagnostic tool, but is time consuming, technically difficult and requires large amounts of genomic DNA for analysis. Therefore, it is not suitable as a rapid routine diagnostic method [14, 48].

Random amplified polymorphic DNA analysis (RAPD)

PCR based random amplification of polymorphic DNA utilizes random fragments of genomic DNA. This method has been used for the identification, differentiation and taxonomic classification of some *Malassezia* species (*M. pachydermatis, M. furfur* and *M. slooffiae*) and for the examination of epidemiological relations. Most of the *Malassezia* species can be differentiated by RAPD, but analysis can be complicated by the presence of intraspecific variations [3]. In spite of limited reproducibility of results and its technical limitations due to the need for specialized equipment and training, RAPD is a sensitive and efficient method for discrimination of closely related strains due to its high specificity. RAPD is still being used for the monitoring of *Malassezia* "carriage" on domestic animals and humans [15, 18, 21].

Amplified fragment length polymorphism (AFLP)

AFLP is a useful diagnostic tool for the identification of *Malassezia* isolates and for the detection and differentiation between clinically relevant variants within *Malassezia* spp. This technique provides highly-specific genotypic information about each strain and helps to understand the genetic relationship among *Malassezia* isolates [8, 32, 60].

AFLP is suitable and apropriate for analyses where detailed information is necessary. This method requires clonal isolates from culture so that the results are usually complicated to interpret. AFLP is relatively time-consuming and sometimes not sufficiently reliable for identification or discrimination of clinically isolated yeasts [2, 14, 18].

Denaturing gradient gel electrophoresis (DGGE)

DGGE is a molecular fingerprinting technique based on differences in the electrophoretic mobility and denaturing properties of double-stranded DNA. DGGE can fully separate and detect DNA fragments of the same size but with different base-pair sequences. The melted DNA is separated and spread through the denaturing polyacrylamide gel and is analyzed for single components [25, 33, 47, 51]. DGGE is useful for the identification of *Malassezia* isolates and is suitable for the analysis of the clinical samples that may include several different species. However, the clinical use may be limited by technical demands [4, 60].

DNA sequence analysis

The rRNA gene complex is often used in identification of clinically important yeasts. The fungal gene is composed of multiple copies of the gene regions: 18S, 5.8S, 26 (28) and 5S. 18S is a small subunit (SSU) and 28S is referred as large subunit (LSU). Other regions, ITS1 and ITS2 region (internal transcribed spacer region) and IGS1 and IGS2 region (intergenic spacer region) are inserted among the subunits. The rRNA gene complex is also used for comparison between phylogenetically distant species. Phylogenetically close species are compared through more variable region, the ITS and IGS regions [5].

Sequencing of ITS1 region of ribosomal DNA is relatively quick and specific analysis and is used for the identification of *Malassezia* species and the strains. Sequence

analysis of IGS1 is not widely used for species identification [55, 57].

RESTRICTION ANALYSIS OF PCR AMPLICONS

Restriction fragment length polymorphism (RFLP)

One of the frequently used molecular method is the polymerase chain reaction (PCR) followed by restriction analysis (RFLP). Enzyme digestion of PCR amplicons has been shown to be useful for the differentiation of *Malassezia* species.

Various authors suggested RFLP for the diagnosis of *Malassezia*. Mirhendi et al. [48] and Gaitanis and Velegraki [17] differentiated 11 species (*M. furfur, M. pachydermatis, M. sympodialis, M. obtusa, M. globosa, M. restricta, M. slooffiae, M. dermatis M. nana, M. japonica* and *M. yamatoensis*) using RFLP. PCR-RFLP analysis is less difficult and more precise than the majority of molecular methods and requires less technical equipment.

Terminal fragment length polymorphism analysis (tFLP)

Terminal restriction fragment length polymorphism (tRFLP) analysis of PCR-amplified genes is a widely used fingerprinting technique. This analysis is based on the restriction endonuclease digestion of fluorescently end-labeled PCR products. tFLP analysis is a sensitive and reproducible method suitable for the rapid and reliable identification of *Malassezia* species. It eliminates the need for prior strain cultivation in direct investigations of *Malassezia* populations on skin samples. The technique, however, is not suitable for epidemiological typing, as its ability to show heterogeneity within a species is limited [23, 32].

Chitin synthase gene sequence analysis

Sequencing of the chs-2 gene has also allowed the differentiation of *Malassezia* spp., in spite of the high similarity (95 %) in sequence among them [36]. A multilocus approach which employed the sequencing of the chs-2 gene, ITS1 and LSU has been applied to *M. pachydermatis* and defined three major *M. pachydermatis genotypes* (A, B and C). Although the multilocus sequencing provides interesting option for epidemiological investigations, it has not yet been employed for studying *Malassezia* from animals other than dogs [12, 13].

In the last few decades, advances in research and technology contributed to partial explanation of the role which *Malassezia* plays in skin diseases. By using the detailed information obtained from genetic analyses, different types of *Malassezia* spp. can be detected and identified [11, 14]. Most of molecular studies point to the presence of numerous *Malassezia* genotypes within species, suggesting a connection to the host, the geographical origin and clinical manifestations. Standardized molecular processes in combination with reliable physiological and biochemical methods are necessary for the definition of species and for consideration of genetic diversity within a species [22].

Despite the usefulness of molecular techniques, there are some disadvantages associated with them, such as: the inability to distinguish all species and questionable reproducibility, the requirements for technical equipment, and higher cost of analysis. Importantly, most of the molecular methods mentioned above require cultivation to enhance sensitivity and to perform the test [14].

Some studies have shown discrepancies between phenotypic and molecular methods for the identification of *Malassezia*. For example, Makimura et al. [43] examined 46 clinical isolates by the phenotypic methods as *M. furfur*. However, by genetic identification 22 of them were identified as *M. sympodialis* and 5 as *M. slooffiae*.

It is possible that differences between phenotypic and molecular methods may reflect the possible mistakes in sampling and culturing, which strengthen the need to perform well-controlled, comparative molecular studies of samples taken directly from the skin, as well as samples after *in vitro* cultivation [14].

CONCLUSIONS

In recent decades, yeast infections have become a significant problem in humans and also in animals. In most cases, they are opportunistic infections because *Malassezia* belongs to the normal commensal skin flora of warm-blooded vertebrates. The increasing trend in the incidence of these infections can be caused mainly because of massive use of broad spectrum antibiotics and the increasing number of immunosuppressed patients.

Identification of yeast is performed by phenotypic and molecular methods. Molecular methods are necessary for identification and differentiation of various *Malassezia* spe-

cies, which can be difficult to characterize by phenotypic methods.

In the future, the identification should focus on the use of reliable molecular methods to achieve a better understanding of the role that *Malassezia* spp. plays as a commensal and as a pathogen.

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