Kafkas Univ Vet Fak Derg
25 (5): 639-643, 2019
DOI: 10.9775/kvfd.2018.21506

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Comparison of Internal Transcribed Spacer Region Sequencing and Conventional Methods Used in the Identification of Fungi Isolated from Domestic Animals

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Article Code: KVFD-2018-21506 Received: 10.12.2018 Accepted: 07.04.2019 Published Online: 07.04.2019

How to Cite This Article

Müştak İB, Sarıçam S, Müştak HK: Comparison of internal transcribed spacer region sequencing and conventional methods used in the identification of fungi isolated from domestic animals. *Kafkas Univ Vet Fak Derg,* 25 (5): 639-643, 2019. DOI: 10.9775/kvfd.2018.21506

Abstract

The aim of this study was to compare gold standard conventional culture method and internal transcribed spacer (ITS) sequence-based analysis in the identification of fungi isolated from domestic animals. A total of 35 animals (15 cats, 11 dogs, 4 horses and 5 chickens) suspected for fungal infection were examined. Of the 35 samples, 20 were found to be positive for fungal culture. Among positive samples 8 (40%) were predominantly found to be dermatophyte species by conventional methods. The ITS regions (ITS1, ITS2 and complete ITS) of fungal isolates were also amplified, sequenced and the results were compared with conventional culture method. The identification results of 18 (90%) fungal species were found to be compatible with both conventional culture and sequencing methods. Comparison of the results demonstrated that, complete ITS regions gene sequencing could be used for the identification of medically important fungi rapidly. Since the results of complete ITS regions gene sequencing were found to be compatible with the results of phenotypic identification, it can be concluded that ITS regions gene sequencing of fungal isolates can be also used as a confirmative tool of conventional culture methods.

Keywords: *Dermatophyte, Fungi, ITS sequencing*

Evcil Hayvanlardan İzole Edilen Mantarların Teşhisinde Kullanılan Konvansiyonel ve ITS Dizi Analizi Metotlarının Karşılaştırılması

Öz

Bu çalışmanın amacı, evcil hayvanlardan izole edilen mantar türlerinin identifikasyonunda altın standart olan konvansiyonel kültür yöntemi ve Internal Ara Bölgeler (ITS) dizi analizi yönteminin karşılaştırılmasıdır. Çalışmada, mantar enfeksiyonlarından şüphelenilen toplam 35 hayvan (15 kedi, 11 köpek, 4 at ve 5 tavuk) incelendi. 35 örnek içinde 20 tanesinin mantar kültürleri pozitif bulundu. Pozitif örnekler arasında 8 (%40) tanesi konvansiyonel yöntemlerle ağırlıklı olarak dermatofit türleri olarak bulundu. İzolatların ITS bölgeleri (ITS1, ITS2 ve ITS bölgesinin tamamı) amplifiye edildi, sekanslandı ve elde edilen sonuçlar kültür yöntemiyle karşılaştırıldı. Hem konvansiyonel hem de dizi analizi yöntemiyle elde edilen identifikasyon sonuçları 18 (%90) örnekte birbiri ile uyumlu bulundu. Bu sonuçlar doğrultusunda, ITS bölgesinin tamamının dizi analizi, mantarların hızlı teşhisi için kullanılabileceğini ortaya koydu. ITS gen bölgesinin tamamına ait dizi analizi sonuçlarının fenotipik identifikasyon sonuçları ile uyumlu olması, ITS gen bölgesi dizi analizinin konvansiyonel yöntemlerin doğrulanmasında bir araç olarak kullanılabileceğini ortaya koymuştur.

Anahtar sözcükler: *Dermatofit, Mantar, ITS dizileme*

INTRODUCTION

The fungi are saprophytic eugenic organisms that can easily spread in the environment [1]. Fungal infections, which have increased over the last three decades, cause severe health problems, from superficial infections to nail and skin, mucocutaneous candidiasis to invasive infections, sometimes with high mortality in humans and animals. High mortality is not only associated with impaired organ function, chronic lung diseases, neurological disorders, blindness, or impaired vision but also associated with late diagnosis of the causative agents [2].

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It is known that only 300 of 100.000 fungal species cause infection in humans or animals. Some fungal species (e.g. *Candida* sp.) can survive both in humans and animals; some types of fungi, such as dermatophytes are both zoonotic and highly contagious [3].

For the treatment and control of fungal diseases, faster and more accurate identification is very important [2]. Accurate identification of fungi is also important for the evaluation of biodiversity, taxonomy and species identification. Conventional identification by morphology and biochemical tests is time consuming and also requires experienced laboratory personnel. Therefore, DNA barcoding, RNA polymerase I and II, translational elongation factor 1-α, β-tubulin and internal transcribed region (ITS) are frequently used for the molecular identification and phylogenetic analysis of fungi [2].

In fungi, the ribosomal RNA operon covers the 5.8S, 18S, 28S ribosomal subunit genes and ITS1, ITS2 un-transcribed regions [1]. For various species of fungi, ITS regions are accepted as standard barcoding site since there is genetic diversity among fungal species [2]. Polymerase chain reaction based methods used to amplify the ITS regions by universal primers and sequence analysis of these regions are currently used for the identification of fungi [4].

The aim of this study is to compare the ITS region (ITS1, ITS2 and complete ITS) sequencing analysis and conventional fungal culture method in the identification of fungi obtained from clinical samples of domestic animals.

MATERIAL and METHODS

Material and Media Used

Skin scraping, swap and hair samples obtained from clinically fungal infection suspected domestic animals (15 cats, 11 dogs, 4 horses and 5 chickens) at different ages and sexes were used as material. After direct microscopic examination with 15% KOH, isolation of fungi was performed by conventional culture method using Saboraud Dextrose Agar (SDA) (Oxoid, USA). All samples were inoculated on SDA and incubated aerobically at 25°C for 7-14 days [5,6].

Identification of Fungi

Slides were prepared from fungal colonies and stained with lacto phenol cotton blue (LFPM) (Merck, Germany). Fungal colonies were identified macroscopically according to the colony characteristics (colony diameter, color, surface appearance, pigment formation etc.) and microscopically according to the conidium, hyphae and spore structures [5,6]. The isolates identified as dermatophytes were also cultured on dermatophyte agar (Becton Dickinson, USA) and Trichophyton agar (Thermo Scientific, USA) to confirm the identification. Tests were performed within two weeks of the initial isolation. Twenty fungal strains identified were cultured onto SDA to be used in molecular analysis.

Standard Strains

Microsporum canis AMF-12, *Trichophyton rubrum* AMF-19 and *Aspergillus niger* AMF-8 strains obtained from the Ankara University Faculty of Veterinary Medicine, Department of Microbiology culture collection were used as reference positive controls in the study.

DNA Extraction

Rapid DNA extraction from colonies on SDA was performed according to the method of Liu et al.[7]. The concentration of the DNA extracts was measured by spectrophotometer (Thermo Scientific NanoDrop 1000). The obtained DNA samples were stored at -20°C until molecular analysis.

Amplification of ITS Regions

The PCR assay was performed in a total 25 mL reaction volume containing 2 mL of template DNA, 0.2 mM dNTPs (10 mM dNTP mix, Thermo Scientific USA), 3 mM of $MgCl_2$ (Thermo Scientific, USA), 2.5 mL PCR reaction buffer, 2U Taq DNA Polymerase (Thermo Scientific, USA) and 0.2 mM of each primer. Thermal cycle conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C, 58°C and 58°C for 30 s (ITS1, ITS2 and complete ITS region respectively) and 72°C for 1 min. Finally, there was 7 min at 72°C for final extension [8].

Sequencing of ITS Regions

The ITS1, ITS2 and complete ITS region were sequenced with the ABI 3500 Genetic Analyzer device using the BigDye Terminator v3.1 Cycle Sequencing Kit with primers that were shown in *Table 1* [8]. Analysis of the sequences was performed using the CLC Main Workbench 7.7.3 (Qiagen) and the obtained data were compared with fungal sequences in NCBI GenBank. The decisions about the fungal genus and species name were given according to the similarity scores obtained from the NCBI GenBank by BLAST analysis [9]. The ITS regions of rRNA gene sequences were also deposited in GenBank as shown in *Table 2*.

RESULTS

A total of 20 samples among 35 samples were found to be positive for the existence of fungi by direct examination and mycological culture. Sequence-based identification of 18 strains among 20 positive isolates were found to be compatible with conventional phenotypic methods.

Based on the structure of conidium, fungal hyphae, spore, mycelium and mycological cultures on media of the fungal colonies, twelve non-dermatophytes species *[A. niger* (n=2), *A. flavus* (n=1), *A. tubingensis* (n=1), *Penicillium expansum* (n=1), *P. chrysogenum* (n=1), *P. dipodomyicola* (n=1),

Table 1. *Primers used for ITS1, ITS1-2 and ITS1-5.8S-ITS2 gene regions amplification*

Name	Primer Sequences (5'-3')	Amplification Region	Amplicon Size
1F 1R	TCCGTAGGTGAACCTGCGG GCTGCGTTCTTCATCGATGC	ITS1	150-250 bp
2F 2R	GCATCGATGAAGAACGCAC TCCTCCGCTTATTGATATGC	ITS2	300-350 bp
1F 2R	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Complete ITS (ITS1-5.8S-ITS2)	380-650 bp

Table 2. *Conventional culture and ITS region sequence-based identification results of fungi*

Strain	Accession	Culture Results	ITS1 Region	ITS2 Region	Complete ITS
AVMF1	MK461906	*A. flavus*	*Aspergillus* sp.	*Aspergillus* sp.	*A. flavus*
AVMF2	MK461907	*A. niger*	*A. niger*	*Aspergillus* sp.	*A. niger*
AVMF3	MK461908	*A. alternata*	*Alternaria* sp.	*Alternaria* sp.	*Alternaria* sp.
AVMF4	MK461909	*A. alternata*	*Alternaria* sp.	*Alternaria* sp.	*Alternaria* sp.
AVMF5	MK461910	*A. niger*	*A. niger*	*Aspergillus* sp.	*A. niger*
AVMF6	MK461911	*A. tubingensis*	*Aspergillus* sp.	*Aspergillus* sp.	*A. tubingensis*
AVMF7	MK461912	*M. nanum*	*M. nanum*	*M. nanum*	*M. nanum*
AVMF8	MK461913	*T. verrucosum*	*T. verrucosum*	*T. verrucosum*	*T. verrucosum*
AVMF9	MK461914	*S. schenckii*	*S. schenckii*	*Sporothrix* sp.	*S.schenckii*
AVMF10	MK461915	*T. tonsurans*	*T. tonsurans*	*Trichophyton* sp.	*T. tonsurans*
AVMF11	MK461916	*T. rubrum*	*T. rubrum*	*T. rubrum*	*T. rubrum*
AVMF12	MK461917	*M. canis*	*Microporum* sp. *A. otae*	*Microporum* sp. *A. otae*	*M. canis* *A. otae*
AVMF13	MK461918	*T. rubrum*	*Trichophyton* sp.	*T. rubrum*	*T. rubrum*
AVMF14	MK461919	*T. schoenleinii*	*T. schoenleinii*	*T. schoenleinii*	*T. schoenleinii*
AVMF15	MK461920	*T. mentagrophytes*	*Trichophyton* sp.	*Trichophyton* sp.	*T. mentagrophytes* *A. vanbreuseghemii*
AVMF16	MK461921	*P. dipodomyicola*	*Penicillium* sp.	*Penicillium* sp.	*P. dipodomyicola*
AVMF17	MK461922	*M. hiemalis*	*Mucor* sp.	*M. hiemalis*	*M. hiemalis*
AVMF18	MK461923	*G. candidum*	*Galactomyces candidum*	*Galactomyces candidum*	*G. candidum* *Galactomyces candidum*
AVMF19	MK461924	*P. expansum*	*Penicillium* sp.	*Penicillium* sp.	*P. expansum*
AVMF20	MK461925	*P. chrysogenum*	*Penicillium* sp.	*Penicillium* sp.	*P. chrysogenum*

Sporothrix schenckii (n=1), *Geotrichum candidum* (n=1) and *Alternaria alternata* (n=2)] and eight dermatophytes species *[M. nanum* (n=1), *T. tonsurans* (n=1), *T. rubrum* (n=2), *T. schoenleinii* (n=1), *T. mentagrophytes* (n=1), *T. verrucosum* (n=1) and *M. canis* (n=2)] were identified.

Four *Aspergillus* species identified phenotypically as *A. niger* (n=2), *A. flavus* (n=1) and *A. tubingensis* (n=1) were also identified to the species level by complete ITS regions sequencing.

Eight dermatophytes conventionally identified as *M. nanum* (n=1), *T. tonsurans* (n=1), *T. rubrum* (n=2), *T. schoenleinii* (n=1), *T. mentagrophytes* (n=1), *T. verrucosum* (n=1) were also identified by complete ITS sequencing except for *M. canis* (n=1) which was identified as *Arthroderma otae* by sequencing.

The non-dermatophyte fungi phenotypically identified as *P. expansum* (n=1), *P. chrysogenum* (n=1), *P. dipodomyicola* (n=1), *S. schenckii* (n=1) and *G. candidum* (n=1) were also identified by complete ITS sequencing. Two *A. alternata* were identified to the species level by conventional methods. However, their sequence-based identification could not be determined at the species level. *Mucor hiemalis* (n=1) which was identified to the species level phenotypically, had sequence-based identification of *Mucor hiemalis* with 99% homology. The results of conventional culture and sequence analysis results of the ITS regions were shown in *Table 2*.

DISCUSSION

Frequently used conventional methods in fungal identification are based on the colony morphology, microscopic examination and several biochemical tests which are also time consuming and subjective. In some cases, there is also

a need for experienced personnel to enable identification to the species level [10].

In order to obtain reliable and faster results, methods based on sequence analysis of ITS regions have been developed and used in routine laboratories [11]. ITS region is accepted and frequently used as a genetic marker for the identification of fungi [12,13]. It is known that ITS regions 1 and 2 are more variable than the other subunit genes among fungi [14,15]. Thus, sequencing of ITS gene regions was chosen for the molecular identification of fungal strains in this study. In regard to this information in this study ITS1, ITS2 and complete ITS region sequencing was used to differentiate the fungi identified by conventional culture methods. Fungal identification results of 6 (30%) samples by ITS1 and ITS2 gene sequencing were found to be compatible with each other and the conventional culture results to the species level. On the other hand, fungal identification results of all samples (100%) by complete ITS region gene sequencing were found to be compatible with conventional culture results.

Molecular techniques were used in several studies for the identification of *Aspergillus* species obtained from environmental and clinical samples [16,17]. In a study, Henry et al.[18] investigated *Aspergillus* species by ITS region sequence analysis and compared the obtained sequences with GenBank sequences using BLAST. They concluded that both ITS1 and ITS2 regions were necessary for the identification of *Aspergillus* isolates to the species level [18]. In another study, Pryce et al.[11] showed that clinical *Aspergillus* isolates had compatible ITS1 and ITS2 sequence results with conventional culture methods. The results of our study found to be compatible with the study of Henry et al.[18] since *A. tubingensis* (n=1), *A. flavus* (n=1) and *A. niger* (n=2) strains were correctly identified to the species level by complete ITS region sequence analysis in comparison to the conventional culture results. It can be said that both ITS regions have to be used for the appropriate identification of the *Aspergillus* sp. because the sequences obtained from both regions exhibited different results. Further studies by increasing the number of samples can demonstrate the discriminative power of ITS1 and ITS2 gene regions for *Aspergillus* species.

CLSI recommends the ITS region sequencing in the identification of *Alternaria* sp. and also several reports supported this statement by using ITS region in the phylogenetic analyses of *Alternaria* species [19-21]. However, it is also stated in the CLSI guideline MM18-A, some *Alternaria* species have nearly identical ITS sequences. In compatible with the CLSI, the results of *Alternaria* ITS sequences exhibited similar BLAST scores with more than one *Alternaria* sp. Thus, in this study ITS sequence-based identification of *Alternaria* strains (n=2) which were identified phenotypically to the species level, was evaluated to the genus level.

Generally common dermatophytes can be identified especially by ITS1 region sequencing [9]. CLSI stated that for the differentiation of *T. rubrum* complex, the sequence analysis of the ITS regions is sufficient to identify only *T. rubrum* and *T. violaceum* species. However, the sequence analysis of rDNA 28S D2 region is required for the other species in the genus [9]. In this study, all dermatophyte isolates except for *M. canis* had compatible conventional and sequence-based identification result. *M. canis* which was identified conventionally, was found as *A. otae* by sequencing. It is known that dermatophytes may have two species names according to their sexual (teleomorph state) and asexual (anamorph state) forms. When *M. canis* find a compatible environment, it changes into sexual form which is called *A. otae* [22]. Similarly, the teleomorph state of *T. mentagrophytes* organism is called *A. vanbreuseghemii*. These results did not change the decision about the results of both complete ITS sequence-based and conventional culture for their compatibility in the identification of common dermatophytes.

Pryce et al.[11] identified a *G. candidum* strain (n=1) to the species level by sequencing of complete ITS region. However, in this study, *G. candidum* strain was identified with 100% homology as *Galactomyces candidum* to the species level by complete ITS, ITS1 and ITS2 regions. As in dermatophytes, *G. candidum* has a synonym name as *Galactomyces candidum* which is the anamorph state of this organism.

Kwiatkowski et al.[23] identified five *Penicillium* species except for *P. marneffei* to the species level by both complete ITS and D1/D2 region sequencing. It is also stated in CLSI guidelines only for the identification of *P. marneffei* alternative DNA targets must be used [9]. In this study for the identification of *Penicillium* genus, while the sequence analysis of ITS1 and ITS2 gene regions were not sufficient, complete ITS region sequencing was found to be discriminative for this genus.

It can be concluded that, the results of conventional culture and complete ITS region sequencing of clinical fungal isolates were found to be compatible. Also in this study it was shown that sequence-based identification is a rapid, accurate and reliable method and may be used for the confirmation of the results obtained from the conventional culture methods which is based on subjective observation in fungal identification.

REFERENCES

1. Xu J: Fungal DNA barcoding. *Genome*, 59, 913-932, 2016. DOI: 10.1139/gen-2016-0046

2. Irinyi L, Serena C, Garcia-Hermoso D, Arabatzis M, Desnos-Ollivier M, Vu D, Cardinali G, Arthur I, Normand AC, Giraldo A, da Cunha KC, Sandoval-Denis M, Hendrickx M, Nishikaku AS, Melo ASD, Merseguel KB, Khan A, Parente Rocha JA, Sampaio P, Briones MRD, Ferreira RCE, Muniz MD, Castanon-Olivares LR, Estrada-Barcenas D, Cassagne C, Mary C, Duan SY, Kong FR, Sun AY, Zeng XY, Zhao ZT, Gantois N,

Botterel F, Robbertse B, Schoch C, Gams W, Ellis D, Halliday C, Chen S, Sorrell TC, Piarroux R, Colombo AL, Pais C, de Hoog S, Zancope-Oliveira RM, Taylor ML, Toriello C, Soares CMD, Delhaes L, Stubbe D, Dromer F, Ranque S, Guarro J, Cano-Lira JF, Robert V, Velegraki A, Meyer W: International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database-the quality controlled standard tool for routine identification of human and animal pathogenic fungi. *Med Mycol,* 53 (4): 313-337, 2015. DOI: 10.1093/mmy/myv008

3. Irınyi L, Lackner M, De Hoog GS, Meyer W: DNA barcoding of fungi causing infections in humans and animals. *Fungal Biol*, 120 (2): 125-136, 2016. DOI: 10.1016/j.funbio.2015.04.007

4. Kiraz N, Oz Y, Aslan H, Erturan Z, Ener B, Akdagli SA, Muslumanoglu H, Cetinkaya Z: Is the extraction by Whatman FTA filter matrix technology and sequencing of large ribosomal subunit D1-D2 region sufficient for identification of clinical fungi? *Mycoses,* 58 (10): 588-597, 2015. DOI: 10.1111/myc.12365

5. Winn WC JR, Allen SD, Janda WM, Koneman E, Procop G, Schreckenberger P, Woods G: Laboratory approach to the diagnosis of fungal infections. **In,** Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed., 1156-1166, Lippincott Williams, Philadelphia, 2006.

6. Winn WC Jr AS, Janda WM, Koneman E, Procop G, Schreckenberger P, Woods G: Hyaline molds and hyalohyphomycosis. **In,** Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed., 1172-1187, Lippincott Williams, Philadelphia, 2006.

7. Liu D, Coloe S, Baird R, Pedersen J: Rapid mini-preparation of fungal DNA for PCR. *J Clin Microbiol*, 38(1): 471, 2000.

8. Kumar M, Shukla PK: Use of PCR targeting of internal transcribed spacer regions and single-stranded conformation polymorphism analysis of sequence variation in different regions of rRNA genes in fungi for rapid diagnosis of mycotic keratitis. *J Clin Microbiol,* 43 (2): 662-668, 2005. DOI: 10.1128/Jcm.43.2.662-668.2005

9. CLSI: Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline. **In,** Institute CaLS, (Ed): CLSI document MM18-A. Wayne, PA ABD 2008.

10. Raja HA, Miller AN, Pearce CJ, Oberlies NH: Fungal identification using molecular tools: A primer for the natural products research community. *JNadProd,* 80, 756-770, 2017. DOI: 10.1021/acs.jnatprod.6b01085

11. Pryce TM, Palladino S, Kay ID, Coombs GW: Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. *Med Mycol,* 41 (5): 369-381, 2003. DOI: 10.1080/13693780310001600435

12. Sulaiman IM, Jacobs E, Simpson S, Kerdahi K: Molecular identification of isolated fungi from unopened containers of greek yogurt by DNA sequencing of internal transcribed spacer region. *Pathogens,* 3 (3): 499-509, 2014. DOI: 10.3390/pathogens3030499

13. Wagner K, Springer B, Pires VP, Keller PM: Molecular detection of fungal pathogens in clinical specimens by 18S rDNA high-throughput screening in comparison to ITS PCR and culture. *Sci Rep,* 8:6964, 2018. DOI: 10.1038/s41598-018-25129-w

14. Ninet B, Jan I, Bontems O, Lechenne B, Jousson O, Panizzon R, Lew D, Monod M: Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. *J Clin Microbiol,* 41 (2): 826-830, 2003. DOI: 10.1128/Jcm.41.2.826-830.2003

15. Bialek R, Ibricevic A, Fothergill A, Begerow D: Small subunit ribosomal DNA sequence shows *Paracoccidioides brasiliensis* closely related to *Blastomyces dermatitidis*. *J Clin Microbiol,* 38 (9): 3190-3193, 2000.

16. Bretagne S, Costa JM, Marmoratkhuong A, Poron F, Cordonnier C, Vidaud M, Fleuryfeith J: Detection of aspergillus species DNA in bronchoalveolar lavage samples by competitive PCR. *J Clin Microbiol,* 33 (5): 1164-1168, 1995.

17. Einsele H, Hebart H, Roller G, Loffler J, Rothenhofer I, Muller CA, Bowden RA, vanBurik JA, Engelhard D, Kanz L, Schumacher U: Detection and identification of fungal pathogens in blood by using molecular probes. *J Clin Microbiol,* 35 (6): 1353-1360, 1997.

18. Henry T, Iwen PC, Hinrichs SH: Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J Clin Microbiol,* 38 (4): 1510-1515, 2000.

19. Chou HH, Wu WS: Phylogenetic analysis of internal transcribed spacer regions of the genus Alternaria, and the significance of filament-beaked conidia. *Mycol Res,* 106, 164-169, 2002. DOI: 10.1017/S0953756201005317

20. Kusaba M, Tsuge T: Phylogeny of Alternaria fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. *Curr Genet,* 28 (5): 491-498, 1995. DOI: 10.1007/Bf00310821

21. Vujanovic V, Labrecque M: Potentially pathogenic and biocontrol Ascomycota associated with green wall structures of basket willow *(Salix viminalis* L.) revealed by phenotypic characters and ITS phylogeny. *Biocontrol,* 53 (2): 413-426, 2008. DOI: 10.1007/s10526-007-9092-2

22. Spicker AR: Dermatophytosis. http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php; 2013. *Accessed:* 05.12.2018

23. Kwiatkowski NP, Babiker WM, Merz WG, Carroll KC, Zhang SX: Evaluation of nucleic acid sequencing of the D1/D2 region of the large subunit of the 28S rDNA and the internal transcribed spacer region using SmartGenes IDNS Software for identification of filamentous fungi in a clinical laboratory. *J Mol Diagn*, 14 (4): 393-401, 2012. DOI: 10.1016/j.jmoldx.2012.02.004