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Phylogenetic analysis of dermatophytes isolated from small domestic animals

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SUMMARY

Dermatophytoses are diseases of skin and its accessory structures that are widely spread worldwide. They are most commonly caused by fungi of the genera *Microsporum* and *Trichophyton*. The identification of the agent's species has a great epidemiological significance and is essential for effective therapy. The aim of the study is the identification and phylogenetic analysis of dermatophytes isolated from dogs and cats in the Republic of Kazakhstan and the Russian Federation by means of molecular techniques. The fungal isolate species were confirmed by sequencing using two rDNA internal transcribed spacer (ITS) primer pairs, and this allowed for their deposition to the GenBank database. Based on the sequencing results, *Microsporum canis* (12 strains) and *Trichophyton benhamiae* (2 strains) were identified. The nucleotide sequences were analysed, and phylogenetic trees were constructed, taking into account the results of the dermatophyte identification using two primer pairs. The constructed phylogenetic trees reflecting the relationships of dermatophytes showed that, irrespective of the primer pairs used, the *Microsporum* and *Trichophyton* pathogens are in all cases reliably assigned to different clades. The analysis of *ITS4F/ITS5R* sequence fragment structures enabled the establishment of genetic relatedness between the *Trichophyton benhamiae* strains first isolated from cats in Russia and the Russian strain recovered from a guinea pig. The comparative analysis of the genomes of the *Microsporum* and *Trichophyton* fungi and reference strains revealed a relatively low level of intraspecies polymorphism and point mutations of the sequences. The data analysis demonstrated a high percentage of nucleotide sequence homology, and this allows using the primers for PCR tests intended for dermatophytosis diagnosis in cats and dogs.

Keywords: dermatophytosis, *Microsporum canis*, *Trichophyton benhamiae*, phylogenetic tree, homology, reference strain, nucleotide sequence

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Филогенетический анализ дерматофитов, выделенных от мелких домашних животных

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РЕЗЮМЕ

Дерматофитозы – широко распространенные во всем мире заболевания кожи и ее производных, чаще всего вызываемые грибами родов *Microsporum* и *Trichophyton*. Идентификация вида возбудителя имеет большое эпидемиологическое значение, а также необходима для проведения эффективной терапии. Цель исследований – идентификация и филогенетический анализ дерматофитов, выделенных от собак и кошек на территории Республики Казахстан и Российской Федерации, с помощью молекулярных методов. Видовая принадлежность изолятов грибов была подтверждена секвенированием по двум парам праймеров внутреннего транскрибируемого спейсерного участка (internal transcribed spacer, ITS) рДНК, что позволило депонировать их в базу данных GenBank. На основании результатов секвенирования были идентифицированы *Microsporum canis* (12 штаммов) и *Trichophyton benhamiae* (2 штамма). Проведен анализ нуклеотидных последовательностей и построены филогенетические деревья с учетом результатов идентификации дерматофитов по двум парам праймеров. Построение филогенетического дерева, основанное на отражении родственных связей дерматофитов, показало, что, независимо от использования разных пар праймеров, возбудители рода *Microsporum* и *Trichophyton* во всех случаях достоверно распределены по разным кладам. Анализ структур фрагментов последовательности *ITS4F/ITS5R* позволил выявить генетическое родство штаммов *Trichophyton benhamiae*, впервые выделенных от кошек на территории России, с российским штаммом, изолированным от морской свинки. Сравнительный анализ геномов грибов рода *Microsporum* и *Trichophyton* с референтными штаммами показал относительно невысокий уровень внутривидового полиморфизма и точечных мутаций

последовательностей. В результате анализа данных был определен высокий процент гомологии нуклеотидных последовательностей, что позволяет использовать праймеры для проведения полимеразной цепной реакции в качестве диагностического теста при дерматофитозах кошек и собак.

Ключевые слова: дерматофитоз, *Microsporum canis*, *Trichophyton benhamiae*, филогенетическое дерево, гомология, референтный штамм, нуклеотидная последовательность

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INTRODUCTION

Dermatophytes are keratinophilic fungi of the family *Arthrodermataceae* (*Onygenales*, *Ascomycota*) that include dozens of related species differentiated mainly by their anamorphs or asexual forms and arranged in three classical genera: *Trichophyton*, *Microsporum* and *Epidermophyton* [1]. The genera *Trichophyton* and *Microsporum* comprise anthropophilic, zoophilic and geophilic dermatophyte species that are able to cause infection mostly in humans or animals and found free-living in soil. The genus *Epidermophyton* includes only one species, *E. floccosum*, which exclusively affects humans.

Dermatophytoses, diseases caused by these fungi, are spread worldwide, with the number of cases in humans and animals increasing annually. The occurrence of dermatophytoses in small domestic animals such as cats and dogs being the companion animals of humans is of particular significance [2–3]. *Microsporum canis* and *Trichophyton mentagrophytes* are the most significant species of dermatophytes isolated from infected dogs, cats and other carnivores [5, 6].

Until recently, the diagnosis of dermatophytoses has been based on the clinical signs of the disease, which are unreliable due to the variable nature of dermatological lesions and similarity with other skin diseases that mimic the symptoms characteristic of dermatophytoses [6]. The direct microscopic examination of biological material samples collected from lesions and the isolation of dermatophyte cultures in nutrient media are the gold standard for dermatophytosis diagnosis. However, species identification may sometimes require further investigation of the biochemical properties of the isolated dermatophyte cultures. Therefore, dermatophyte species identification based on studying their phenotypic characteristics is a labour-intensive and time-consuming process that requires skilled researchers [7].

Molecular techniques are promising for the direct detection of fungal DNAs in the clinical samples and their species identification [8]. At present, methods based on ribosomal gene nucleotide sequencing are utilized for dermatophyte species identification in some countries [9]. Data on fully or partially sequenced rRNA genes of various microorganisms are submitted to the international databases and can be used as reference ones. The comparative analysis of sequences of genes and individual gene regions encoding ribosomal RNAs may contribute to the detection of dermatophyte relationships [10]. Multilocus microsatellite typing was applied for tracking the routes of spread and transmission of *M. canis* in Japan [11]. The findings from a study on *M. canis* occurrence in cats, dogs and humans by means of molecular genetic typing using forward (*ITS1* 5′-TC CGTAGGTGAACCTGCGG-3′) and reverse (*ITS4* 5′-TCCTCCGCTTATTGATATGC-3′) primers showed that indoor and outdoor animals, as well as cats and dogs with or without the disease symptoms are the main dermatophyte sources for humans [12]. The application of molecular techniques allowed for the determination of the etiological structure of dermatophytoses in Iran, which was represented by the following species: *M. canis* – 78.5%, *M. gypseum* – 10.7% and *T. mentagrophytes* – 10.7% [13].

The study was aimed at the assessment of the possibility of using molecular techniques for the identification and phylogenetic analysis of dermatophytes isolated from dogs and cats in the Republic of Kazakhstan and the Russian Federation.

MATERIALS AND METHODS

The objects of the study were the intergenic internal transcribed spacer (*ITS*) region 5.8, 18, 28S rRNA nucleotide sequences of the representatives of two dermatophyte genera, *Microsporum* ($n = 12$) and *Trichophyton* ($n = 2$), isolated from dogs and cats in the Republic of Kazakhstan and the Russian Federation (Table).

The amplification of marker genes (*ITS*) was carried out in the final reaction volume of 25 µL containing 1× Phusion HF-buffer, 2.5 mM of $MgCl_2$, 1U Phusion DNA-polymerase and 200 µM of dNTP (New England BioLabs Inc., USA), 25 pmol of each primer and 20 ng of extracted DNA from one sample.

Thermal cycling conditions for polymerase chain reaction (PCR) were as follows: initial DNA denaturation at 95 °C for 5 minutes, then 35 cycles at 95 °C for 30 seconds,

at 58 °C for 40 seconds, at 72 °C for 50 seconds and final extension at 72 °C for 5 minutes. The amplified DNA products were analysed with horizontal 1.5% agarose gel electrophoresis using 1× TAE buffer and EtBr. The electrophoresis parameters were 120 V, 250 mA, 50 W, the reaction time was 30 minutes. The amplified DNA fragments were sequenced using the Sanger method and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

The primer sequences used were the same as for PCR.

To ensure the accuracy of results, the amplified fragments were sequenced using two primer pairs: the forward primer *ITS1F* (TCCGTAGGTGAACCTGCGG) and the reverse primer *ITS4R* (TCCTCCGCTTATTGATATGC) [12]; the forward primer *ITS4F* (TCCTCCGCTTATTGATATGC) and the reverse primer *ITS5R* (GGAAGTAAAAGTCGTAACAAGG) [14].

The sequencing products were analysed using an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA). The chromatograms were analysed and edited using Sequencing Analysis Software v5.2, Patch 2 (Applied Biosystems, USA). The resulting sequences were deposited to the international GenBank database. The multiple sequence alignment was carried out using MUSCLE and ClustalW algorithms.

The phylogenetic analysis was performed using the maximum likelihood and nearest neighbour methods, as well as MEGA (v11) bioinformatic analysis software.

RESULTS AND DISCUSSION

M. canis and *T. benhamiae,* the agents of dermatophytoses in cats and dogs, were identified based on the results of sequencing by the Sanger method using the primer pair *ITS1F/ITS4R* (12 *Microsporum* and 2 *Trichophyton* isolates) and the primer pair *ITS4F/ITS5R* (6 *Microsporum* isolates and 2 *Trichophyton* strains).

The analysed isolates showed a high percentage of nucleotide sequence homology with reference strains, and this allowed for their deposition to the international NCBI database. The following unique identifiers were assigned based on the *ITS1F/ITS4R* genotyping results: *M. canis* – OQ592853.1, OQ592883.1, OQ592896.1, OQ592901.1, OQ593382.1, OQ593383.1, OQ593387.1, OQ593395.1, OQ593394.1, OQ594023.1, OQ594046.1, OQ594324.1; *T. benhamiae* – OQ592797.1, OQ600605.1.

The following unique identifiers were assigned based on the *ITS4F/ITS5R* genotyping results: *M. canis* – ON527772.1, ON527773.1, ON527774.1, ON527775.1,

Table
The list of dermatophyte isolates recovered from dogs and cats

No.	Suspected pathogen	Isolate No.	Date of isolation in nutrient media	Animal species	Region
1	*Microsporum* spp.	5	04.05.2021	female cat	Republic of Kazakhstan
2		8	31.05.2021		
3		22	14.09.2021		
4		27	17.09.2021		
5		29	21.09.2021	male cat	
6		33	10.10.2021		
7		35	11.10.2021		
8		48	28.10.2021	female cat	
9		58	21.12.2021		
10		61	25.12.2021	dog	
11		64	25.12.2021		
12	*Trichophyton* spp.	68	12.01.2022	female cat	
13	*Trichophyton* spp.	19	02.12.2021	female cat	Russian Federation
14		20	02.12.2021	male cat	

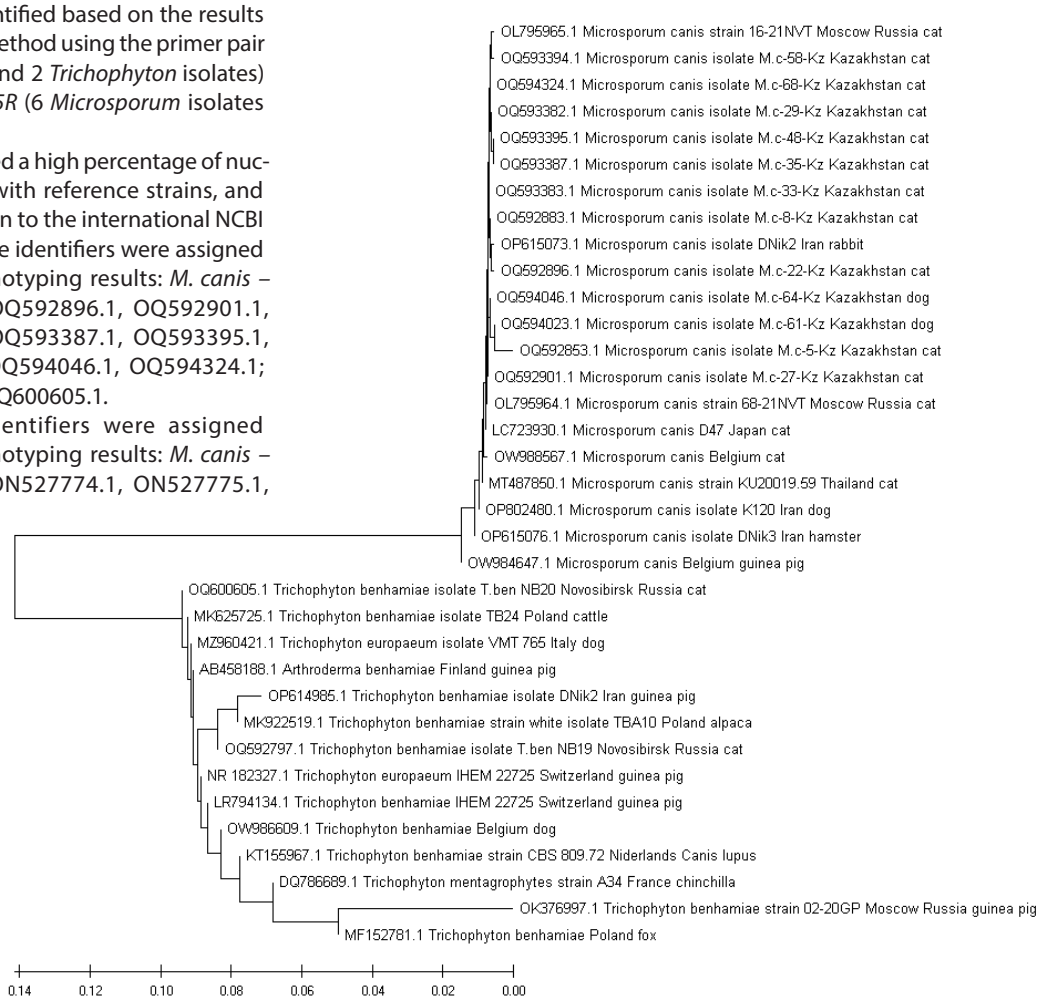

Fig. 1. Phylogenetic tree of Microsporum and Trichophyton spp. dermatophytes based on ITS1F/ITS4R sequence fragment structure analysis

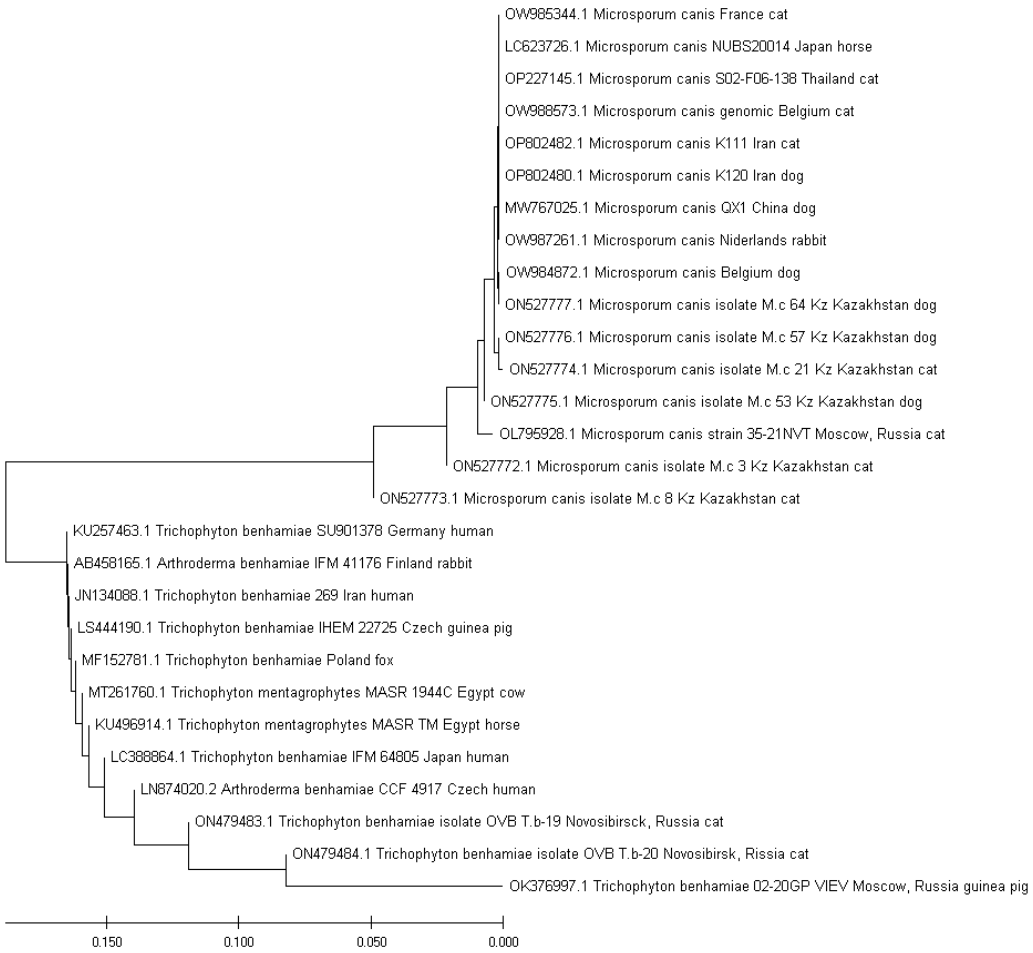

Fig. 2. Phylogenetic tree of Microsporum and Trichophyton spp. dermatophytes based on ITS4F/ITS5R sequence fragment structure analysis

ON527776.1, ON527777.1; *T. benhamiae* – ON479483.1, ON479484.1.

To construct a phylogenetic tree, the database of complete intergenic region 5.8, 18, 28S rRNA nucleotide sequences was searched for the typical representatives of *Microsporum* and *Trichophyton* dermatophytes to be used as reference strains for the comparative phylogenetic analysis of the identified strains.

The evolutionary relationship of the strains was inferred using the neighbour-joining method [15]. The phylogenetic tree was drawn to scale, with the evolutionary distance corresponding to 14 substitutions per 100 nucleotides. The evolutionary distances were computed using the maximum composite likelihood method [16] and expressed in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (the pairwise-deletion option). There was a total of 1,419 positions in the final dataset. The phylogenetic trees based on the *ITS1F/ITS4R* and *ITS4F/ITS5R* sequence fragment structure analysis and reflecting the relationships of *Microsporum* and *Trichophyton* dermatophytes are presented in Figures 1 and 2, respectively.

The optimal tree presented in Figure 2 is drawn to scale, with the evolutionary distance corresponding to 15–20 substitutions per 100 nucleotides. The analysis included 28 nucleotide sequences. All ambiguous positions were removed for each sequence pair (the pairwise-deletion option). There was a total of 1,152 positions in the final dataset. The evolutionary analysis was carried out using MEGA (v11) bioinformatic analysis software [17].

The constructed phylogenetic tree reflecting the relationships of dermatophytes showed that, irrespective of the primer pairs used, the *Microsporum* and *Trichophyton* pathogens are in all cases reliably assigned to different clades. The analysis of *ITS4F/ITS5R* sequence fragment structures enabled the establishment of genetic relatedness between all *T. benhamiae* strains first isolated from cats in Russia and the Russian strain recovered from a guinea pig.

The evolutionary relationship of the strains inferred by the analysis of the genome data for each dermatophyte strain obtained using the primers *ITS1F/ITS4R* is shown in Figures 3 and 4.

Sequences used for comparison of the relevant *M. canis* strain intergenic region rDNA sequence were selected from those of the outgroup strains.

Figure 3 shows the high homology of *M. canis* strain intergenic region 5.8, 18, 28S rDNA sequences. The LC723930.1 and MT487850.1 sequences have only one nucleotide substitution each, which is indicative of point mutation. OL795964.1 was found to have three mononucleotide deletions (i.e. polymorphism). The OW988567.1 sequence and that of the Kazakhstan OQ594324.1 strain (marked with an asterisk *) were found to have only one deletion.

Similar methods were used for the analysis of the relevant *T. benhamiae* strain intergenic region rDNA sequence.

Data presented in Figure 4 show that the OQ592797.1 strain sequence (marked with an asterisk *) has additional nucleotide insertions indicative of polymorphism. The second *T. benhamiae* strain, OQ600605.1, was homologous to the reference strains. Point mutations were detected in the OP614985.1, MK922519.1, MK625725.1 strain sequences. There were two substitutions in the OK376997.1 strain sequence, and this is also indicative of point mutations.

The evolutionary relationship of the strains inferred by the analysis of the genome data for each dermatophyte strain obtained using the primers *ITS4F/ITS5R* is shown in Figures 5 and 6.

Sequences used for comparison of the relevant *M. canis* strain intergenic region rDNA sequence were selected from those of the outgroup strains.

Figure 5 shows that nine out of eleven sequences presented have a single polymorphism, one sequence has a mutation. The analysed sequence (marked with an asterisk *) has three nucleotide substitutions.

The comparative analysis of the relevant *T. benhamiae* strain intergenic region rDNA sequences using the primers *ITS4F/ITS5R* is presented in Figure 6.

As we can see, all the nucleotide sequences of the *T. benhamiae* strains are homologous. The sequences of three strains (MT261760.1, KU496914.1 and OK376997.1) have one mutation each. *T. mentagrophytes* MT261760.1 and KU496914.1 strains were found to have two nucleotide deletions. These strains belong to the same clade and are not the members of the species *T. benhamiae*, and this reliably increases the specificity of the diagnostic test and enables differentiation between the representatives of two species of the same genus.

OK376997.1 strain is the representative of the species *T. benhamiae* isolated from a guinea pig. In comparison with the analysed T. *benhamiae* strains isolated from cats, its nucleotide sequences have one difference, namely a point mutation.

In view of these findings, we would emphasize the importance of dermatophytosis pathogen species identification. This is particularly important for the species *T. benhamiae,* a new causative agent of dermatophytosis in cats, first isolated by us in Russia [18].

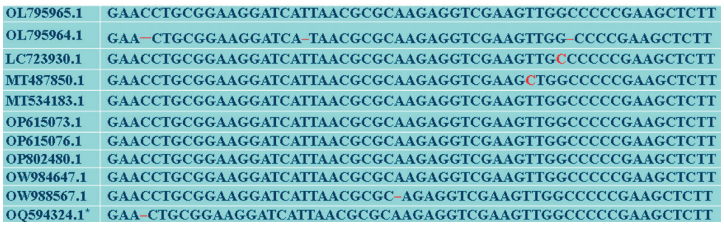

Fig. 3. M. canis strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

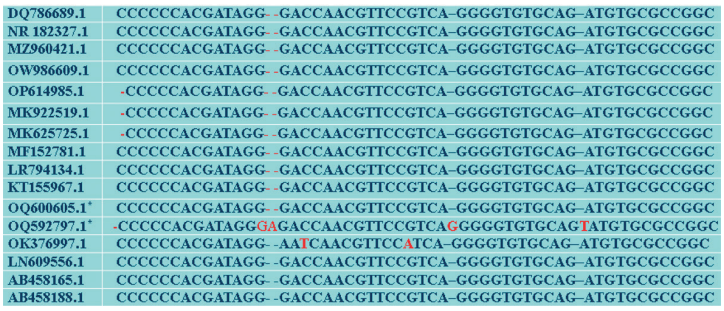

Fig. 4. T. benhamiae strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

Fig. 5. M. canis strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

Fig. 6. Trichophyton spp. strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

CONCLUSION

The phylogenetic analysis of 12 *Microsporum* and 2 *Trichophyton* strains demonstrated a high percentage of their nucleotide sequence homology. The comparative analysis of the ribosomal RNA gene fragments of the *Microsporum* and *Trichophyton* fungi and reference strains revealed in each case a relatively low level of intraspecies polymorphism and point mutations of the sequences. This shows that *ITS*-region 5.8, 18, 28S rDNA gene nucleotide sequencing can be considered as a rapid and reliable technique for the identification of closely related dermatophytes of the genera *Trichophyton* and *Microsporum.* The detected similarity of the nucleotide sequences of the analysed and reference strains of dermatophytes is indicative of the reliability of the results obtained and the possibility of using molecular diagnostic techniques for their species identification.

Thus, *ITS*-PCR is a reliable and robust method for the identification of closely related dermatophyte species and can therefore be used for dermatophytosis diagnosis in cats and dogs.

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