1 **Molecular Sequencing and Phylogenetic Analysis of *Malassezia pachydermatis* Isolates from**
2 **Dogs *and Cats***

3 **Running title: ITS-Based Phylogenetic Analysis of *Malassezia pachydermatis***

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17 **Abstract**
18 Introduction: *Malassezia pachydermatis* is a lipophilic yeast that is part of the normal skin
19 microbiota of dogs and cats but can act as an opportunistic pathogen associated with dermatitis and
20 otitis externa. Molecular characterization is essential for understanding its genetic diversity and
21 epidemiology. This study aimed to perform ITS-1-based sequencing and phylogenetic analysis of
22 *M. pachydermatis* isolates from companion animals.

23 Materials and Methods: A total of 300 clinical samples were collected from dogs and cats with
24 dermatological lesions or otitis externa in northern Iran. Isolates were identified by culture on
25 modified Dixon agar and ITS-1 PCR. Ten representative isolates were sequenced using the
26 Sanger method. Sequence similarity was analyzed using BLAST, and phylogenetic relationships
27 were inferred using the Neighbour-Joining method with the Kimura two-parameter model and
28 1000 bootstrap replications.

29 Results: Eighty isolates were identified as *Malassezia* spp., including 87.5% *M. pachydermatis* and
30 12.5% *M. nana*. All sequenced isolates showed ≥98% similarity with reference strains.
31 Phylogenetic analysis revealed clustering mainly within genotype B. No host-specific clustering
32 was observed between canine and feline isolates. Most isolates showed identical or closely related
33 haplotypes, with high bootstrap support (>70%).

34 Conclusion:The results indicate high genetic homogeneity among *M. pachydermatis* isolates. ITS-
35 1 sequencing is a reliable method for molecular identification and phylogenetic analysis. The lack

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of host-specific clustering suggests a shared population structure between canine and feline isolates.

Keywords: Companion animals; ITS-1*; Malassezia pachydermatis;* Molecular sequencing; Phylogenetic analysis

1. Introduction

M. pachydermatis is a lipophilic yeast that is part of the normal skin microbiota of dogs and cats. However, under certain predisposing conditions, it can become an opportunistic pathogen. This yeast is often associated with otitis externa and dermatitis in companion animals. These conditions represent a significant portion of dermatological disorders encountered in veterinary clinical practice and are typically characterized by recurrent or chronic episodes [1,2].

Accurate identification of Malassezia species is critical for understanding their epidemiology, pathogenic potential, and transmission dynamics. Conventional identification methods that rely on phenotypic, biochemical, and physiological characteristics are still commonly used. However, these methods can be limited by variations in phenotype, overlapping morphological traits, and inconsistent expression of diagnostic features. Such limitations can result in misidentification, especially when atypical isolates are encountered [3,4].

Molecular techniques are increasingly being used to accurately identify Malassezia species. One of the most reliable methods for fungal taxonomy is sequencing the internal transcribed spacer (ITS) region of ribosomal DNA. The ITS-1 region, in particular, offers enough resolution for species-level identification of M. pachydermatis and enables the assessment of genetic relationships within the species [5].

Phylogenetic analyses based on ITS sequences have shown that isolates of M. pachydermatis can be categorized into distinct genotypes, which display different geographic and epidemiological distributions. Notably, Genotype B has been identified as the predominant genotype among veterinary clinical isolates worldwide, indicating it is a widely distributed and genetically conserved lineage. However, there is still limited molecular data available regarding the phylogenetic structure of M. pachydermatis isolates in several regions [6].

The present study aimed to conduct molecular sequencing and phylogenetic analysis of M. pachydermatis isolates obtained from dogs and cats through ITS-1 sequencing. By comparing the generated sequences with reference strains available in GenBank, this study sought to evaluate the genetic relatedness among the isolates and clarify their phylogenetic placement, without duplicating phenotypic or epidemiological data reported in other studies [7,8].

2. Materials and Methods

2.1. Sample Collection and Identification

This study is descriptive and cross-sectional in nature. A total of 300 clinical samples—comprising 190 ear swabs, 50 skin scrapings, and 60 hair samples—were collected from dogs and cats exhibiting dermatological or otic lesions at four veterinary centers in northern Iran: Gilan

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73 Pet, Rafa Hospital, Lahijan Pet, and Fereshtegan Clinic. The samples were examined
74 microscopically using Gram and methylene blue staining to identify yeast-like cells. Culture was
75 then performed on modified Dixon agar (Oxoid, UK), which contained chloramphenicol (0.05
76 g/L) and cycloheximide (0.5 g/L). The plates were incubated at 32 °C for 3 to 5 days [8].

77 **2.2.DNA Extraction**

78 Genomic DNA was extracted using the manual phenol–chloroform method. Fresh yeast colonies
79 were suspended in a lysis buffer containing SDS and proteinase K, then incubated at 56 °C. The
80 DNA was extracted using a phenol–chloroform–isoamyl alcohol solution. Following extraction,
81 the DNA was precipitated with ethanol, washed with 70% ethanol, air-dried, and finally
82 resuspended in TE buffer. The purity and concentration of the extracted DNA were assessed
83 spectrophotometrically by measuring the absorbance at A260/A280. Species-specific PCR assays
84 were performed to identify *M. pachydermatis* isolates using primer sets that target the ITS
85 region of rDNA. The specific primers for *M. pachydermatis* were Mp-F (5′-
86 GATTTGCTGCGTTCTTCATC-3′) and Mp-R (5′-AGCTGGCAGTTGAGGTCATT-3′), which
87 amplify a 483 base pair fragment.

88 Each PCR reaction was conducted in a final volume of 25 µL, consisting of 12.5 µL of 2× PCR
89 Master Mix (Thermo Fisher Scientific, USA), 1 µL of each primer (10 pmol/µL), 2 µL of
90 template DNA, and nuclease-free water to reach the final volume. Amplification was performed
91 in a thermal cycler (Eppendorf Mastercycler, Germany) under the following conditions: initial
92 denaturation at 95 °C for 5 minutes; followed by 35 cycles of denaturation at 94 °C for 30
93 seconds, annealing at 56 °C for 30 seconds, and extension at 72 °C for 45 seconds; concluding
94 with a final extension at 72 °C for 7 minutes. PCR products were visualized by electrophoresis on
95 a 1.5% agarose gel stained with ethidium bromide and observed under UV illumination[2].

96 **2.3.PCR Amplification of the ITS-1 Region**

97 The internal transcribed spacer 1 (ITS-1) region was amplified using universal fungal primers.
98 The PCR reactions were conducted in a final volume that included template DNA, primers, PCR
99 master mix, and nuclease-free water. Amplification was performed under standard cycling
100 conditions. The PCR products were then visualized through agarose gel electrophoresis to
101 confirm the presence of specific bands.

102 **2.4. Sequencing and Molecular Identification**

103 PCR products exhibiting clear, specific bands were subjected to sequencing.
104 The ITS-1 sequences generated in the present study were used for molecular identification and
105 phylogenetic analysis.
106 Obtained sequences were compared with reference sequences available in the GenBank database
107 using the BLAST algorithm to confirm species-level identification.

108 **2.5. Sequence Alignment and Genetic Distance Analysis**

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109 Multiple sequence alignment of ITS-1 nucleotide sequences was performed using the ClustalW
110 algorithm. Genetic distances among sequences were calculated using the Kimura two-parameter
111 (K2P) model to evaluate intraspecific variation.

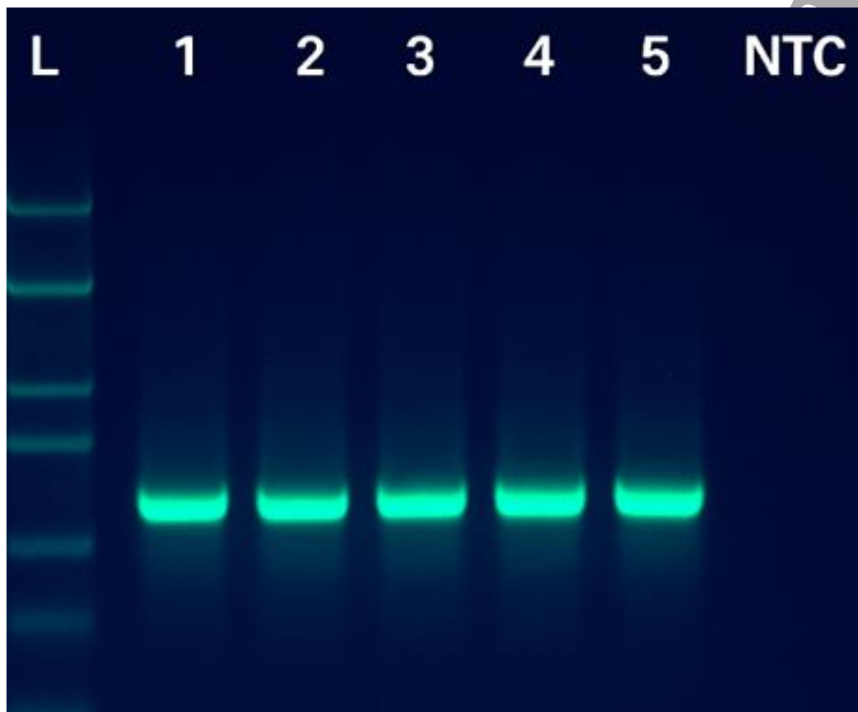
112 **2.6. Phylogenetic Analysis**

113 Phylogenetic relationships were inferred using the Neighbour-Joining method based on the K2P
114 model. Bootstrap analysis with 1000 replicates was performed to assess the robustness of the tree
115 topology. Phylogenetic analysis was conducted using standard bioinformatics software.

116 **3. Results**

117 **3.1. ITS-1 Sequencing and Molecular Identification**

118 Amplification of the ITS-1 region produced a single, specific PCR band that was suitable for
119 sequencing. High-quality ITS-1 sequences were successfully obtained for all ten selected isolates.
120 A BLASTn analysis showed ≥98% nucleotide similarity with validated *M. pachydermatis*
121 reference sequences available in GenBank, confirming the species-level identification.



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123 Figure 1. Agarose gel electrophoresis of ITS-1 PCR products amplified from *Malassezia*
124 *pachydermatis* isolates, showing single specific bands suitable for sequencing.

125 **3.2. Genetic Distance Analysis**

126 The K2P distance analysis revealed low levels of sequence divergence among the isolates
127 examined, indicating a high degree of genetic homogeneity within the ITS-1 region. One isolate
128 exhibited a slightly higher genetic distance; however, this variation still fell within the expected
129 range for intraspecific diversity.

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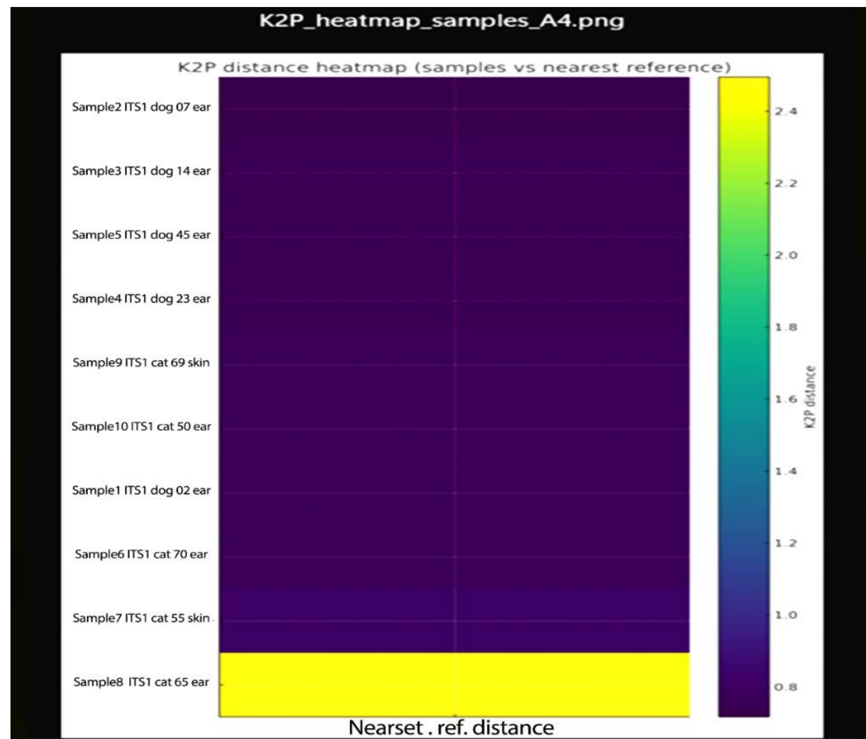

131 Figure 2. Heatmap representation of Kimura two-parameter genetic distances among ITS-1
132 sequences of *Malassezia pachydermatis* isolates and the closest reference sequence (DQ915504).

133 **3.3. Phylogenetic Analysis**

134 Phylogenetic reconstruction grouped all isolates within the M. pachydermatis clade. Most isolates
135 were classified under genotype B and showed a close genetic relationship to the reference
136 sequences DQ915504 and DQ915505. No clustering based on host species was observed, as
137 canine and feline isolates were found intermixed within the same phylogenetic groups. Bootstrap
138 values indicated strong support for the stability of the major branches.

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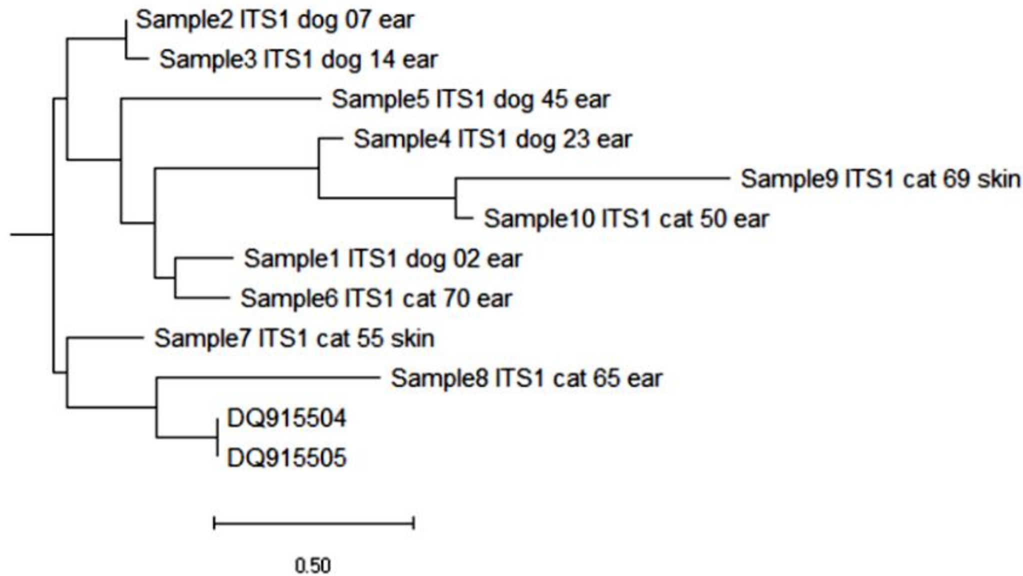

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140 **Figure 3.** Neighbour-Joining phylogenetic tree based on ITS-1 sequences of *Malassezia*
141 *pachydermatis* isolates constructed using the Kimura two-parameter model with 1000 bootstrap
142 replications.

143 The multiple sequence alignment of ITS-1 nucleotide sequences is provided as Supplementary
144 File 1.

145 **4. Discussion**

146 The current study offers both molecular and epidemiological insights into *Malassezia*
147 *pachydermatis* isolates collected from dogs and cats in northern Iran. The overall isolation rate of
148 Malassezia species is 45%, with *M. pachydermatis* being the predominant isolate. These findings
149 are in line with recent reports from Iran and other parts of the world [1,9].

150 In the current study, *M. pachydermatis* accounted for the majority of the identified isolates. This
151 finding is consistent with recent veterinary research that identifies this species as the predominant
152 type of Malassezia in dogs, and to a lesser extent, in cats. Recent studies have reported
153 prevalence rates of *M. pachydermatis* ranging from approximately 30% to 70% in dogs affected
154 by otitis externa and dermatitis [10]. The notable proportion observed in this study may be due to
155 the humid climate of northern Iran, which creates favorable conditions for yeast growth.[2].

156 Recent studies from various regions of Iran have shown a high prevalence of *M. pachydermatis*
157 in canine samples, though some variations in prevalence rates have been noted [11]. These
158 differences can be attributed to environmental conditions, animal management practices, and
159 sampling methods. In contrast, several European studies have reported lower prevalence rates,
160 likely due to variations in climate and healthcare systems [12].

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161 The lack of a clear distinction between canine and feline isolates in this study aligns with recent
162 findings that *M. pachydermatis* does not exhibit strict host specificity [1, 12]. Studies in Asia and
163 Europe indicate possible interspecies transmission or shared environmental reservoirs [12].

164 Phylogenetic analysis using ITS-1 sequences revealed a high nucleotide similarity (≥98%),
165 indicating a genetically homogeneous population. This observation aligns with recent molecular
166 studies that show limited intraspecific variation in *M. pachydermatis* [13, 14]. The prevalence of
167 genotype B in this study aligns with recent findings, which indicate this genotype as the most
168 common among clinical isolates [15].

169 The clustering of isolates from both dogs and cats within the same phylogenetic branches
170 provides further evidence that there is no host-specific genetic structuring. Similar phylogenetic
171 patterns have been observed in recent studies conducted in Europe and East Asia, where isolates
172 from different host species have been found to group [14]. The presence of identical haplotypes
173 among certain isolates suggests the possibility of clonal expansion or shared sources of infection.
174 On the other hand, the existence of a slightly divergent isolate indicates minor genetic variation
175 within the population. Additionally, comparisons with recent studies from Iran reveal a similar
176 pattern of genetic homogeneity, although some minor regional differences may be present [5].
177 These variations may be due to environmental factors or specific local evolutionary pressures.

178 While ITS-1 sequencing is useful, its resolution at the intra-species level is limited [16,17].
179 Overall, the findings of this study align with recent global data, which show that *M.*
180 *pachydermatis* is a genetically conserved species with a broad host distribution and exhibits
181 limited genetic divergence [1,14].

182 **5. Conclusion**

183 ITS-1 sequencing showed a high degree of genetic uniformity among *M. pachydermatis* isolates
184 from dogs and cats. Phylogenetic analysis indicated that genotype B was the most prevalent, with
185 no indications of host-specific clustering. These findings confirm the conserved genetic structure
186 of *M. pachydermatis* in veterinary clinical settings and support the use of ITS-based sequencing
187 for molecular identification and phylogenetic assessment.

188 **Ethical considerations**
189 The samples used in this study were obtained during routine veterinary diagnostic procedures
190 from dogs and cats. No animals were subjected to experimental manipulation, and no additional
191 sampling was performed solely for research purposes. Therefore, ethical approval from an
192 institutional animal care and use committee was not required.

193 **Authors Contribution**

194 Nima Niaee Barmachi: conceived and designed the study, Nima Niaee Barmachi :performed
195 sample collection and laboratory experiments, Nakisa Sohrabi Haghdoost: performed molecular
196 analyses and sequencing, Leila Eini Farahani: conducted phylogenetic and bioinformatics
197 analyses, Nima Niaee Barmachi: analyzed and interpreted the data, Batool Ghorbaniyekta :

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drafted the initial version of the manuscript, Mansour Bayat :critically revised the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest

Use of Artificial Intelligence

Artificial intelligence tools were used solely for language editing and grammatical refinement. No AI tools were used for data generation, analysis, or interpretation.

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