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Molecular Characterization of *Malassezia* Species Isolated from Dog with and Without Otitis and Seborrhoeic Dermatitis

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Abstract: The lipophilic yeasts of the genus *Malassezia* are opportunistic microorganisms of the skin microflora, but they can be agents of various dermatomycoses. In this study, random amplification of polymorphic DNA (RAPD)-PCR technique was applied to the genetic typing of *Malassezia* species isolated from dog with otitis and seborrhoeic dermatitis and healthy dog. The analysis of electrophoretic profiles on 1/5% agarose gel showed a total 890 clearly amplified PCR band in 176 different positions. The phenogram constructed from the pairwise similarity among all *Malassezia* isolates demonstrated that the tested isolates of *Malassezia* are grouped into 22 distinct groups. This study was able to assess some DNA polymorphism of different *Malassezia* isolates in dogs. The detection of these differences between the RAPD band patterns from dogs observed could facilitate the monitoring of spread and pathogenicity of *Malassezia* infections in these animals.

Key words: *Malassezia* spp. · Otitis · Seborrhoeic dermatitis · RAPD analysis

INTRODUCTION

The species of the genus *Malassezia* are described as members of the normal microbiota of human and animal skin. Thirteen *Malassezia* species have been described and the species *M. pachydermatis*, *M. nana*, *M. equina* and *M. caprae* could be considered zoophilic [1-5]. Lipid-dependent species have been associated with pathologies in humans including pityriasis versicolor (PV), folliculitis, seborrheic dermatitis, otitis and even fungaemia found in new born infants [6, 7]. *Malassezia pachydermatis* has often been isolated from the ear canal and fur of healthy dogs and cats or in association with seborrheic dermatitis and external otitis [8-10]. This species is occasionally isolated from humans and has caused nosocomial infections in neonates suggesting the transference from pet animals [6, 11, 12].

In contrast, lipid- dependent *Malassezia* species yeasts were considered to be strictly anthropophilic. However, several authors have recently cultured these species from animal specimens [13-17].

Malassezia species can be identified through their morphological features and biochemical characterization [18]. However, these phenotypic methods are usually time consuming, lack sufficient discriminatory power and are unable to unambiguously differentiate newly identified species. Various DNA-based molecular methods have been described to overcome this problem [19-21]. Several techniques have been used to acquire epidemiological information of dermatological disorders caused by *Malassezia* yeast. These include karyotyping by pulse field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP), polymerase chain reaction followed restriction enzyme analysis (PCR-REA) and nested PCR [3, 22-27]. The intra-species subtypes obtained with RAPD from samples of *Malassezia* species suggest the presence of genetic population differences that may be an important tool for epidemiological investigation [21, 22].

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In the present study, RAPD-PCR technique was applied to the genetic typing of *Malassezia* species isolated from dog with otitis and seborrhoeic dermatitis and healthy dog.

MATERIAL AND METHODS

Forty five strains of *Malassezia* isolated from dogs with otitis and seborrhoeic dermatitis and healthy dogs are presented in table 1. These strains were identified previously by the conventional techniques based on the morphological, biochemical and physiological characteristics.

Table 1: Isolates of *Malassezia* species from dog with otitis and seborrhoeic dermatitis and healthy dog used in RAPD genetic analysis

Strains	Source (SD/Otitis /External ear canal)	*Malassezia* spp.
m1	Otitis	*M. pachydermatis + M. sympodialis*
m2	Otitis	*M. pachydermatis*
m3	Otitis	*M. pachydermatis*
m4	SD	*M. pachydermatis*
m5	SD	*M. sympodialis + M. furfur*
m6	Otitis	*M. pachydermatis + M. sympodialis*
m7	Otitis	*M. sympodialis + M. furfur*
m8	Otitis	*M. pachydermatis*
m9	Otitis	*M. pachydermatis*
m10	Otitis	*M. pachydermatis*
m11	SD	*M. pachydermatis+ M. sympodialis*
m12	Otitis	*M. pachydermatis*
m13	Otitis	*M. pachydermatis + M. sympodialis*
m14	SD	*M. pachydermatis + M. obtusa*
m15	Otitis	*M. pachydermatis + M. obtusa*
m16	SD	*M. pachydermatis + M. sympodialis*
m17	Otitis	*M. pachydermatis*
m18	Otitis	*M. pachydermatis + M. sympodialis*
m19	Otitis	*M. pachydermatis + M. sympodialis*
m20	SD	*M. pachydermatis + M. sympodialis*
m21	Otitis	*M. pachydermatis + M. obtusa*
m22	Otitis	*M. pachydermatis*
m23	Otitis	*M. pachydermatis + M. sympodialis*
m24	Otitis	*M. sympodialis*
m25	SD	*M. pachydermatis+ M. sympodialis*
m26	Otitis	*M. pachydermatis + M. sympodialis*
m27	Otitis	*M. pachydermatis*
m28	Otitis	*M. globosa*
m29	Otitis	*M. pachydermatis*
m30	Otitis	*M. pachydermatis + M. sympodialis*
m31	SD	*M. furfur*
m32	Otitis	*M. pachydermatis + M. restricta*
m33	Otitis	*M. pachydermatis*
m34	SD	*M. pachydermatis*
m35	External ear canal	*M. pachydermatis + M. obtusa*
m36	External ear canal	*M. pachydermatis + M. sympodialis*
m37	External ear canal	*M. sympodialis*
m38	External ear canal	*M. pachydermatis + M. sympodialis*
m39	External ear canal	*M. pachydermatis + M. sympodialis*
m40	External ear canal	*M. pachydermatis + M. sympodialis*
m41	External ear canal	*M. pachydermatis*
m42	External ear canal	*M. pachydermatis*
m43	External ear canal	*M. sympodialis*
m44	External ear canal	*M. pachydermatis*
m45	External ear canal	*M. pachydermatis*

Following species identification, the isolated strains were seeded on solid Dixon medium and incubated for 5 days at 32°C to obtain microorganisms at the exponential growth phase. DNA was extracted as described by Liu *et al*. [28]. DNA concentration in the each sample was measured by use of spectrophotometer at λ=260 nm. To confirm the quality of the nucleic acid extracted, DNA was resolved by electrophoresis on a 1% (w/v) agarose gel. Total DNA bands were visualized after stain with ethidium bromide using molecular Analyst™ software (1.4.1. version, Bio-Rad) in the GEL DOC 1000 equipment. The DNA extracts were stored at -20°C.

The following primers were used: B_2, FM_1, M_{13}, P_{10}, A_4 and A_{10} for RAPD reaction. The PCR was carried out in 25 µl volumes containing 100 ng genomic DNA (approximately 1.2 µl), 12.5 µl master mix (10 mM Tris-HCl (pH 8.60), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM of each dNTP and 2.5 units of Taq DNA polymerase) (Sinagen Company), 0.4 µM primer (approximately 1.3 µl) (Sinagen Company) and 10 µl distilled water. RAPD-PCR was performed in a Biotech Thermalcycler system programmed for optimal amplification conditions for every primer. The details of the sequences of the primers and amplification programs for RAPD-PCR are listed in table 2.

RAPD products were analyzed by electrophoresis on a 1/5% (w/v) agarose gel and 100bp ladder DNA (Fermentans) was used as the molecular weight marker. Gels were stained with ethidiumbromide, visualized UV illumination and photographed with a Polaroid camera.

For data analysis, The RAPD-PCR was performed at least twice. The presence or absence of a marker was scored as 1 or 0, respectively. Similarity coefficient between two isolates were calculated according to the formula of Nei and Li [29] as $F = 2N_{xy}/N_x + N_y$, where N_{xy} was the number of common fragments between two isolates and N_x and N_y were the number of fragments in isolates X and Y, respectively. The genetic distance was evaluated through euclidean distance. The dendrogram was constructed based on the unweighted pair-group method using arithmetic averages (UPGMA) [30]. Pooled data from six primers were used for this calculation.

Table 2: Sequences of primers and amplification program for RAPD-PCR

		Amplification program						
Target	Sequence (5' to 3')	1	2	3	4	5	6	
FM_1	AGCCGCCTCCATGGCCCCAGG	95°C	94°C	40°C	72°C	72°C	4°C	
		1min	1min	2min	1min	5min		2~4 40 cycle
M_{13}	GAGGGTGGCGGTTCT	95°C	94°C	32°C	72°C	72°C	4°C	
		6min	45sec	90sec	90sec	8min		2~4 40 cycle
B_2	ATGGATCGG	95°C	94°C	36°C	72°C	72°C	4°C	
		5min	45sec	45sec	90sec	10min		2~4 40 cycle
P_{10}	GACAGACGCG	95°C	95°C	39/7°C	72°C	72°C	4°C	
		1min	15sec	45sec	90sec	5min		2~4 30 cycle
A_4	AATCGGGCTG	95°C	94°C	36°C	72°C	72°C	4°C	
		5min	45sec	45sec	90sec	10min		2~4 40 cycle
A_{10}	GTGATCGCAG	95°C	94°C	40°C	72°C	72°C	4°C	
		5min	45sec	45sec	90sec	10min		2~4 40 cycle

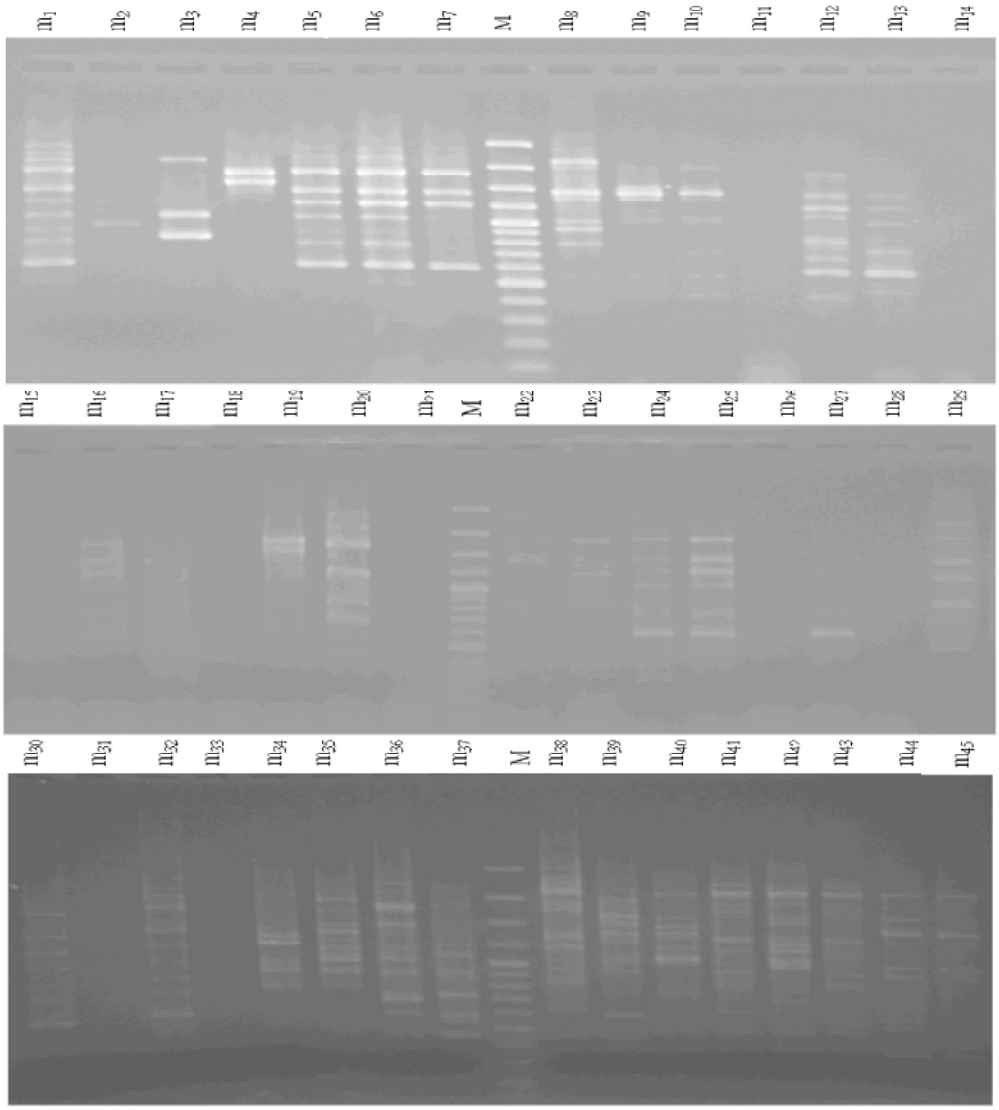


Fig. 1: Electrophoretic profiles of different *Malassezia* species from dogs generated by RAPD–PCR with M_{13} primer. Line M: 100 bp DNA Ladder and from number 1 to 45 were samples (A): m_1-m_{14}, (B): m_{15}- m_{29}, (C): m_{30}- m_{45}.

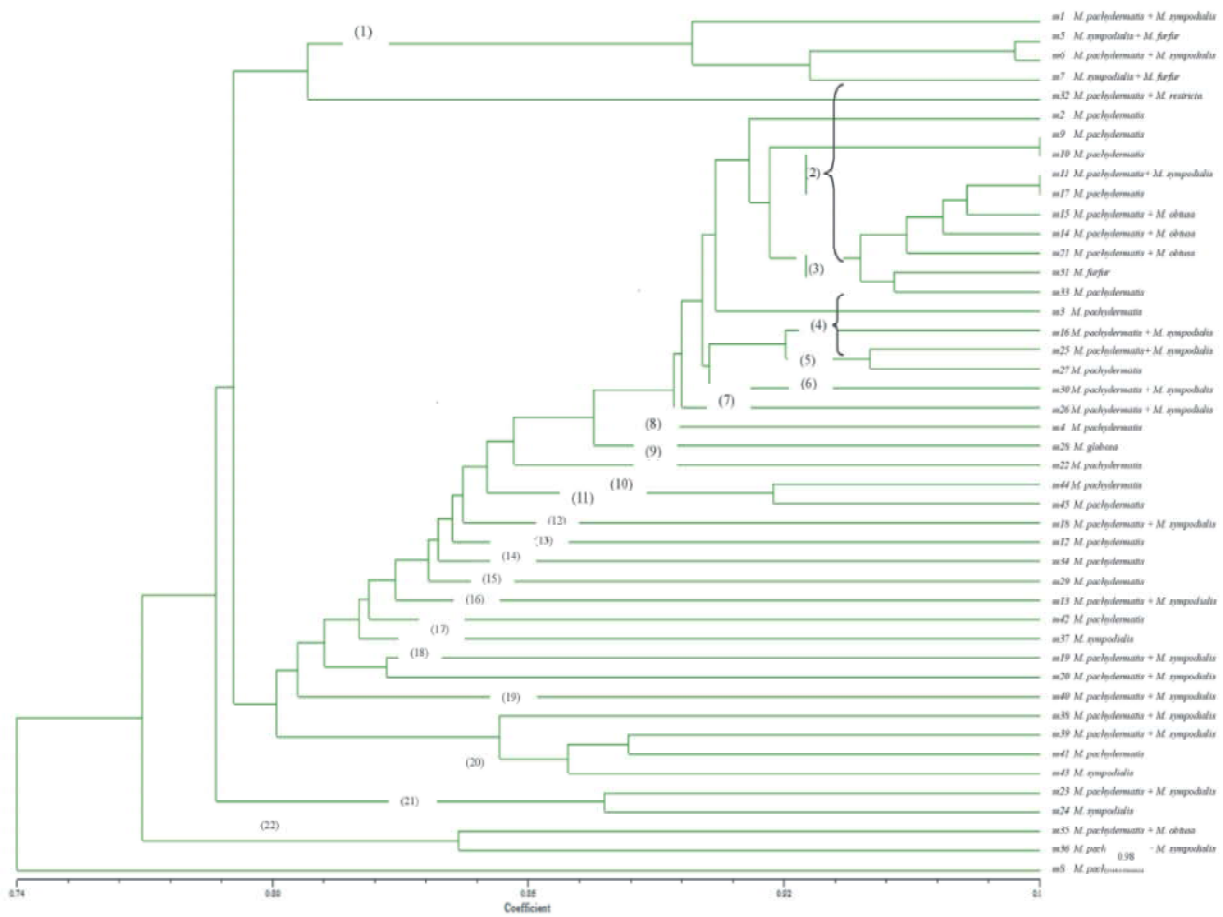


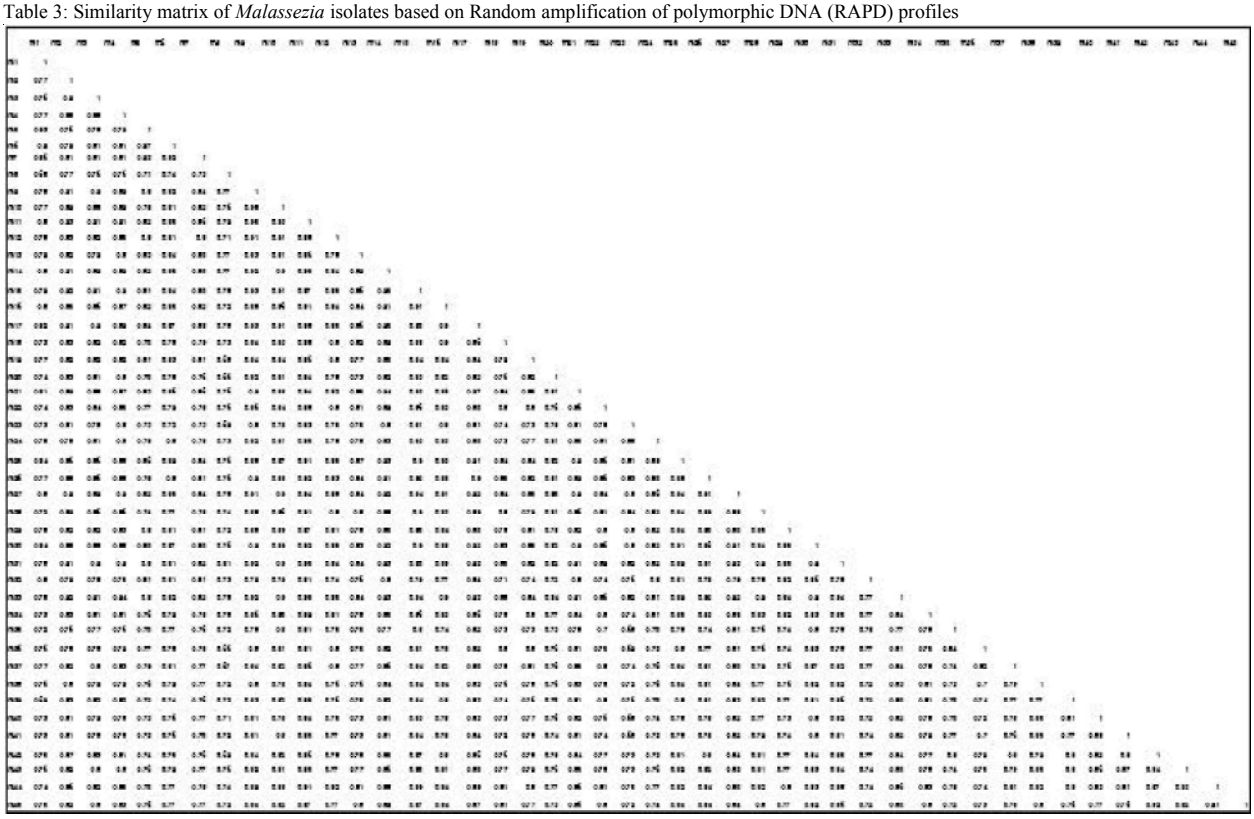
Fig. 2: Dendrogram of 45 isolates of *Malassezia* species based on RAPD data set.

RESULTS

Six primers gave consistent results and produced a reasonable number of identification and polymorphic pattern of the PCR products from independent genomic DNA preparation of each isolate. Pooled data from six primers gave a total 890 clearly amplified PCR band in 176 different positions. The primers P_{10} and B_2 permitted the observation of the most and least intra-specific variation between the genomic profiles obtained for the analyzed strains of *Malassezia* species, respectively; so that data obtained showed the primers B_2, A_{10}, A_4, M_{13}, FM_1 and P_{10} produced 77 bands in 26 different positions, 110 bands in 25 different positions, 155 bands in 34 different positions, 173 bands in 28 different positions, 180 bands in 27 different positions and 195 bands in 36 different positions, respectively. Our data support previous finding that the discriminatory power of RAPD analysis depended upon the primer used [31]. The size of the fragments ranged from 0/18 to 53 Kb and 51/14% bands were larger than 1 Kb. An example of an RAPD pattern generated by Primer M_{13} is shown in fig. 1.

All PCR fragments obtained were used for genetic distance analysis. The dendrogram constructed from the pairwise similarity among all *Malassezia* isolates demonstrated that the tested isolates of *Malassezia* were grouped into 22 distinct groups (Fig. 2). Within each group, members share more than 80% similarity of the RAPD bands pattern; i.e. the average similarity of groups 2, 3, 4 and 9 were more than 90%. The average similarities were shown in Table 3. Furthermore, with in the groups, a cluster of 98% identity was found: i.e. cluster 2, m_9 and m_{10}, m_{11} and m_{17}. Group 2, the largest group, contains 10 of 45 isolates. The next large groups are 1, 4 and 19, containing 5, 4 and 4 of 45 isolates, respectively. The other groups were minor, containing 1or 2 *Malassezia* isolates per group.

Table 3: Similarity matrix of *Malassezia* isolates based on Random amplification of polymorphic DNA (RAPD) profiles



DISCUSSION

The expansion of the genus *Malassezia* has generated interest in the epidemiological investigation of the distribution of new species in a range of dermatomycoses. Recently, lipid-dependent *Malassezia* species have been frequently cultured from veterinary specimens and the identification of this species from animals is important to clarify the epidemiology of the malasseziosis in animals and humans [32]. Classical identification of *Malassezia* species is based on morphological, biochemical and physiological characteristics. In the present study, from 34 samples in dog with otitis and seborrhoeic dermatitis, 52 isolates were obtained, including 29 isolates (55.77%) related to *M. pachydermatis* and 23 isolates (44.23%) related to lipid-dependent *Malassezia* species. Furthermore, from 11 samples in the healthy dogs, 16 isolates were obtained, including 9 isolates (56.25%) related to *M. pachydermatis* and 7 isolates (43.75%) related to the lipid-dependent *Malassezia* species. The isolation of lipid-dependent species from dogs in this study suggests a potential role of these animals as carriers for humans. By using the combination of different biochemical and physiological methods, it is reported that *Malassezia* species in mixed cultures are frequently found both in animals and in humans [16, 33]. In doing so, we were able to isolate mixed cultures from 62.5% and 74.54% of the healthy and diseased dogs, respectively (Table1).

Several typing methods for differentiating *Malassezia* isolates have been recently used as epidemiological tools. According to Schiottfeldt *et al*. [34], species differentiation based on molecular characteristics can be performed by a number of tests, among which PFGE (Pulse-Field Gel Electrophoresis) and RAPD are the most used. PFGE is very useful for species identification but it has little value for epidemiological investigation, since each species has revealed different karyotypes, which in most cases have lacked strain-specific variation [22, 35]; for while, RAPD provides information about molecular changes within the species, identifying subtypes. In this work the assessment of randomly amplified polymorphic DNA was established as a molecular epidemiological tool. Major advantages of the RAPD-PCR are flexibility, technical simplicity and high discriminatory power. To perform RAPD-PCR assay is unnecessary figure out of the nucleotide sequence of DNA target. Low levels of misclassification and high levels of specificity make

RAPD-PCR an efficient, sensitive and suitable mean of distinguishing closely related strains. The important of RAPD typing method have been demonstrated in several studies of *Malassezia* genera and other fungi that are poorly characterized allowing a rapid evaluation of genetic diversity of these species [36].

Phenogram of *Malassezia* species based on UPGMA method derived from RAPD assays and generated by using six primers show that *M. pachydermatis* isolated from dogs with otitis and seborrhoeic dermatitis is clustered to nine (clusters 2, 3, 4, 6, 8, 11, 12, 13 and 22) and eight (clusters 1, 2, 4, 5, 10, 14, 17 and 20) alone or in combination with.

M. sympodialis, *M. obtusa* or *M. restricta*, respectively; whereas Aizawa *et al*. [37] in the investigation of 16 strains of *M. pachydermatis* isolated from dogs with otitis and seborrhoeic dermatitis by RAPD analysis, using the FM_1 primer, reported that this species was classified into three clusters. Castella *et al.* [38] noted that 38 strains of *M. pachydermatis* recovered from dogs with and without otitis by RAPD technique, using M_{13} and OPT_{20} primers, were distributed in four genetic types. Duarte and Hamdan [32] showed that *M. pachydermatis* isolated from dogs with otitis by RAPD genotyping, using the OPA_2 primer, was classified into three clusters.

In the present study, *M. sympodialis* isolated from diseased dogs is clustered to one (cluster 20) and seven (clusters 1, 4, 5, 10, 14, 17 and 20) alone or in combination with *M. pachydermatis* or *M. furfur*, respectively. Likewise, *M. furfur* isolated from diseased dogs studied is grouped to one (group 2) and one (group 1) alone or in combination with *M. sympodialis*, respectively. *M. globosa* isolated from this group of dogs is also clustered to one (cluster 7) lonely. In addition, *M. pachydermatis* isolated from healthy dogs is grouped to three (groups 9, 15 and 19) and three (groups 18, 19 and 21) alone or in combination with *M. sympodialis* or *M. obtusa*, respectively. In a recent research by Hossain *et al*. [39] revealed that the RAPD analysis of 114 *M. pachydermatis* isolated from canine ear and skin, using FM_1 primer, were distributed into 28 distinct genotypes. In another study, Aizawa *et al*. [40], differentiated four RAPD profiles among 110 clinical isolates of *M. pachydermatis* from healthy and diseased dogs and cats by using the identical primer FM_1.

Besides, in our research, *M. sympodialis* isolated from healthy dogs is clustered to two (clusters 16 and 19) and three (clusters 18, 19 and 21) alone or in combination with *M. pachydermatis*, respectively.

Although *M. pachydermatis* is part of the commensal microbiota of canine skin, it has been reported to play a secondary pathogenic role on the skin of dogs affected by numerous dermatitis [41]. Recently, lipid-dependent *Malassezia* species have been frequently cultured from veterinary specimens as reported by our study and other authors [13, 15, 17, 42]. The normal communal yeasts may become a pathogen whenever alteration on the skin surface microclimate or host defenses occurs [8]. The factors, which favour proliferation of *Malassezia* species and its transition from a commensal organism to an apparent pathogen on canine skin, are poorly understood. A study of monitoring the spread of *Malassezia* infections in a neonatal intensive care unit (NICU) was performed by RAPD–PCR. Fourteen *M. pachydermatis* isolates that normally are zoophilic were cultivated from newborn children and incubators. The isolates were genetically indistinguishable over time showing homogeneity of the fingerprints during the nosocomial epidemy [6]. In an outbreak, it was suggested that the source of contamination was pet dogs owned by nursing staff who worked at NICU [11]. A recent research described that dog owners can transmit *M. pachydermatis* indicating the importance of good hand hygiene by healthcare professionals [43]. The analysis of these studies showed the importance of research on the differences in pathogenicity and specificity among the genetic types of dogs and it may contribute relevant insights for a better understanding of the aetiology or ecological role of the genus *Malassezia*. The animal-to-human or human-to-animal carriage is still not clearly known and its risk factors must be determined. RAPD–PCR and DNA sequence analysis have been efficient epidemiological instruments to make clear the origins of the *Malassezia* infections.

In conclusion, this study was able to assess some DNA polymorphism of different *Malassezia* isolates in dogs. The detection of these differences between the RAPD band patterns from dogs observed could facilitate the monitoring of spread and pathogenicity of *Malassezia* infections in these animals.

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