Detection and identification of *Malassezia* species in domestic animals and aquatic birds by PCR-RFLP

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Summary

The present study aimed at detection and species-level identification of the *Malassezia* yeasts in domestic animals and aquatic birds by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Samples were collected using tape strips and swabs from 471 animals including 97 horses, 102 cattle, 105 sheep, 20 camels, 60 dogs, 30 cats, 1 hamster, 1 squirrel, 50 aquatic birds and 5 turkeys. Tape-strip samples were examined by direct microscopy. All samples were inoculated on modified Leeming and Notman agar medium. DNA extracted from the yeast colonies was amplified by PCR using primers specific for 26S rDNA. RFLP of the PCR products was performed using *Hin*6I enzyme, and PCR and RFLP products were visualized by agarose gel electrophoresis. *Malassezia* yeasts were detected at the following frequencies: 15.46% in horses, 12.74% in cattle, 12.38% in sheep, 28.33% in dogs, 26.66% in cats and 26% in aquatic birds. Eighty colonies of 6 species were isolated: *Malassezia globosa* 41.25%, *Malassezia furfur* 22.5%, *Malassezia restricta* 15%, *Malassezia sympodialis* 15%, *Malassezia pachydermatis* 5% and *Malassezia slooffiae* 1.25%. Therefore different lipophilic *Malassezia* species are found in a wide diversity of animals and aquatic birds. PCR-RFLP is a suitable technique for identification of different *Malassezia* species.

Key words: *Malassezia* yeasts, PCR-RFLP, Domestic animals, Aquatic birds

Introduction

Yeasts belonging to the genus *Malassezia* colonize the skin of a variety of mammals and birds. These lipophilic yeasts inhabit the stratum corneum of the skin of humans and animals, because this layer is rich in lipids. For reasons currently unknown, these yeasts can change their saprophytic state and invade the stratum corneum as pathogens (Guisiano *et al*., 2010).

However, the new findings on virulence factors of *Malassezia* have been reported based on their enzymatic lipolytic activity resulting in the production of distinct metabolites and special cell wall features (Hort and Mayser, 2011). *Malassezia* can adhere to the surfaces of different materials and form biofilms. Compared with other yeasts, its cell wall is exceptionally thick, with an additional lipid layer on the outside that appears to be important for the organism's ability to suppress cytokine release and down regulate pathogenic uptake and killing. Also, it can produce enzymes and metabolites such as azelaic acid, which has been shown to decrease the production of reactive oxygen species in neutrophils. These factors support the general ability of yeast to cause invasive disease (Dokos *et al*., 2012).

However, these yeasts cause opportunistic dermatological infections in both humans and animals, such as pityriasis versicolor, seborrhoeic dermatitis, folliculitis, and systemic infections in humans; and *Malassezia* dermatitis and otitis in domestic animals (Crespo *et al*., 2000; Bensignor *et al*., 2002; Morris *et al*., 2005; Rincon *et al*., 2005; Cafarchia *et al*., 2006; Hort *et al*., 2006). Proliferation of these yeasts appears to be the preliminary step in *Malassezia* dermatitis. The lipid-dependent species were normally isolated only from human skin, but these species are nowadays reported to colonize the skin of various domestic animals (Garau *et al*., 2005).

At present *Malassezia* species are identified on the basis of morphological, ultrastructural, physiological, and molecular analyses and include the following 7 species: *Malassezia furfur*, *Malassezia pachydermatis*, *Malassezia sympodialis*, *Malassezia globosa*, *Malassezia obtusa*, *Malassezia slooffiae*, and *Malassezia restricta* (Crespo *et al*., 2002). Recently, on the basis of DNA relatedness, new species, *Malassezia dermatis*, *Malassezia nana*, *Malassezia japonica*, and *Malassezia yamatoensis* have been recognized and added to this genus (Castella *et al*., 2005; Mirhendi *et al*., 2005; Zomorodian *et al*., 2008). Three additional distinct genetic entities are now accepted as species of *Malassezia* including *M. caprae*, *M. equine* (Cabanes *et al*., 2007) and *M. cuniculi* (Cabanes *et al*., 2011; Gaitanis *et al*., 2012; Sei, 2012). With the exception of *Malassezia pachydermatis*, no other lipid dependent species of this genus can synthesize C_{14} or C_{16} fatty acids (Ashbee, 2007). The findings of phenotypic analyses may cause ambiguity in the interpretation of the results because different species of this genus have an almost

similar morphology, and isolation and maintenance of some species such as *M. restricta* and *M. globosa* is difficult (Gaitanis *et al.*, 2002). In addition, these phenotypic methods are usually time consuming, lack sufficient discriminatory power, and cannot be used to detect new species. A variety of molecular methods such as nested polymerase chain reaction (PCR), real time PCR, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), etc have been used for identification of *Malassezia* (Morishita *et al.*, 2006; Sugita *et al.*, 2010; Eidi *et al.*, 2011; Ko *et al.*, 2011). Molecular typing methods, and DNA sequences of the internal transcribed spacer 1 (ITS1) region located between the 18S and 5.8S regions in the 5′ end region of the large-subunit (26S) ribosomal DNA (D1/D2 region) and other gene segments-expressing the ribosomal RNA have been utilized for the identification of *Malassezia* species (Hirai *et al.*, 2002).

Considering the importance of *Malassezia* yeasts in medical mycology and their ability to cause skin and other disorders in humans, animals, and birds, we decided to perform this study on the different *Malassezia* species infecting animals and aquatic birds.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a reliable and easy-to-perform technique and is used in epidemiological and research studies. Many researchers have studied about the frequency rate of *Malassezia* species. The aim of the present study was to utilize this method to identify various *Malassezia* species infecting domestic animals and aquatic birds.

Materials and Methods

For this study, samples were collected from 471 animals including 97 horses, 102 cattle, 105 sheep, 20 camels, 60 dogs, 30 cats, 1 hamster, 1 squirrel, 50 aquatic birds and 5 turkeys. Samples collection was performed using 2 techniques:
(A) The tape-strip technique: first, the skin was cleaned by ethanol 70% and then a 3-cm piece of adhesive tape was placed on the skin and then removed. Therefore, the tape piece was placed with its adhesive-side down on a clean and sterile slide. Direct microscopic examination using sterile distilled water and methylene blue for each tape-strip was carried out to identify areas with yeast cells.
(B) The swab sampling technique: cotton swabs maintained in a tube containing 0.5 ml of sterile distilled water were used for sampling the ears of the domestic animals and the skin of birds.

The samples (n=2111 samples) were collected from the skin in the different portions of the body (the dorsum, abdomen, and groins) and external ear canals of each animal. All samples (including tape-strip and swab samples) were inoculated on modified Leeming and Notman agar (MLNA) medium (composition: pepton 10 g, glucose 10 g, yeast extract 2 g, desiccated ox bile 8 g, glycerol monostearate 0.5 g, agar 15 g, glycerol 10 ml,

Tween 60 5 ml, olive oil 20 ml, chloramphenicol 0.5%, cycloheximide 0.5%, and deionized distilled water 1 L) and incubated at 32°C for 7-14 days. The confirmation of *Malassezia* genus was carried out by the study of morphologic and microscopic features of yeast colonies.

DNA was extracted and purified from the pure cultures of the 80 colonies of *Malassezia* species according to the following protocol.

First, yeast colony was suspended in 200 μLI of sterile deionized distilled water in a microfuge tube, and the suspension was centrifuged at 5000 g for 5 min. Thereafter, supernatant was discarded, and the pellet was suspended in 250 μLI of lysis buffer, and 250 μLI of phenol:chloroform (1:1) solution, and glass beads were added to this solution. The solution was then shaken at full speed for 5 min and centrifuged at 5000 g for 5 min. The upper aqueous layer of the supernatant was transferred to a new microfuge tube, and an equal volume of phenol:chloroform (1:1) solution was added to this DNA solution. This DNA-containing solution was shaken at full speed for 10 s and centrifuged at 5000 g for 5 min; the top layer of supernatant was again transferred to a new microfuge tube, and 0.1 volume of sodium acetate and equal volume of 2-propanol was added. This solution was then vortexed incubated at -20ºC for 10 min, and centrifuged at 12000 g for 12 min; a pellet was formed at the bottom of tube, and the aqueous layer was carefully removed so as to ensure that the pellet is not disturbed. Thereafter, the pellet was washed by resuspending it in 250 μL of 70% ethanol and centrifuging at 5000 g for 5 min. The supernatant was carefully removed, and the remaining of alcohol was dried off the pellet at room temperature. Thereafter, 100 μL of sterile deionized distilled water was added to the pellet, the mixture was vortexed and centrifuged for 1 s. Finally, the DNA thus obtained was stored at -20°C until use (Yamada *et al.*, 2002; Mirhendi *et al.*, 2005).

PCR amplification was carried out in 50 μLI final volume of the reaction mixture. Each reaction mixture contained 5 μLI template DNA, 0.5 μL deoxyribonucleotides (dNTPs), 0.5 μLI of each of the forward (Mal1F: 5′-TAA CAA GGA TTC CCC TAG TA-3′) and reverse (Mal1R: 5′-ATT ACG CCA GCA TCC TAA G-3′) primers as described in different studies (Mirhendi *et al.*, 2005; Jang *et al.*, 2009; Ko *et al.*, 2011). 5 μL PCR buffer, 0.25 μL Taq DNA polymerase, 1.5 μL magnesium chloride, and 31.75 μL distilled water. PCR amplification was carried out in a thermal cycler (Applied Biosystems) with an initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7 min, as follows (30 cycles): denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min (Mirhendi *et al.*, 2005).

For visualization of the amplified products, 5 μL of each product was run on 1.5% (w/v) agarose gel; the electrophoresis was carried out in TBE buffer {having the following composition: Tris (molecular weight [MW] = 121.44 [109 g]) 0.9 M, boric acid (MW = 61.83 [55.64 g]) 0.9 M, ethylenediaminetetraacetic acid (EDTA; MW = 372.24 [7.44 g]) 20 mM, and distilled water. After

electrophoresis, the gel was stained with ethidium bromide (0.5 µg ml⁻¹)}, and photographed under UV transillumination.

For RFLP analysis a master mixture was prepared using the following ingredients:

*Hin*6I (*Cfo1*) enzyme (0.5 µL), enzyme buffer (1.5 µL), distilled water (8 µL) and amplified product (5 µL). The reaction mixture was incubated at 37°C for 2 h, and agarose gel electrophoresis in TBE buffer was conducted for detection of the PCR-RFLP products as follows: each RFLP product was run on 2% (w/v) agarose gel, stained with ethidium bromide (0.5 µg ml⁻¹), and photographed under UV transillumination.

Results

PCR amplification of DNA from colonies of *Malassezia* species was successfully accomplished. Figure 1 shows photograph of agarose gel electrophoresis of the PCR-amplified products.
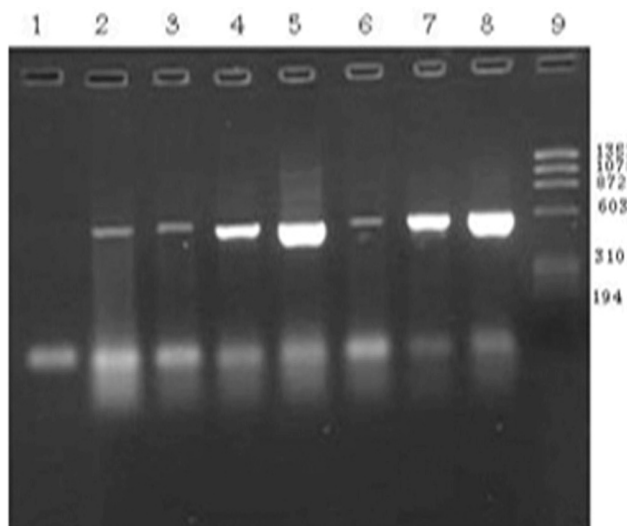
Fig. 1: Electrophoresis of PCR-amplification products. As can be seen, all samples yielded a single band of approximately 580 bp. 1: Negative control; 2, 3: *M. globosa*; 4: *M. restricta*; 5: Mixture of *M. globosa* and *M. sympodialis*; 6: *M. furfur*; 7: *M. sympodialis*; 8: *M. restricta*; 9: Molecular marker No. 9

After restriction digestion of the PCR products, different band patterns were observed on the electrophoresed gel. Figure 2 represent a photograph of agarose gel electrophoresis of RFLP products.
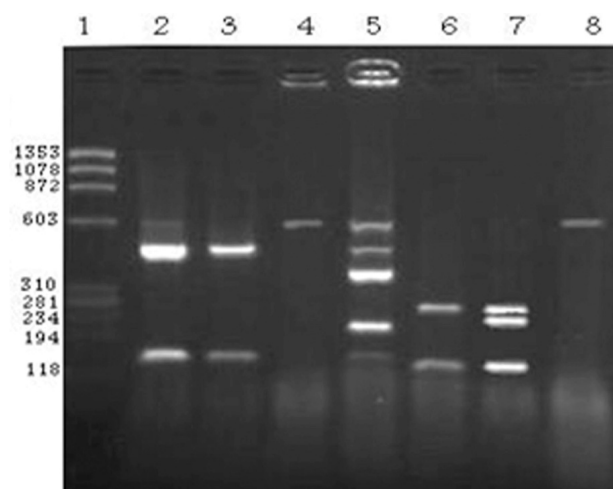
Fig. 2: Electrophoresis of PCR-RFLP products after restriction digestion of the products with *Hin*6I. 1: Molecular marker No. 9; 2, 3: *M. globosa*; 4: *M. restricta*, 5: Mixture of *M. globosa* and *M. sympodialis*; 6: *M. furfur*; 7: *M. pachydermatis*; 8: *M. restricta*

Malassezia yeasts were detected in different animals and aquatic birds at the following rates: 15.46% horses; 12.74% cattle; 12.38% sheep; 5% camel; 28.33% dogs; 26.66% cats; 26% aquatic birds. Tables 1 and 2 show the frequency rate of each species in different animals and different body sites of each animal respectively.

In addition, DNA from the yeast colony isolated from a hamster, yielded a 580 bp band after PCR amplification using specific primers for *Malassezia* species. However, after RFLP using *Hin*6I, the same sample yielded bands of 300 and 180 bp. This band pattern appears to be a novel one, not yet reported in previous studies.

Eighty colonies of 6 species were isolated from these animals and birds; each species occurred in these subjects at the following frequencies: *M. globosa* 41.25%; *M. furfur* 22.5%; *M. restricta* 15%; *M. sympodialis* 15%; *M. pachydermatis* 5%; and *M. slooffiae* 1.25%.

One sheep was noted to have simultaneously been infected by *M. globosa* and *M. sympodialis*.

Malassezia slooffiae (1 subject) was isolated from a

Table 1: Occurrence rate of *Malassezia* species in different animals and birds as detected by PCR-RFLP

Animal	*Malassezia* species						Total
	M. globosa	*M. furfur*	*M. sympodialis*	*M. restricta*	*M. pachydermatis*	*M. sloofiae*	
	n (p)	n (p)	n (p)	n (p)	n (p)	n (p)	n (p)
Horse	5 (33.33)	4 (26.67)	2 (13.33)	3 (20)	1 (6.67)	0 (0)	15 (100)
Cattle	5 (38.46)	3 (23.08)	2 (15.38)	3 (23.08)	0 (0)	0 (0)	13 (100)
*Sheep	10 (76.90)	1 (7.70)	1 (7.70)	1 (7.70)	0 (0)	0 (0)	13 (100)
Camel	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Dog	7 (41.18)	3 (17.65)	2 (11.76)	3 (17.65)	2ˢʷ (11.76)	0 (0)	17 (100)
Cat	1 (12.50)	3 (37.50)	3 (37.50)	0 (0)	1ˢʷ (12.50)	0 (0)	8 (100)
Aquatic birds	5 (38.46)	3 (23.08)	2ˢʷ (15.38)	2 (15.38)	0 (0)	1ˢʷ (7.70)	13 (100)
Total	33 (41.25)	18 (22.50)	12 (15)	12 (15)	4 (5)	1 (1.25)	80 (100)

* Mix of *M. globosa* and *M. sympodilis*. sw: Obtained from swab samples, n: Number, and p: Percent

Table 2: Occurrence rate of *Malassezia* species in different body sites of animals

Animal	Body site	*Malassezia* species					Total
		M. globosa	*M. furfur*	*M. sympodialis*	*M. restricta*	*M. pachydermatis*	
		n (p)	n (p)	n (p)	n (p)	n (p)	n (p)
Horse	Groin	3 (20)	3 (20)	2 (13.33)	1 (6.67)	1 (6.67)	15 (100)
	Abdomen	2 (13.33)	1 (6.67)	0 (0)	1 (6.67)	0 (0)	
	Dorsum	0 (0)	0 (0)	0 (0)	1 (6.67)	0 (0)	
Cattle	Groin	2 (15.38)	2 (15.38)	1 (7.69)	0 (0)	0 (0)	13 (100)
	Abdomen	1 (7.69)	1 (7.69)	1 (7.69)	1 (7.69)	0 (0)	
	Dorsum	2 (15.38)	0 (0)	0 (0)	2 (15.38)	0 (0)	
*Sheep	Groin	5 (38.45)	1 (7.70)	1 (7.70)	0 (0)	0 (0)	13 (100)
	Abdomen	5 (38.45)	0 (0)	0 (0)	0 (0)	0 (0)	
	Dorsum	0 (0)	0 (0)	0 (0)	1 (7.70)	0 (0)	
Camel	Groin	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)
	Abdomen	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
	Dorsum	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Dog	Groin	3 (17.65)	1 (5.88)	1 (5.88)	0 (0)	2[sw] (11.76)	17 (100)
	Abdomen	3 (17.65)	1 (5.88)	1 (5.88)	1 (5.88)	0 (0)	
	Dorsum	1 (5.88)	1 (5.88)	0 (0)	2 (11.76)	0 (0)	
Cat	Groin	1 (12.50)	1 (12.50)	3 (37.50)	0 (0)	1[sw] (12.50)	8 (100)
	Abdomen	0 (0)	1 (12.50)	0 (0)	0 (0)	0 (0)	
	Dorsum	0 (0)	1 (12.50)	0 (0)	0 (0)	0 (0)	
Total		28 (41.79)	15 (22.39)	10 (14.92)	10 (14.92)	4 (5.98)	67 (100)

* Mix of *M. globosa* and *M. sympodilis*. sw: Obtained from swab samples, n: Number, and p: Percent

pelican, and other species isolated from the aquatic birds were as follows: *M. furfur* (1 subject) from a mute swan, *M. globosa* (4 subjects), *M. furfur* (2 subjects), *M. sympodialis* (1 subject) and *M. restricta* (2 subjects) from different duck races.

Discussion

Few studies have been conducted to identify and assess the prevalence of *Malassezia* yeasts in different types of animals; therefore, in the present research, we focused on isolating *Malassezia* yeasts on different portions of the body (the dorsum, abdomen, and groin) of different animals including horses, cattle, sheep, camels, dogs, cats and some types of aquatic birds.

One of the main causes of the difficulty faced in the characterization of these lipophilic yeast species is the lack of suitable methods for their isolation and identification; therefore, the PCR-RFLP technique was used in the present study to determine the occurrence rate of *Malassezia* yeasts in domestic animals and aquatic birds. This method has been successfully used in a previous study (Mirhendi *et al.*, 2005), appears to be reliable for the identification of nearly all the known *Malassezia* species, requires only PCR and enzymatic digestion by 1 or 2 enzyme(s), and is technically less demanding than most other molecular biological methods (Mirhendi *et al.*, 2005; Gaitanis *et al.*, 2006).

Gupta *et al.* (2001) reported that pure cultures of *Malassezia* species were successfully recovered from the lesions in only ≤50% of the cases.

In this study, the rate of obtaining isolated colonies from the MLNA medium of these yeasts was only 6.5% (103 of 2111 cultured samples). Of all the yeast cultures

isolated, 80 colonies (77.7%) were identified as *Malassezia* yeasts. It should be noted that *M. obtusa* was not isolated from any of the animal samples, probably because of the difficulty in isolating *M. obtusa* on solid media.

In a study on the occurrence of lipid-dependent *Malassezia* species in domestic animals, Cabanes *et al.* (2007) described 2 such new species, *Malassezia caprae* isolated from goats and *Malassezia equina* from horses. In our study, yeast colonies were observed to resemble those of *Malassezia* yeasts morphologically and microscopically; however, because their molecular patterns did not resemble those of *Malassezia* yeasts, these colonies were not studied further.

Durate *et al.* (1999) studied cultures isolated from asymptomatic cattle and cattle with otitis and found that 54.7% of the cultures from bovines with otitis and 34.6% from healthy bovines were positive for *Malassezia* species. In our study commonly isolated species were highly related to *M. globosa*, *M. furfur*, *M. slooffiae*, *M. sympodialis*, *M. obtusa* and *M. pachydermatis* (Durate *et al.*, 1999).

Gaitanis *et al.* (2002) have used PCR-RFLP for detection and identification of *Malassezia* species from the skin scales of human patients and reported that this technique is sensitive and enables rapid detection and identification of *Malassezia* species. In addition, in our study, we were able to detect simultaneous occurrence of *M. globosa* and *M. sympodilis* in a sheep sample.

Further, results of nucleotide sequence analysis of yeast isolated derived from a hamster indicate that this isolate may be a novel *Malassezia* species, signifying that this technique may be useful in identifying new species.

This study was conducted because there is a lack of studies on the rate of the occurrence of *Malassezia* species in different animals, particularly in aquatic birds and also considering the elimination of earlier restrictions on the use of molecular techniques for identification of different fungi in Iran.

The findings of our study showed that *Malassezia* species are found to colonize a wide range of animals.

Crespo *et al*. (2002) have studied the occurrence of *Malassezia* species in 112 animals including 50 horses, 25 sheep, 25 goats, and 12 cows and reported that *Malassezia* yeasts occurred in 60% of horses, 28% of sheep, 44% of goats, and 58% of cows and that occurrence of lipid-dependent species was much greater than that of *M. pachydermatis*. However, in our study, *Malassezia* species occurred in 15.46% of horses, 12.38% of sheep, and 12.74% of cattle; the differences in rates of occurrence between 2 studies may be related to factors such as geographic area, sample number, and sampling conditions.

Nardoni *et al*. (2007) reported the distribution of *Malassezia* yeasts on different cutaneous areas in atopic dogs; they stated that these yeasts were isolated at a higher frequency from the interdigital areas (70.7%) and ears (63.4%).

Raabe *et al*. (1999) studied the skin, hair, and ears of 371 healthy dogs and 120 healthy cats and isolated *M. pachydermatis* (83%), *M. sympodialis* (75%) and *M. furfur* (45%). In our study, the frequency of isolation from interdigital areas was 28% and that from the ears was only one case 2% (1 subject). The reason for this difference between 2 studies may be related to the absence of lesions in dogs in our study.

Few studies have, however, reported the occurrence of *Malassezia* yeasts in aquatic birds. In the present study, *M. globosa*, *M. furfur*, *M. sympodialis*, *M. restricta*, and *M. sloofiae* were successfully isolated from aquatic birds; the species most commonly isolated was *M. globosa* (38.46%), which is similar to the results obtained in other studies.

Ko *et al*. (2011) used a PCR-RFLP method for detection of the distribution of *Malassezia* yeasts in patients with *Malassezia* folliculitis successfully and showed that PCR-RFLP is a suitable technique for diagnosis and differentiation of *Malassezia* species.

Hence, although only a few molecular studies on *Malassezia* yeasts in animals have been reported, we believe that PCR-RFLP is an easy-to-perform and inexpensive method for identification of *Malassezia* species and epidemiological studies in animals.

Acknowledgements

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