

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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12 **Abstract**

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

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

24 Results: Eighty isolates were identified as *Malassezia* spp., including 87.5% *M. pachydermatis* and
25 12.5% *M. nana*. All sequenced isolates showed $\geq 98\%$ similarity with reference strains.
26 Phylogenetic analysis revealed clustering mainly within genotype B. No host-specific clustering
27 was observed between canine and feline isolates. Most isolates showed identical or closely related
28 haplotypes, with high bootstrap support ($>70\%$).

29 Conclusion: The results indicate high genetic homogeneity among *M. pachydermatis* isolates. ITS-
30 1 sequencing is a reliable method for molecular identification and phylogenetic analysis. The lack
31 of host-specific clustering suggests a shared population structure between canine and feline
32 isolates.

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

35 **1. Introduction**

36 *M. pachydermatis* is a lipophilic yeast that is part of the normal skin microbiota of dogs and cats.
37 However, under certain predisposing conditions, it can become an opportunistic pathogen. This

38 yeast is often associated with otitis externa and dermatitis in companion animals. These
39 conditions represent a significant portion of dermatological disorders encountered in veterinary
40 clinical practice and are typically characterized by recurrent or chronic episodes [1,2].

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

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

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

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

63 **2. Materials and Methods**

64 **2.1. Sample Collection and Identification**

65 This study is descriptive and cross-sectional in nature. A total of 300 clinical samples—
66 comprising 190 ear swabs, 50 skin scrapings, and 60 hair samples—were collected from dogs and
67 cats exhibiting dermatological or otic lesions at four veterinary centers in northern Iran: Gilan
68 Pet, Rafa Hospital, Lahijan Pet, and Fereshtegan Clinic. The samples were examined
69 microscopically using Gram and methylene blue staining to identify yeast-like cells. Culture was
70 then performed on modified Dixon agar (Oxoid, UK), which contained chloramphenicol (0.05
71 g/L) and cycloheximide (0.5 g/L). The plates were incubated at 32 °C for 3 to 5 days [8].

72 **2.2. DNA Extraction**

73 Genomic DNA was extracted using the manual phenol–chloroform method. Fresh yeast colonies
74 were suspended in a lysis buffer containing SDS and proteinase K, then incubated at 56 °C. The
75 DNA was extracted using a phenol–chloroform–isoamyl alcohol solution. Following extraction,

76 the DNA was precipitated with ethanol, washed with 70% ethanol, air-dried, and finally
77 resuspended in TE buffer. The purity and concentration of the extracted DNA were assessed
78 spectrophotometrically by measuring the absorbance at A260/A280. Species-specific PCR assays
79 were performed to identify *M. pachydermatis* isolates using primer sets that target the ITS
80 region of rDNA. The specific primers for *M. pachydermatis* were Mp-F (5'-
81 GATTTGCTGCGTTCTTCATC-3') and Mp-R (5'-AGCTGGCAGTTGAGGTCATT-3'), which
82 amplify a 483 base pair fragment.

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

91 **2.3.PCR Amplification of the ITS-1 Region**

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

97 **2.4. Sequencing and Molecular Identification**

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

103 **2.5. Sequence Alignment and Genetic Distance Analysis**

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

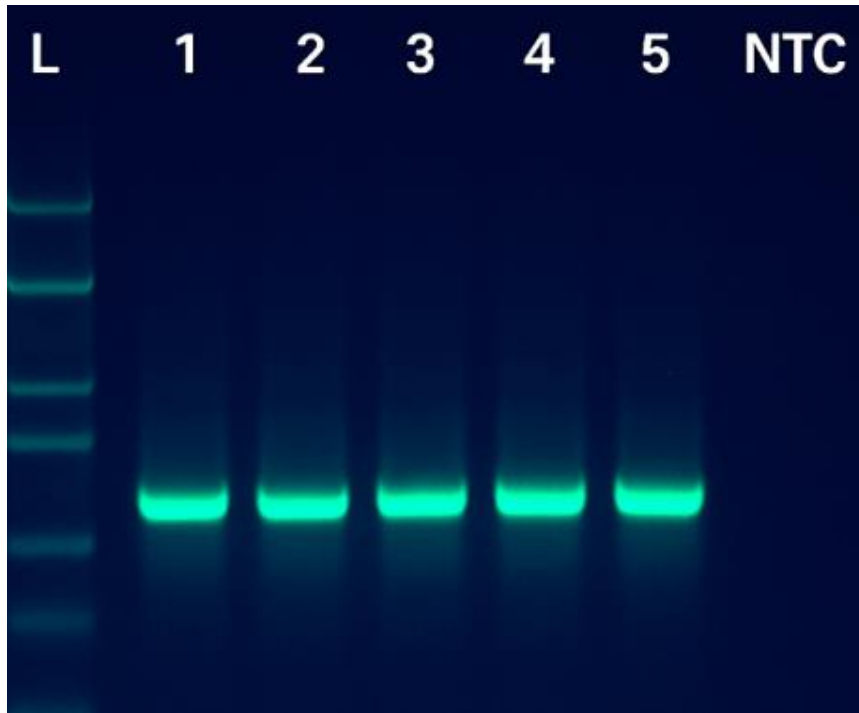
107 **2.6. Phylogenetic Analysis**

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

111 **3. Results**

112 **3.1. ITS-1 Sequencing and Molecular Identification**

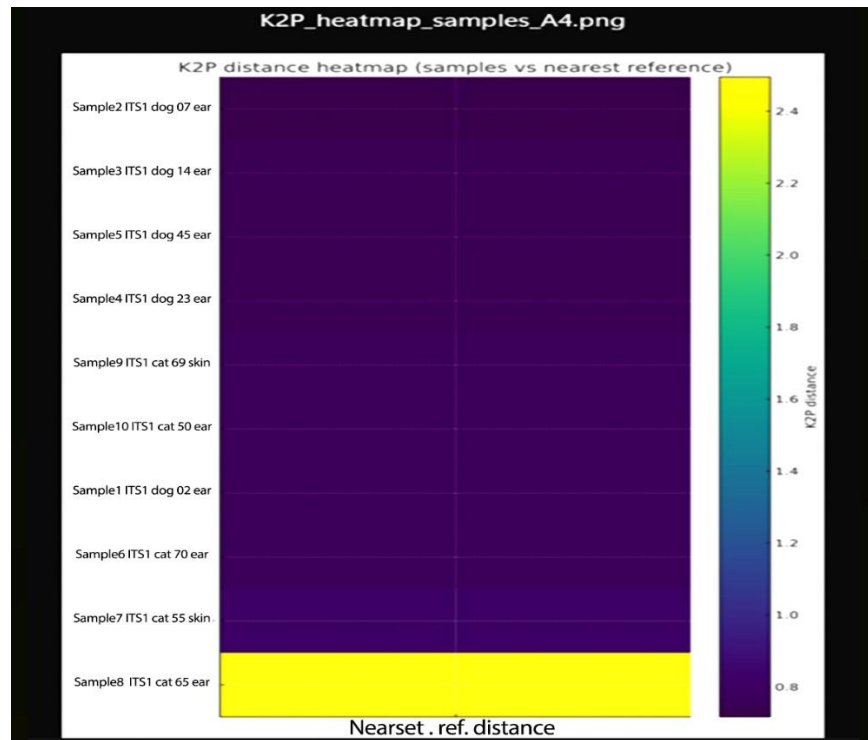
113 Amplification of the ITS-1 region produced a single, specific PCR band that was suitable for
114 sequencing. High-quality ITS-1 sequences were successfully obtained for all ten selected isolates.
115 A BLASTn analysis showed $\geq 98\%$ nucleotide similarity with validated *M. pachydermatis*
116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



117
118 Figure 1. Agarose gel electrophoresis of ITS-1 PCR products amplified from *Malassezia*
119 *pachydermatis* isolates, showing single specific bands suitable for sequencing.

120 **3.2. Genetic Distance Analysis**

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

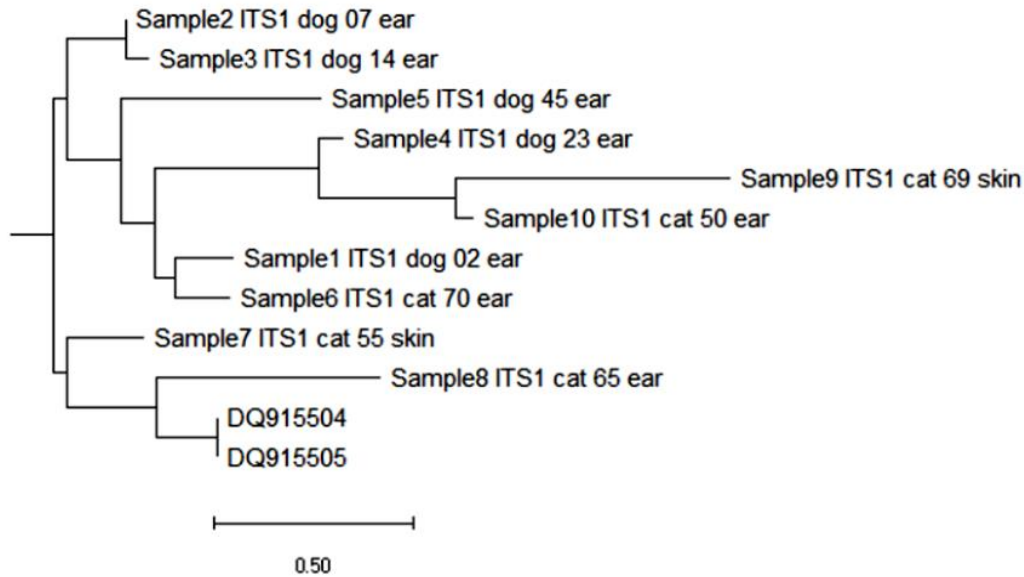


125

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

128 **3.3. Phylogenetic Analysis**

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



134
 135 **Figure 3.** Neighbour-Joining phylogenetic tree based on ITS-1 sequences of *Malassezia*
 136 *pachydermatis* isolates constructed using the Kimura two-parameter model with 1000 bootstrap
 137 replications.

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

140 **4. Discussion**

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

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

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

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

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

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

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

177 **5. Conclusion**

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

183 **Ethical considerations**

184 The samples used in this study were obtained during routine veterinary diagnostic procedures
185 from dogs and cats. No animals were subjected to experimental manipulation, and no additional
186 sampling was performed solely for research purposes. Therefore, ethical approval from an
187 institutional animal care and use committee was not required.

188 **Authors Contribution**

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

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

195 **Conflict of Interest**

196 The authors declare that there is no conflict of interest

197 **Use of Artificial Intelligence**

198 *Artificial intelligence tools were used solely for language editing and grammatical refinement.*

199 *No AI tools were used for data generation, analysis, or interpretation.*

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