J Vet Clin 23(4) : 405-410 (2006)

Journal of Veterinary Clinics
www.ksvc.or.kr

Molecular Phylogenetic Classification of Dermatophytes Isolated from Dogs and Cats

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(Accepted: December 11, 2006)

Abstract: Using internal transcribed spacer 1 (ITS1) region ribosomal DNA sequences from 9 strains of *Microsporum canis* and 5 strains of *Microsporum gypseum* isolated from dogs and a cat with dermatophytosis, we demonstrated the mutual phylogenetic relationship of these strains. Nucleotide sequence analysis of the ITS1 gene fragments from the 9 strains of *M canis* had the 100% nucleotide sequence similarities and the 5 strains of *M gypseum* also had the 100% nucleotide sequence similarities. The phylogenetic analysis of the nucleotide sequences of the 9 strains of *M canis* formed a nested cluster with the reference strains of *M canis* originating from USA, Australia, Japan, and Europe. *M canis* were genetically distinct from the other reference strains of *Microsporum* spp, but *M distortum, M equinum,* and *M ferrugineum* were genetically very close to *M canis*. *M gypseum* form a cluster in the phylogenetic tree with *M canis* as an outgroup. The molecular analysis of ITS1 genes provided the useful information for the identification of these *Microsporum* species and the understanding of their relationship.

Key words : *Microsporum* spp, ITS1, phylogeny, dog and cat.

Introduction

Microsporum spp is one of the most ubiquitous dermatophytes. This species has a worldwide distribution and is the most common cause of dermatophytosis of dogs and cats (15). The phylogeny of dermatophytes remains unclear because their members are phylogenetically and taxonomically very closely related, their phenotypic features are sometimes poor, and many isolates from medical and veterinary samples have lost their sexual activity (17). From a clinical point of view, for definition of species or for performance of an epidemiological study, it is important to have a reliable method for identification of dermatophyte species. Molecular tools provide a means of an epidemiological study. The phylogenetic classification of dermatophytes has been achieved using the G+C content of chromosomal DNA (3), total DNA homology (4), restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) (10,14), and the base sequence of the 18S (7) or 28S rRNA (11) or ribosomal DNA (rDNA) (12). However, for dermatophytes the phylogenic relationship or species-specific sequences cannot be defined by these methods, because the dermatophytes are phylogenetically and taxonomocally very closely related. The internal transcribed spacer 1 region (ITS1) is located between the 18S and 5.8S rDNAs. As reported previously, the variable ITS regions have proven useful in resolving relationships between close taxonomic relatives (1,2). Makimura *et al* (13) determined and phylogenetically analyzed successfully the specific DNA sequences of the ITS1 of the rDNA in the *Trichophyton mentagrophytes* complex strains.

In the present study, we have used ITS1 typing technique to analyze *Microsporum* spp strains from animals (dogs and cat) with dermatophytosis. We also demonstrated their phylogenetic relationship of *Microsporum* spp by base pair comparisons of ITS1 regions.

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Materials and Methods

Fungal strains

The 9 strains of *M canis* and 5 strains of *M gypseum* were isolated from dogs and a cat with dermatophytosis in Korea and were identified by their morphological features and biochemical tests. The reference strains of several species of *Microsporum* used for comparison were from the GenBank database (Table 1).

Preparation of DNA from fungal cells

All fungal strains were grown on Sabouraud dextrose agar (1% [wt/vol] peptone, 1% [wt/vol] glucose, 1.5% [wt/vol] agar) at 27 or 37°C for 1 to 5 days. Rapid preparation of DNA from fungi was performed by a modification of the described method (13). A small amount of mycelium grown on Sabouraud dextrose agar was placed in 100 μl of lysis buffer (200 mM Tris-HCl [pH 8.0], 0.5% [wt/vol] sodium dodecyl sulfate, 250 mM NaCl, 25 mM EDTA) and crushed with a

Table 1. Clinical isolates of *Microsporum* spp and reference strains used in this study

No.	Species	Strain	Origin	GenBank accession no.
1	*Microsporum canis*	K009	Dog in this study	-
2		K040	"	-
3		K077	"	-
4		K098	Cat in this study	-
5		K134	Dog in this study	-
6		K161	"	-
7		K174	"	-
8		K195	"	-
9		K217	"	-
10	*M audouinii*	CBS215.47	Germany	AJ252331
11	*Microsporum canis*	-	Human in Australia	AF168127
12		-	Germany	AJ222622
13		-	Human in UK	AM183275
14		ATCC23828	USA	AY213657
15		CBS545.93	Germany	AJ000623
16		IFM46053	Human in Japan	AB193637
17	*M distotum*	CBS101514	Japan	AB042607
18	*M equinum*	CBS556.80	Germany	AJ252330
19	*M ferrugineum*	CBS449.61	Japan	AB042605
20	*M langeronii*	CBS404.61	Germany	AJ000624
21	*M otae*	IFM40767	Japan	AB193612
22	*M rivalieri*	CBS409.51	Germany	AJ000625
23	*M gypseum*	K012	Dog in this study	-
24		K024	"	-
25		K069	"	-
26		K188	"	-
27		K199	"	-
28	*M fulvum*	CBS 287.55	Germany	AJ000627
29	*M gypseum*	-	Germany	AJ970150
30		CBS170.64	Australia	AF168128
31		CBS258.61	Germany	AJ970141
32		IFM40728	Human in Japan	AB193675
33		IFM 52633	Human in Japan	AB193708
34		VUT4004	Japan	AB049924
35		WM04.495	Australia	AJ853774
36	*M nanum*	CBS 322.61	Germany	AJ970149
37	*M persicolor*	-	Austria	DQ002902

cornical grinder. It was incubated at 100°C for 15 min, mixed with 150 μl of 3.0 M sodium acetate, kept at -20°C for 10 min, and then centrifuged at 10,000 ×g for 5 min. The supernatant was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and subsequently extracted once with chloroform. DNA was precipitated with equal volume of isopropanol at -20°C for 10 min, washed with 0.5 ml of 99% ethanol, dried, and suspended in 50 μl of ultrapure water (Milli-Q Synthesis A 10; Millipore, USA).

Polymerase chain reaction (PCR)

The PCR was carried out with a minor modification of previous protocol (13) to detect ITS1 region of dermatophytes. Oligonucleotide primers used for amplification of the ITS1 region were one foreword primer (18SF1, 5'-AGGTTTCCGTAGGTGAACCT-3') and the other reverse primer (58SR1, 5'-TTCGCTGCGTTCTTCATCGA-3'). Each PCR mixture contained 10 μl of 10× reaction buffer (Pharmacia, USA); 100 μm of each dATP, dCTP, dGTP, and dGTP (Pharmacia); 2.5U of Taq polymerase (Pharmacia); 30 pmol of each primer;

and DNA template solution. Ultrapure water was added to increase the volume to 100 μl. Each mixture was heated to 94°C for 5 min, and PCR was then performed under the following conditions: denaturation at 94°C for 1 min, annealing at 60°C for 30 s, extension at 72°C for 30 s for 35 cycles. Thermal cycling was terminated by polymerization at 72°C for 10 min. Two percent agarose gel electrophoresis was performed to examine the quality of the PCR products. These products were then stained with ethidium bromide and visualized by UV irradiation.

ITS1 DNA sequencing and phylogenetic analysis

PCR products were directly sequenced by using a DNA sequencing kit (Perkin-Elmer, USA) with primers 18SF1 and 58SR1 and an automatic sequencer (ABI Genetic Analyzer 310; Perkin-Elmer) according to the manufacturer's instructions. The ITS1 sequences were aligned by using the Clustal W computer program (DNAStar, USA). The phylogenetic tree was then constructed by using maximum parsimony analysis with 100 bootstrap replicates in a heuristic search with the PAUP 4.0 software program (Sinauer Associates Inc, USA).

Results

Dermatophytes, the 14 clinical isolates of *M canis* and *M gypseum*, were analyzed for their relationship. Amplication of 9 strains of *M canis* and 5 strains of *M gypseum* DNAs with ITS1 primers yielded fragment of about 300 and 225 bp, respectively (data not shown). Nucleotide sequence analysis of the ITS1 gene fragments from the 9 strains of *M canis* isolated in this study indicated the sequence similarities of 100% (Table 2). And a high degree of nucleotide sequence similarity, more than 99%, was noted between the ITS1 gene fragments of *M audouinii*, *M canis*, *M equinum*, *M ferrugineum*, *M langeronii* and *M rivalieri*. The 5 strains of *M gypseum* isolated in this study had the 100% nucleotide sequence similarities (Table 3). A phylogenetic tree was constructed from 9 strains of *M canis* and 5 strains of *M gypseum* with the reference strains (Fig 1). The phylogenetic analysis of the nucleotide sequences of ITS1 gene fragments of the 9 strains of *M canis* formed a cluster with the reference *M canis* strains originating from USA, Australia, Japan, and Europe. However, *M distortum*, *M equinum*, and *M ferrugineum* were genetically very close to *M canis* (Fig 1). Differentiation between *M equinum* and *M canis* by nucleotide sequence was not possible because their nucleotide sequences were identical (Table 2). *M gypseum* formed a cluster in the phylogenetic tree with *M canis* as an outgroup (Fig 1). The molecular analysis of ITS1 genes provided the useful information for the identification of these *Microsporum* spp and the understanding of their relationship.

Discussion

The animal-associated dermatophytes encompass a number of organisms with a very recent phylogenetic history. Frequently, individual clusters are discernable which contain mutually closely related, zoophilic next to anthropophilic species (9). Most of the entities are morphologically slightly different from each other (8). The clusters are hypothesized to encompass an ancestral, sexually reproducing species, plus a number of derives, clonal, strictly asexual lineages that have host-specificities different from that of the ancestral species (16). Molecular and polyphasic approaches to species recognition, being based on average molecular distances between defined DNA regions and averaged combinations of dissimilar data sets, respectively, have been applied (6). Most of the phenotypically described taxa were thus confirmed, as they proved to be supported by reproducible distances in ITS rDNA and in fingerprint differences.

Using ITS1 rDNA sequences from 14 strains of dermatophytes, we demonstrated the phylogeny of members of the *Microsporum* spp. The phylogenetic relationship based on the alignment of the ITS1 DNA sequences of perfect and imperfect states of the *M canis* agreed with the proposed taxomonic connection in its sexual compatibility and RFLP analysis of mtDNA (17). The group consists of a cluster of phylogenetically closely related anamorphs; the zoophilic taxon *M canis, M distortum* and *M equinum* and the anthrophilic taxa *M ferrugineum.* The phylogenetic analysis of the nucleotide sequences of ITS1 gene fragments of *Microsporum* spp were genetically distinct from one another, but *M distortum, M equinum,* and *M ferrugineum* were genetically very close to *M canis* (Fig 1). The results obtained in this study seem to be similar to those in a previous phylogenetic study with microsatellite loci to suggest that *M canis, M distortum*, and *M equinum* are genetically closely related (9). Therefore, *M equinum* can be considered to be very close or identical to *M canis* from various genetic analysis. This suggests that these medically important dermatophytes have been overclassified. However, since each of the "species" has a unique phenotype, pathogenicity, and host specific affinity, it is reasonable to retain their "species" identification in other to identify the pathogen.

M gypseum was distantly related to the other *Microsporum* species. The PCR fingerprinting analysis using a single primer also showed that *M gypseum* strains form a cluster distant from the other species (5). Therefore, all of the genetic analyses including those obtained in this study suggest similar relationships of *M gypseum* among the *Microsporum* spp.

We demonstrated the applicability of ITS1 DNA sequences to phylogenetic analysis of closely related strains of the *Microsporum* spp. This ITS1-based system will prepare the way for the complicated phylogenetic classification of dermatophytes and other fungi. And the ITS1-based identification system saves time (it takes 2 to 3 days) and is accurate and applicable even to strains with atypical morphological features (13). The classical method of species identification takes at least several weeks and then is sometimes unsuccessful. The method described in the present study would allow veterinar-

Table 2. Comparison of nucleotide and deduced amino acid sequences of ITS1 gene of *Microsporum canis*. Fungi source was referred to serial numbers of Table 1

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	K009 (*M canis*, Korea)	***	100[a]	100	100	100	100	100	100	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
2	K040 (*M canis*, Korea)	0.0[b]	***	100	100	100	100	100	100	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
3	K077 (*M canis*, Korea)	0.0	0.0	***	100	100	100	100	100	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
4	K098 (*M canis*, Korea)	0.0	0.0	0.0	***	100	100	100	100	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
5	K134 (*M canis*, Korea)	0.0	0.0	0.0	0.0	***	100	100	100	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
6	K161 (*M canis*, Korea)	0.0	0.0	0.0	0.0	0.0	***	100	100	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
7	K174 (*M canis*, Korea)	0.0	0.0	0.0	0.0	0.0	0.0	***	100	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
8	K195 (*M canis*, Korea)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	***	100	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
9	K217 (*M canis*, Korea)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	***	99.3	100	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
10	*M audouinii* (AJ252331, Germany)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	***	99.3	99.3	99.3	99.3	97.9	99.3	97.6	99.3	99.0	99.7	98.6	99.7
11	*M canis* (AF168127, Australia)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	***	100	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
12	*M canis* (AJ222622, Germany)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	***	100	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
13	*M canis* (AM183275, UK)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	***	100	97.9	100	98.3	100	99.7	99.7	98.6	99.7
14	*M canis* (AY213657, USA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	***	97.9	100	98.3	100	99.7	99.7	98.6	99.7
15	*M canis* (AJ000623, Germany)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.3	0.3	0.3	0.3	***	97.9	96.2	97.9	97.6	98.3	97.2	98.3
16	*M canis* (AB193637, Japan)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.3	***	98.3	100	99.7	99.7	98.6	99.7
17	*M distortum* (AB042607, Japan)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.3	0.0	***	98.3	97.9	97.9	96.9	97.9
18	*M equinum* (AJ252330, Germany)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.3	0.0	0.0	***	99.7	99.7	98.6	99.7
19	*M ferrugineum* (AB042605, Japan)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.7	0.3	0.3	0.3	0.3	0.7	0.3	0.3	0.3	***	99.3	98.3	99.3
20	*M langeronii* (AJ000624, Germany	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.3	0.3	0.3	0.3	0.0	0.3	0.3	0.3	0.7	***	99.0	100
21	*M otae* (AB193612, Japan)	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.0	1.4	1.4	1.4	1.4	1.0	1.4	1.4	1.4	1.8	1.0	***	99.0
22	*M rivalieri* (AJ000625, Germany)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.3	0.3	0.3	0.3	0.0	0.3	0.3	0.3	0.7	0.0	1.0	***

[a] Percentage of nucleotide similarity is indicated in upper right triangle.
[b] Percentage of nucleotide dissimilarity is indicated in upper right triangle.

Table 3. Comparison of nucleotide and deduced amino acid sequences of ITS1 gene of *Microsporum gypseum*. Fungi source was referred to serial numbers of Table 1

		23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
23	K012 (*M gypseum*, Korea)	***	100[a]	100	100	100	82.8	98.2	97.8	100	96.9	100	100	100	53.6	83.9
24	K024 (*M gypseum*, Korea)	0.0[b]	***	100	100	100	82.8	98.2	97.8	100	96.9	100	100	100	53.6	83.9
25	K069 (*M gypseum*, Korea)	0.0	0.0	***	100	100	82.3	97.8	97.8	100	96.3	100	100	100	53.4	83.9
26	K188 (*M gypseum*, Korea)	0.0	0.0	0.0	***	100	82.8	98.2	97.8	100	96.9	100	100	100	53.6	83.9
27	K199 (*M gypseum*, Korea)	0.0	0.0	0.0	0.0	***	82.8	98.2	97.8	100	96.9	100	100	100	53.6	83.9
28	*M fulvum* (AJ000627, Germany)	8.3	8.3	8.3	8.3	8.3	***	82.8	80.9	82.8	74.8	83.7	83.7	82.8	56.9	77.0
29	*M gypseum* (AJ970150, Germany)	1.8	1.8	1.8	1.8	1.8	7.8	***	96.0	98.2	94.5	98.2	98.2	98.2	54.3	83.9
30	*M gypseum* (AF168128 , Australia)	0.0	0.0	0.0	0.0	0.0	8.3	1.8	***	97.8	93.9	97.3	97.3	97.8	52.0	82.2
31	*M gypseum* (AJ970141, Germany)	0.0	0.0	0.0	0.0	0.0	8.3	1.8	0.0	***	96.9	100	100	100	53.6	83.9
32	*M gypseum* (AB193675, Japan)	0.0	0.0	0.0	0.0	0.0	8.9	2.5	0.0	0.0	***	96.9	96.9	96.9	60.1	77.3
33	*M gypseum* (AB193708, Japan)	0.0	0.0	0.0	0.0	0.0	8.2	1.8	0.0	0.0	0.0	***	100	100	62.3	87.0
34	*M gypseum* (AB049924, Japan)	0.0	0.0	0.0	0.0	0.0	8.2	1.8	0.0	0.0	0.0	0.0	***	100	53.5	84.1
35	*M gypseum* (AJ853774, Australia)	0.0	0.0	0.0	0.0	0.0	8.3	1.8	0.0	0.0	0.0	0.0	0.0	***	53.6	83.9
36	*M nanum* (AJ970149, Germany)	11.1	11.1	11.2	11.1	11.1	10.5	10.5	11.0	11.1	3.9	8.8	11.7	11.1	***	80.4
37	*M persicolor* (DQ002902, Austria)	13.3	13.3	13.4	13.3	13.3	12.0	12.3	13.3	13.3	16.0	10.6	13.2	13.3	6.5	***

[a] Percentage of nucleotide similarity is indicated in upper right triangle.
[b] Percentage of nucleotide dissimilarity is indicated in upper right triangle.

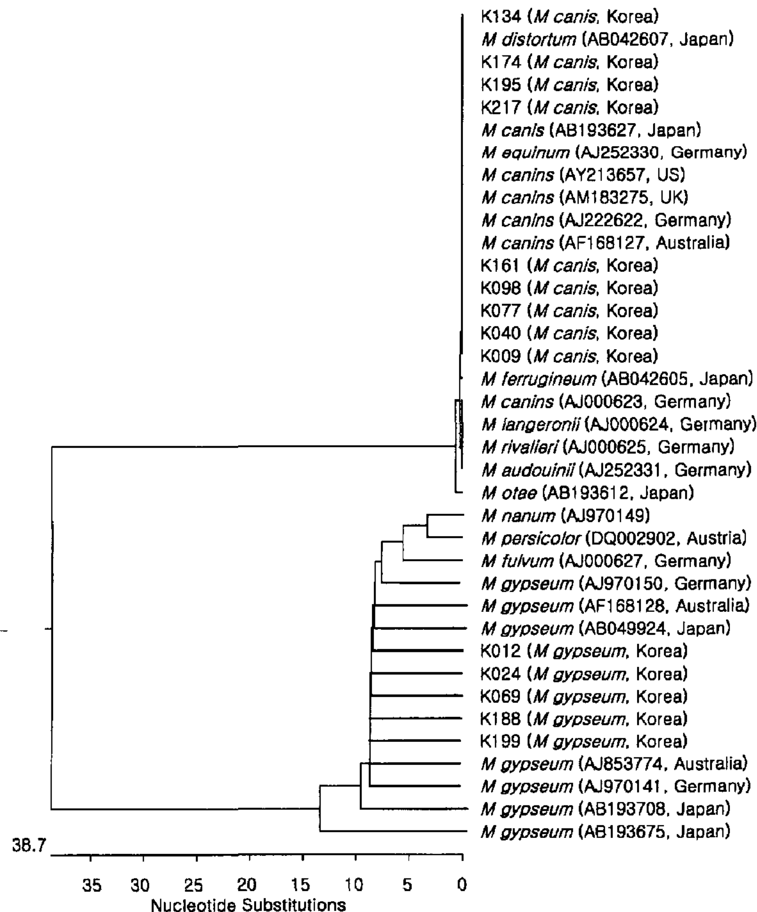

Fig 1. Phylogenetic relationship of the *Microsporum* spp based on ITS1 rDNA sequences. Phylogenetic tree was generated by the maximum parsimony methods in a heuristic search with 100 bootstrap replicates. The length of each branch represents the numbers of nucleotide differences between sequences. The tree was constructed with data for reference strains of *Microsporum* spp (see Table 1).

ians to determine the involved pathogen and to advise the owner of the proper way to control against infection by the fungus, epidemiologically or etiologically.

Acknowledgement

This study was supported by the Research Grant from Kangwon National University

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개와 고양이 유래 피부사상균의 분자생물학적 계통 분석

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요 약 : 피부사상균증이 있는 개와 고양이에서 분리한 9주의 *Microsporum canis*와 5주의 *Microsporum gypseum*에서 ribosomal DNA를 추출하여 internal transcribed spacer 1 (ITS1) gene을 PCR로 증폭한 후 sequencing을 실시하여, 각 사상균의 계통학적 관계를 조사하였다. *M canis* 분리주 9주의 ITS1 gene의 nucleotide sequence는 100% 일치하였으며 *M gypseum* 분리주 5주의 nucleotide sequence도 100% 일치하였다. *M canis* 분리주 9주의 계통분석 결과 미국, 일본, 호주 및 유럽에서 분리된 *M canis*와 같은 cluster에 속하였으며 다른 *Microsporum* spp와는 유전적으로 다른 cluster를 형성하였다. 그러나 *M canis*와 *M distortum, M equinum, M ferrugineum*은 유전적으로 매우 가까운 위치에 있었다. *M gypseum* 분리주는 *M canis*와는 다른 cluster를 형성하였다. ITS1 gene의 분자생물학적 분석은 *Microsporum* spp를 확인하고 그들의 유전학적 관계를 이해하는 유용한 정보를 제공하는 것으로 생각된다.

주요어 : *Microsporum* spp, ITS1, 계통분석, 개와 고양이.