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RESEARCH ARTICLE

PCR-Restriction Fragment Length Polymorphism and DNA Sequencing for Identification of *Malassezia* species Isolated from Animals in Egypt

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Abstract

Malassezia is one of the most significant yeast genera causing Malasseziosis in different animals. In the present study, the phenotypic methods, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing were applied for identification of *Malassezia* species isolated from 160 ear swabs and skin scrapings of apparently healthy and diseased dogs, cats, horses and buffaloes (40 animals, each). Of the 82 ear swabs as well as 78 skin scrapings, 24 (29.3%) and 25 (32.1%) yielded a positive growth on mycobiotic agar, respectively. The forty-nine *Malassezia* isolates were subjected for phenotypic identification based on macro- and micro-morphological characters on mycobiotic agar medium, growth on Dixon`s medium at different temperatures, and the physiological characters (tween assimilation, esculin hydrolysis, tryptophan utilization, and production of catalase enzyme). Polymerase chain reaction (PCR) amplification of 26S rDNA gene, followed by restriction analysis using *HhaI* restriction enzyme and DNA sequencing were employed. Forty-eight and one isolates were phenotypically identified as *M. pachydermatis* and *M. globose*, respectively. The PCR-RFLP assay for 21 representative isolates revealed the identification of *M. pachydermatis* (n=17), *M. furfur* (n=1), *M. globosa* (n=2) and *M. restricta* (n=1). Furthermore, the DNA sequencing showed a maximum identity (100%) of the tested isolates to *Malassezia* spp. available on the Genbank database. The most frequently identified *Malassezia* spp. by genotypic method was *M. pachydermatis* (80.95%). It was isolated from 33.3%, 23.8%, 14.28% and 9.52% of examined dogs, cats, horses and buffaloes, respectively. The second frequent identified species was *M. globosa* (9.52%). It was isolated only from horses and buffaloes (4.76% each), meanwhile *M. furfur* was recovered from buffaloes and *M. restricta* was isolated from dogs (4.76% each). In conclusion, PCR-RFLP assay and DNA sequencing proved to be more accurate and reliable methods for *Malassezia* spp. identification and are complementary for phenotypic methods.

Keywords: *Malassezia* species, PCR-RFLP, Phenotypic identification, 26S rDNA sequencing, Animals.

Introduction

Malassezia is one of the most significant basidiomycetous yeast genera, which is characterized by its lipid dependence [1]. *Malassezia* spp. are mostly established on the scalp, face, neck, top of the chest and back. It is one of the mycobiome of human skin that is rich in sebum production and its colonization increases after puberty, presumably due to the increased sebaceous gland activity [2-4]. Moreover, it presents as a microflora of most animals and sometimes acts as an opportunistic pathogen [5, 6].

Twelve *Malassezia* spp. including *M. dermatis*, *M. furfur*, *M. globosa*, *M. japonica*,

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M. nana, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, *M. yamatoensis*, *M. caprae* and *M. equine* have been recognized to be lipid dependents, whereas *M. pachydermatis* doesn't require lipid supplementation for growth [7, 8].

The frequency of *M. pachydermatis* differs markedly between dogs with or without skin lesions, usually being larger on the affected skin compared with the healthy one [9, 10]. *M. pachydermatis* settles the stratum corneum of normal dogs with healthy skin in very low numbers [11]; while, dramatically increase of the number was found in the ear canals and allergic skin diseases in dogs [12]. Therefore, there is a great potential for human exposure to this organism. Most of the lipid dependent *Malassezia* spp. has been recovered from the healthy skin of cats [13-15], horses and different domestic ruminants, especially *M. nana*; from cats and cows [16].

Although the incidence of external otitis in horses is low, the presence of *Malassezia* spp. in the ear canal microbiome gives indication that these yeasts can cause infections when immune suppression occurs, or host has condition that favor excessive growth of *Malassezia* spp. [17].

Malassezia spp. can be identified on the basis of morphological and biochemical features [18]. While phenotypic methods are time consuming and can't differentiate the newly identified spp., molecular methods are more rapid and accurate for the identification of *Malassezia* yeasts due to their simplicity, specificity and sensitivity [19, 20]. The recent molecular methods that were employed for differentiation of *Malassezia* spp. include single PCR restriction endonuclease analysis (REA) [21], PCR of 26S rDNA gene, followed by RFLP using the restriction enzymes *HhaI* [17, 22] and real-time PCR [23]. Analysis of 26S, ITS regions of rRNA gene, and chitin synthase gene sequencing and amplified fragment length polymorphism (AFLP) was used to identify *M. caprae and M. equina* from domestic animals [8]. Therefore, the purpose of this study was to evaluate both the phenotypic and genotypic methods for accurate identification of *Malassezia* spp. isolated from apparently healthy and diseased dogs, cats, horses and buffaloes.

Materials and Methods

Samples and examination procedures

A total of 160 samples (82 ear swabs and 78 skin scrapings) were obtained from apparently healthy (55) and diseased (105) animals. Samples were collected from dogs, cats, horses and buffaloes (40 each) attending private clinics and farms in Cairo and Sharkia Governorates, Egypt, during the period from September 2016 to December 2017. All the diseased animals were examined for recording the skin lesions of Malasseziosis (erythematous patches of alopecia and hair loss at the site of infection).

After cleaning the lesions by sterile gauze moistened with 70% ethanol, skin samples were obtained by scraping of the healthy skin or the lesion with sterile blades. Fine particles of skin scrapings were subjected for direct microscopic examination under the high power (40 x) objective lens of the light microscope (Binocular Biological Microscope, Xsz-2108, China) after treating with 20% potassium hydroxide. Ear swabs were collected by sterile cotton wool swabs moistened with sterile saline from the external auditory meatus of clinically suspected cases of otitis externa as well as from normal cases [24].

Fungal culture and phenotypic identification of Malassezia spp.

Fungal culture was carried out by inoculating the prepared samples onto mycobiotic agar media (CONDA, Spain, CAT: 1072); 4 slope agar tubes for each sample (2 with olive oil drops and 2 without oil). The tubes were incubated at 32°C for fourteen days and examined for growth every three days. The isolates have been identified using phenotypic methods; macro and microscopic features in addition to growth on Dixon`s medium at different temperatures; 32, 37 and 41°C [17]. The macro-morphological characteristics on mycobiotic agar medium as the isolates growth rates and colonies colors were recorded [18]. Moreover, microscopical examination of colonies by Gram`s stains were performed and the data were analyzed [1].

All the recovered isolates were subjected to catalase test. Moreover, tweens assimilation tests; 20, 40, 60, and 80 using well diffusion method was performed for detection of the physiological characters of *Malassezia* spp. [25]. Esculin splitting was also used to distinguish *M. furfur, M. slooffiae* and *M. sympodialis* from other *Malassezia* spp. [24]. Finally, tryptophan utilization test was used for identification of the brown pigments specific for *M. furfur* only [7].

Genotypic identification of Malassezia spp.

PCR-RFLP assay

Extraction of genomic DNA from representative *Malassezia* isolates was performed using QIAamp DNA Mini Kit (Sigma, USA, Catalogue no. 51304) according to manufacturer's instructions. PCR targeting 26S rDNA gene was done using the oligonucleotide primers 5´- TAA CAA GGA TTC CCC TAG TA-3´ and 5´- ATT ACG CCA GCA TCC TAA G-3´ [22]. The amplification was carried out in Applied Biosystem thermal cycler, with a final volume of 25µL of the following reaction mixture: 12.5 µL Emerald Amp GT PCR mastermix (Takara, Code No. RR310A), 1 µL of each primer (20 pmol), 5 µL of template DNA, and 5.5 µL PCR grade water. The following cycling conditions were conducted; 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 s, 56˚C for 40 s, and 72˚C for 45 sec, and a final extension at 72˚C for 10 min.

Furthermore, RFLP analysis was performed by incubating a 10 µL aliquot of each PCR product with 1 µL of *Hha1* restriction enzyme (Catalog number: FD1854 Thermo Fisher, Germany), 2 µl related buffer, and 17 µL nuclease-free water for 3h at 37 ºC [26].

DNA Sequencing and phylogenetic analysis

PCR products of analyzed *Malassezia* spp. were purified using QIA quick Spin Columns (Qiagen Corp., Chatsworth, Calif.) and sequenced in the forward and reverse directions by Solgent Co. Ltd (South Korea). The obtained sequences were analyzed by DNA baser software (http:// www. dnabaser. com/index.html). The sequences were compared against those published at GenBank using online Basic Local Alignment Search Tool (BLAST) (http:// blast. ncbi. nlm. nih. gov/Blast.cgi). The genetic relatedness of the isolates was investigated via constructing a phylogenetic tree using neighbor joining method. This analysis was done using the MEGA software (V.5).

Statistical analysis

Statistical package for social science (SPSS ver. 20) was employed for data analysis using Chi_Square tests. Values were considered statistically significant at P value < 0.05.

Results

Small bottle shaped yeast cells of *Malassezia* spp. were observed in 32.05% (25/78) of skin scrapings from dogs, cats, horses and buffaloes by direct microscopy. Mycobiotic agar medium was utilized to determine the lipid dependent species. Out of 160 analyzed samples, 49 (25 skin scraping and 24 ear swabs) yielded positive growths onto mycobiotic agar medium (30.63%). On mycobiotic agar with olive oil, lipid dependent species showed creamy and rough colonies, whereas without olive oil, non-lipid dependent species suspected to be *M. pachydermatis* revealed raised, smooth and creamy colonies. The recovery rates of *Malassezia* spp. from the collected samples from all animal species are listed in Table1. *Malassezia* yeasts were detected in 49% of apparently healthy animals and 51% of diseased one.

Table 1: Total recovery rate of *Malassezia* spp. in ear swabs and skin scrapings from apparently healthy and diseased animals in Egypt

Animals species*	No. of *Malassezia* isolates / No. of collected samples (%)				Total number of isolates (%)
	Ear swabs		Skin scrapings		
	Apparently Healthy	Diseased	Apparently Healthy	Diseased	
Dogs	5/6 (83.3)	3/11(27.2)	4/5 (80)	7/18 (38.8)	19/40 (47.5)
Cats	6/7 (85.7)	4/18 (22.2)	3/5 (60)	2/10 (20)	15/40 (37.5)
Horses	1/3 (33.3)	2/13 (15.3)	1/9 (11.1)	5/15 (33.3)	9/40 (22.5)
Buffaloes	2/9 (22.2)	1/15 (6.6)	2/11 (18.1)	1/5 (20)	6/40 (15)
Total (160)	14/25 (56)	10/57 (37.0)	10/30 (33.3)	15/48 (31.2)	49/160 (30.62)
	24/82 (29.26)		25/78 (32.05)		

* Forty animals from each animal species were examined

Phenotypic identification of Malassezia isolates

Microscopical examination of the developed colonies onto mycobiotic slope agar revealed cylindrical to oval yeast cells with broad base buds (bottle-shaped appearance) and spherical yeast cells with narrow based buds.

Out of 49 positive *Malassezia* isolates, two different *Malassezia* spp. were identified; *M. pachydermatis* and *M. globosa* depending on their phenotypic criteria. *M. pachydermatis* was the most frequent isolated species with a percentage of 97.96% (48/49), while *M. globosa* was identified only in one isolate (2.04%). The isolates suspected to be *M. pachydermatis* revealed cylindrical to oval yeast cells with broad base buds under light microscope, grew at 31, 37 and 40˚C on Dixon`s medium, assimilated all tweens and all were negative for both tryptophan utilization and esculin hydrolysis tests. The only *M. globosa* isolate yielded spherical yeast cell with narrow based buds under light microscope, failed to grow on Dixon`s medium at 40˚C, gave positive results for catalase test, did not assimilate all tweens and was negative for both tryptophan utilization and esculin hydrolysis tests.

PCR-RFLP assay for Malassezia spp. identification

Twenty-one representative *Malassezia* isolates formally identified according to their phenotypic characters (20 were identified as *M. pachydermatis* and one *M. globosa*) were subjected to PCR-RFLP assay. PCR amplification of 26S rDNA gene from all tested *Malassezia* spp. revealed a single PCR product of the expected size at 580 bp. Digestion of the amplicons with *Hha1* restriction enzyme revealed four restriction patterns specific for *M. pachydermatis* (n=17), *M. furfur* (n=1), *M. globosa* (n=2) and *M. restricta* (n=1) as shown in Figure (1). Plainly, 17 out of 20 *M. pachydermatis*, were correctly identified, meanwhile three isolates were identified as *M. globosa*, *M. furfur* and *M. restricta*. Moreover, the isolate of *M. globosa* was successfully identified by PCR-RFLP assay.

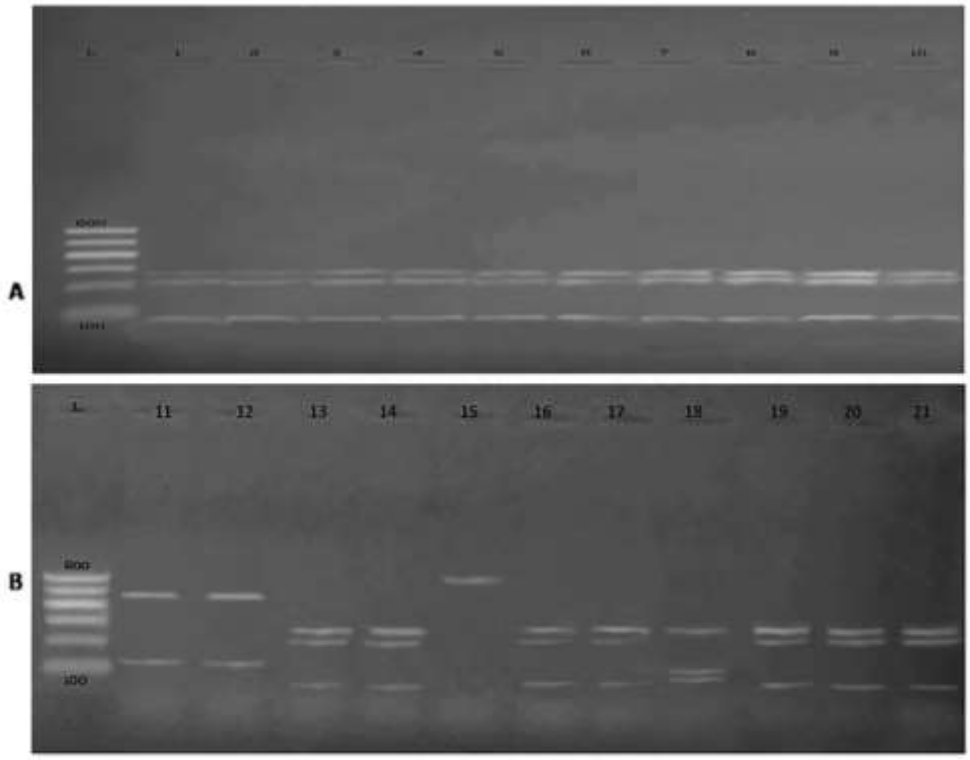

Figure1: Agarose gel electrophoresis of PCR restriction patterns of 21 *Malassezia* spp. using *Hha1*. Lane L1-10 (A), 13, 14, 16, 17, 19, 20 and 21 (B) *M. pachydermatis* (97, 221, and 250), Lanes 11 and 12 (B): *M. globosa* (129 and 455), Lanes 15 (B): *M. restricta* (580), and Lane 18 *M. furfur* (107, 113 and 250).

DNA sequencing of the 26S rDNA regions of Malassezia spp.

The GenBank accession numbers of nucleotide sequences were MK351279 for *M. furfur* that was isolated from skin scrapings of diseased buffalo, MK351310 and MK351317 for *M. globosa*, from skin scrapings of diseased horse and apparently healthy buffalo, respectively. The accession number MK351319 was for *M. pachydermatis* from ear swab of apparently healthy cat and MK351315 for *M. restricta* from skin scrapings of diseased dog. The alignment of the nucleotide sequence of 26S rDNA gene of five representative *Malassezia* spp. with the published sequences in GenBank was presented in Figure (2). Concordance between PCR-RFLP and DNA sequencing was 100%. A Phylogenetic tree built from the obtained sequences showed different clusters for each species, indicating variation in their sequences. The identified sequences for all species were clustered with those previously deposited at GenBank for the same species (Figure 3).

Phenotypic methods identified only *M. pachydermatis* and *M. globosa*, while molecular method successfully identified *M. pachydermatis, M. globosa, M. restricta* and *M. furfur* (Table 2).

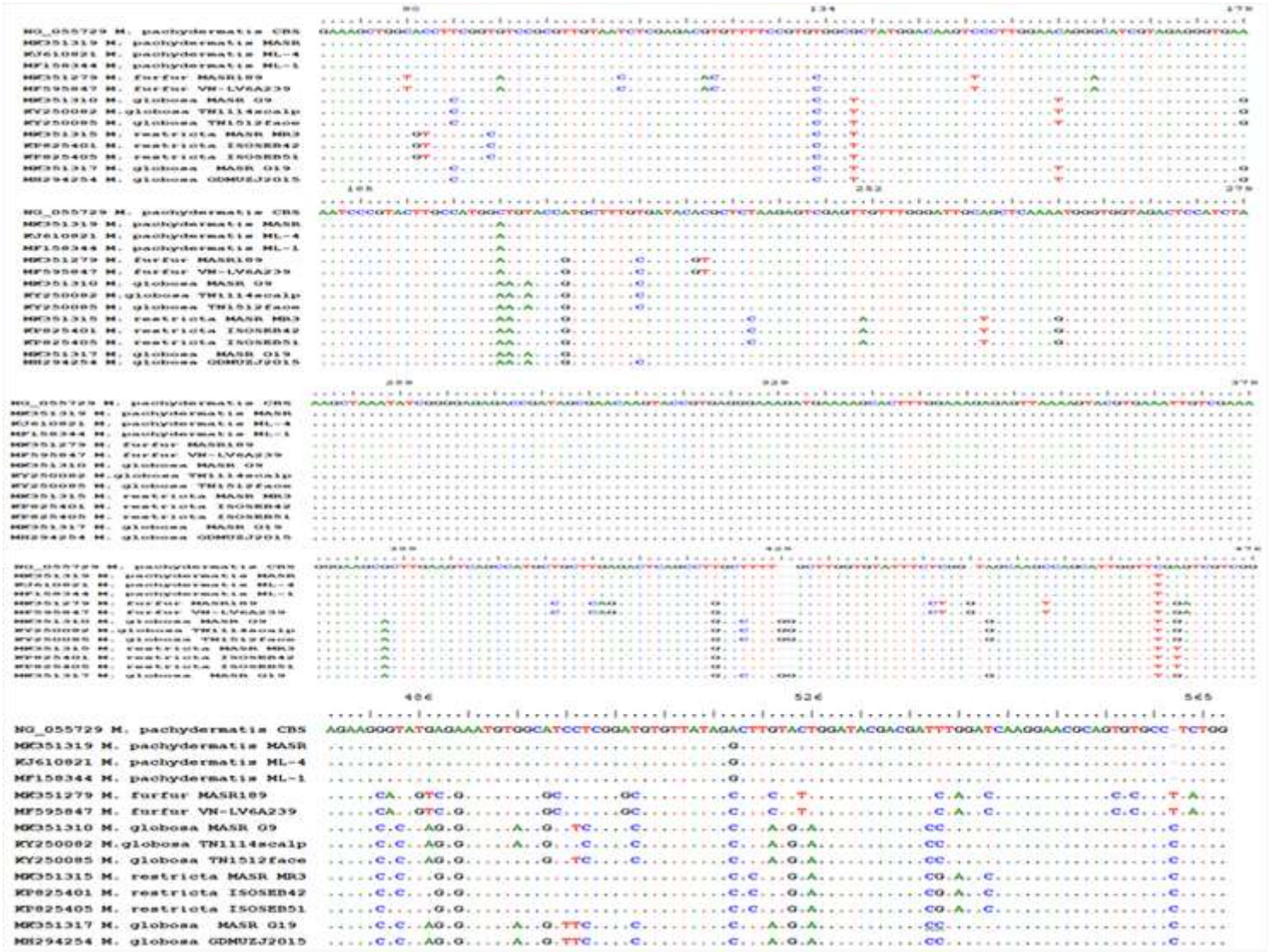

Figure 2: An alignment of the 26S rDNA region sequences of *M. furfur* (MK351279), *M. globosa* (MK351310 and MK351317), *M. pachydermatis* (MK351319), and *M. restricta* (MK351315) with published sequences in GenBank by online blast search. Numbers refer to the nucleotide positions and dots indicate nucleotide positions are identical to the corresponding sequence.

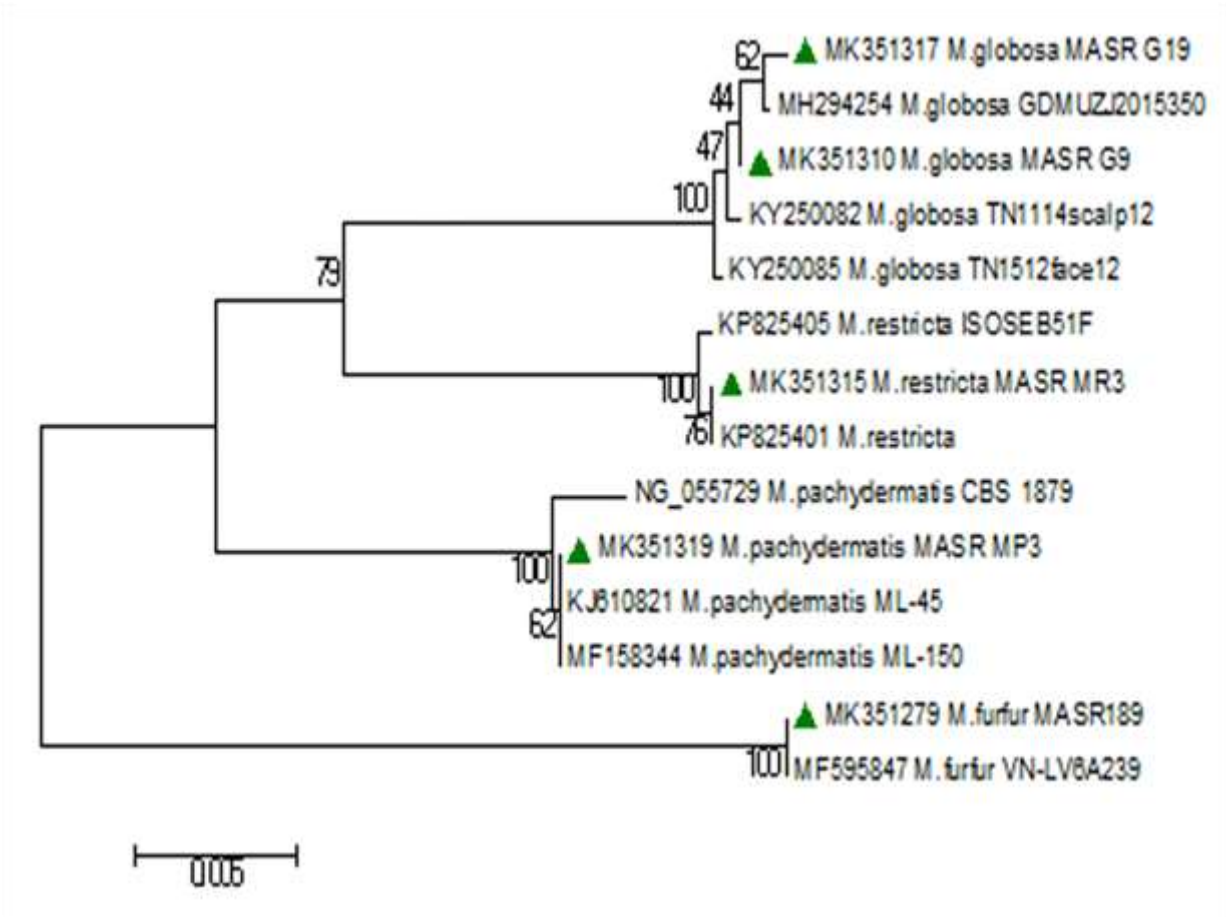

Figure 3: Phylogenetic tree based on 26S rDNA region sequences for *Malassezia* spp. obtained in this study with their reference strains in NCBI GenBank database. Bar indicates two base changes per 1000 nucleotide position.

Table 2: Correlation between phenotypic and genotypic identification of 21 representative *Malassezia* spp.

Code no. of isolate	Host (sample)	Locality				*Malassezia* spp. by phenotypic	*Malassezia* spp. by genotypic
		Sharkia		Cairo			
		Apparently Healthy	Diseased	Apparently Healthy	Diseased		
D2	Dog (Ear swab)	-	+	-	-	*M. pachydermatis*	*M. pachydermatis*
D3	Dog (Ear swab)	-	+	-	-	*M. pachydermatis*	*M. pachydermatis*
D5	Dog (Skin scraping)	+	-	-	-	*M. pachydermatis*	*M. pachydermatis*
D7	Dog (Skin scraping)	+	-	-	-	*M. pachydermatis*	*M. pachydermatis*
D13	Dog (Skin scraping)	-	+	-	-	*M. pachydermatis*	*M. pachydermatis*
D15	Dog (Skin scraping)	-	+	-	-	*M. pachydermatis*	*M. restricta*
D29	Dog (Ear swab)	+	-	-	-	*M. pachydermatis*	*M. pachydermatis*
D31	Dog (Ear swab)	+	-	-	-	*M. pachydermatis*	*M. pachydermatis*
C7	Cat (Ear swab)	+	-	-	-	*M. pachydermatis*	*M. pachydermatis*

C8	Cat (Ear swab)	-	-	+		*M. pachydermatis*	*M. pachydermatis*
C9	Cat (Ear swab)	-	-	-	+	*M. pachydermatis*	*M. pachydermatis*
C11	Cat (Ear swab)	-	-	+	-	*M. pachydermatis*	*M. pachydermatis*
C35	Cat (Skin scraping)	-	-	+	-	*M. pachydermatis*	*M. pachydermatis*
H7	Horse (Skin scraping)	-	-	-	+	*M. pachydermatis*	*M. globosa*
H11	Horse (Skin scraping)	+	-	-	-	*M. pachydermatis*	*M. pachydermatis*
H29	Horse (Skin scraping)	-	-	-	+	*M. pachydermatis*	*M. pachydermatis*
H38	Horse (Ear swab)	-	+	-	-	*M. pachydermatis*	*M. pachydermatis*
B24	Buffalo (Skin scraping)	-	+	-	-	*M. pachydermatis*	*M. furfur*
B28	Buffalo (Ear swab)	-	+	-	-	*M. pachydermatis*	*M. pachydermatis*
B33	Buffalo (Skin scraping)	+	-	-	-	*M. pachydermatis*	*M. pachydermatis*
B40	Buffalo (Skin scraping)	+	-	-	-	*M. globosa*	*M. globosa*

There are significant differences between phenotypic and PCR results *(P* value = 0 .019). The relative sensitivity and accuracy of PCR-RFLP assay were 100% and 86% respectively (data not tabulated).

Discussion

Yeasts of the genus *Malassezia* are considered as both commensal and pathogens on the humans and animals' skin. Rare cases of life threatening fungemia in people have been attributed to *M. pachydermatis*, for which dogs are a natural host. Zoonotic transfer has been documented from dogs to immunocompromised patients by healthcare workers who own dogs. [27]. The present study inspected the phenotypic and genotypic methods for identification of *Malassezia* spp. isolated from dogs, cats, horses and buffaloes in Egypt. From 160 samples of skin scrapings and ear swabs collected from different animals, 49 isolates (30.63%) were identified as *Malassezia* spp. Macro-morphology of 49 isolates of *Malassezia* spp. on mycobiotic agar medium as well as the micro-morphology revealed characteristic features of *M. pachydermatis* and *M. globosa*, that agreed with previously published studies [28-30].

As presented in Table (1), *Malassezia* yeasts were detected in 49% of apparently healthy animals and 51% of diseased one. In support of our findings, Durate *et al.* [31] isolated *Malassezia* spp. at 40% from healthy animals and 64% from diseased one.

Malassezia spp. was isolated from dogs, cats, horses and buffalos at percentages of 47.5%, 37.5%, 22.5% and 15% respectively. Lower percentages were declared by Zia and his co-workers in which *Malassezia* yeasts were detected in different animals at the following rates: 28.33%, 26.66%, 15.46% and 12.74% from dogs, cats, horses and cattle, respectively [32]. Nevertheless, Crespo *et al.* [13] and Rani *et al.* [33] reported the occurrence of *Malassezia* spp. from 60% of horses and 47.5% of buffalos, respectively.

In Egypt, the most frequently detected *Malassezia* spp. among human patient were firstly *M. furfur, M. globosa* and *M. restricta* [28, 34]. Nonetheless, these species were detected among animals by Crespo and his coworkers [35]. In the present study, one *M. furfur* isolate was obtained from affected skin scrapings of buffalo, two *M. globosa* were

identified from the affected skin of a horse and apparently healthy skin of a buffalo and one *M. restricta* was recovered from skin scraping of a diseased dog.

Molecular methods have been established to furnish rapid and precise identification of *Malassezia* spp. as compared to phenotypic methods [21]. The PCR for 26S rDNA gene and RFLP analysis using *HhaI* enzyme have been used extensively for molecular analysis of *Malassezia* spp. [22, 31]. In this study, PCR for 26S rDNA gene showed identical bands for *Malassezia* genus at 580 bp. The restriction pattern of the isolates identified them at the species level of *M. globosa* that showed 2 bands (129 and 455 bp), *M. pachydermatis* showed 3 bands (97, 221 and 250 bp), *M. restricta* showed one band (580 bp), and *M. furfur* showed 3 bands (107, 113 and 250 bp), the obtained findings were in harmony with previous researches [22, 26]. In the case of *Malassezia* spp., Gupta *et al.*, [36] observed that PCR-RFLP analysis of the internal transcribed spacer (ITS) region was sufficient to resolve the differences between the physiologically similar species *M. sympodialis*, *M. furfur* and *M. slooffiae*. Further, sequence diversity within various species has been observed, which suggests the presence of several genotypes within the species [37]. ITS sequencing has likewise been demonstrated as valuable in discriminating the phylogenetically related *Malassezia* spp. [38]. The pairwise differences among sequences of the new genotypes from lipid-dependent *Malassezia* strains and the previously described genotypes ranged from 0.1 to 7.0% and 0.1 to 3.4% for ITS and beta-tubulin genes, respectively. These genetic analyses confirmed the identification of the lipid-dependent strains as *M. pachydermatis* [39]. In this study, the phylogenetic tree was inferred from the sequences of closest strains in light of 26S rDNA gene sequences. The phylogenetic tree showed different clusters for each species indicating variation in their sequences. In essence, molecular methods are necessary for identification and differentiation of various *Malassezia* species, which can be difficult to characterize by phenotypic methods [40].

Conclusion

Although the phenotypic methods could identify some *Malassezia* spp., the PCR-RFLP assay using *Hha1* restriction enzyme and DNA sequencing are complementary and mandatory for *Malassezia* spp. identification from animals.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

تفاعل البلمرة المتسلسل -وتعدد أطوال جزء الحصرو تحليل التتابع الجينى لتصنيف انواع الملاسيزيا المعزولة من الحيوانات

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الملاسيزيا هى واحدة من أهم الخمائر التي تسبب أمراض جلدية في مختلف الحيوانات. وقد تم الكشف عن الفطر بكلاً من الطرق الظاهرية والجينية لعزلات الملاسيزيا من الكلاب ، القطط ، الخيول والجاموس السليمة ظاهرياً والمريضة . تم جمع ١٦٠ عينة من مسحات الاذن وكشطات من الجلد من الحيوانات السليمة والحيوانات المصابة . من بين ٨٢ عينة من مسحات ألاذن و٧٨ عينة كشطات من الجلد، فقد اظهرت ٢٤ (٢٩.٢٧%) و ٢٥ (٣٢.٠٥%) عينة نمو ايجابى على وسط المايكوبيوتيك على التوالى. تم فحص الخصائص الظاهرية والمجهرية الدقيقة لعدد ٤٩ عزلة بعد انمائها على وسط المايكوبيوتيك وكذلك وسط الديكسون عند درجات حرارة مختلفة و كذلك تم تعيين بعض الخصائص الفسيولوجية والتى تشمل استهلاك التوين، تحليل الاوسكلين، استهلاك التربتوفان وانتاج انزيم الكاتاليز كما تم استخدام الطرق الجينية (تفاعل البلمرة المتسلسل ويلية التقطيع باستخدام انزيم قطع *Hha*1) للتاكد من العزلات المعرفة مسبقاً على انها ملاسيزيا. تم تصنيف العزلات ظاهريا الى ملاسيزيا جلوبوزا وملاسيزيا باكيديرميتس بينما تم التعرف على ملاسيزيا ريستريكتا (١) وملاسيزيا فيرفر (١) وملاسيزيا جلوبوزا (٢) وملاسيزيا باكيديرميتس (١٧) من واحد وعشرون عزلة ممثلة بتفاعل البلمرة المتسلسل وتعدد اطوال جزء الحصر وعلاوة على ذلك فقد تم القيام بعمل تسلسل الحمض النووى الناتج وقد وجد نسبة تشابة ١٠٠% للمعزولات المختبرة مع تلك المتاحة في بنك الجينات وأظهرت النتائج أن الانواع الاكثر شيوعاً والتي تم تحديدها هي ملاسيزيا باكيديرميتس بنسبة ٨٠.٩٥% . وقد تم عزلها من ٣٣.٣%، ٢٣.٨%، ١٤.٢٨% و٩.٥٢% من الكلاب ، القطط ، الخيول والجاموس على التوالى ، وتعد الملاسيزيا جلوبوزا ثانى اكثر الانواع شيوعا بنسة ٩.٥٢% وقد عزلت فقط بنسبة ٤.٧٦% من كلاً من الخيول والجاموس بينما عزلت الملاسيزيا فيرفر بنسبة ٤.٧٦% من الجاموس وملاسيزيا ريستريكتا بنسبة ٤.٧٦% من الكلاب. مما سبق يتضح ان تفاعل البلمرة المتسلسل وتعدد أطوال جزء الحصر وتحليل التتابع الجينى أكثر دقة و موثوقية للتعرف علي انواع الملاسيزيا و مكملة للطرق الظاهرية.