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Identification of Dermatomycoses Pathogens by Multilocus Sequence Typing Method

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Identification of dermatomycetes with the use of traditional methods is often problematic. This is due to microscopic or macroscopic atypical morphological features that occur when cultured on nutrient media. The purpose of this study was to evaluate the possibility of using multilocus sequence typing using primers ITS region (ITS4/ITS5) and SSU region (NS1/NS4), RPB1, RPB2 to identify dermatomycoses pathogens. Regions of DNA dermatomycetes were amplified by PCR and it was sequenced. Nucleotide sequences were analyzed and combined in a common sequence which was identified in the GeneBank by BLAST algorithm. According to fungi identification except classical dermatomycoses pathogens, they are mold and yeast.

Key words: Multilocus sequence typing, Dermatomycetes, Identification, Primers, ITS region.

Dermatomycetes are keratinophilic fungi which are capable to cause animals' and humans' dermatomycoses. Fungi of Epidermophyton, Microsporum and Trichophyton genera belong to dermatomycetes[1].

Clinical signs, culture characteristic, microscopy of morphological structures and analysis of physiological features are classical methods for identification of dermatophytes. However, identification of dermatomycetes is obstructed due to their variability and pleomorphism which is greatly varies phenotypic characteristics of fungi[2, 3, 4].

Progress has been achieved in the methods of dermatophyte genodiagnostic which has been developed since the late 1990s. These methods include gene-specific PCR (Kamiya *et al*., 2004; Kanbe *et al*., 2003a; Liu *et al*., 2001; Yoshida *et al*., 2006), RFLP analysis (Kamiya *et al*., 2004; Kanbe *et al* ., 2003b; Leon-Mateos *et al*., 2006; Mochizuki *et al*., 2003), sequencing of the large submit rRNA gene (Ninet *et al*., 2003) or the chitin synthase-encoding gene (Kano *et al*., 2000), PCR fingerprinting (Gräser *et al*., 2000a, b) and DNA hybridization (El Fari *et al*., 1999). Sequencing of the internal transcribed spacer (ITS) regions (Gräser *et al*., 1999a, b, 2000a, b; Kaszubiak *et al*., 2004; Makimura *et al*., 1998, 1999, 2001; Mochizuki *et al*., 1999; Sharma *et al*., 2006) has proved to be a useful method for phylogenetic analysis and identification of dermatophytes[5, 6].

Holding of genotypic characteristics of dermatomycetes isolated from patients with humans' and animals' dermatomycoses with sequencing ITS (ITS4/ITS5) and SSU region (NS1/NS4), RPB1, RPB2 regions DNA clinical isolates is the purpose of this study.

MATERIALS AND METHODS

Fungal strains. In total 28 clinical isolates of dermatophytes and two reference strains were analyzed in this study. Reference strains were obtained from the collections of microorganisms of the State Institution "National Center of

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Monitoring, Reference, Laboratory Diagnostics and Methodology in Veterinary Medicine" (Astana, Kazakhstan). Clinical isolates were isolated from pathological material of various samples including skin, hair and nails. Preliminarily microscopic and macroscopic characteristics of fungi were determined[7].

The names of the species used in this study were consistent with the current taxonomy[8, 9]. Fungal strains were grown on dextrose agar Sabouraud (Sabouraud dextrose agar, "Titan biotech LTD" India) and incubated at 28 °C to complete formation of colonies. Identification of pure cultures was carried out using appropriate atlases and determinants[10-15].

Amplification and sequencing of ITS-regions. Buffer solution by protocol[16] and the method described by O.E. Amer *et al.*[17] in this modification was used for DNA extraction. Amplification of DNA regions, sequencing and purification of PCR products were performed as described previously[18, 19, 20].

Construction and analysis of sequences of the database. Nucleotide sequences were analyzed and grouped in general sequence in the software SeqMan (DNA Star) after which terminal fragments were removed. The sequences obtained were identified in GeneBank algorithm BLAST[21].

Identification was carried out relatively to GeneBank accession numbers of the first three nucleotide sequences having the best match.

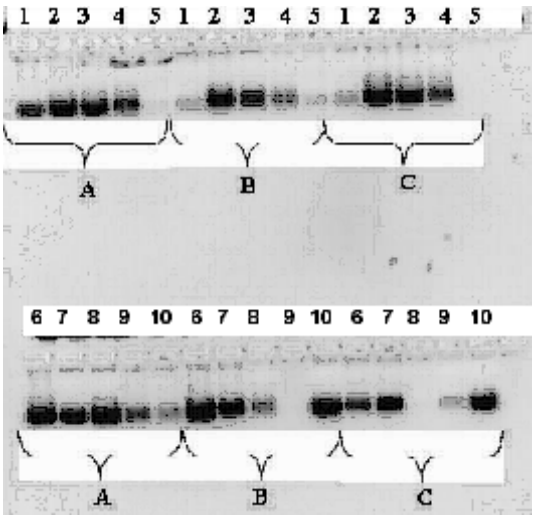

Fig. 1. Electrophoregram of qualitative analysis of DNA

Building dendrogramm were performed by using software Mega 3.1[22], the alignment of nucleotide sequences was performed using the algorithm Clusta l.W. as well as construction of phylogenetic trees was performed using the method of nearest neighbors (Neiighbor-Joining N.J.)[23-24].

Documentation of obtained results was performed using gel documentation GelDoc (Bio-Rad) with software QuantityOne (Bio-Rad). Molecular sizes of the analyzed DNA samples were determined by comparing their electrophoretic mobility in gel mobility markers – DNA fragment of known molecular weight. "DNA Ladder 1kb" (Ferments) was used as a molecular weight marker.

RESULTS AND DISCUSSION

Trichophytia bovine is found throughout the territory of Kazakhstan. Mass lesions of the skin, caused by Ringworm pathogens: cattle's Trichophyton and Microsporum infected by dermatomycoses are registered among imported beef livestock. Moreover, the disease often occurs in the malignant form, causing depletion and skin defects, shows atypical clinical picture and is not always treatable by vaccine preparations.

54 pure cultures of microorganisms – dermatomycoses pathogens isolated from animals in Astana and Akmola regions were selected to obtain DNA samples. Qualitative analysis of some DNA samples is shown in Figure 1.

DNA concentration of all 54 crops is listed in Table 1. As it can be seen from the results of a qualitative assessment of DNA samples shown in the table, the concentration of DNA isolated from dermatomycetes, molds and fungi has high performance and the DNA concentration above 10 ng/mL which is occurred in 91% of cases.

This indicates that used modification of DNA isolation method based on increasing concentrations of the buffer solutions was effective for dermatomycetes, yeasts and molds, so for further work the method of DNA isolation protocol[17] was used in this modification.

Specific fragment of molecular weight from 500 to 850 n.p. (nucleotide pair) was amplified at setting PCR with ITS4 and ITS5 primers. In Figure 2, the samples marked with the numbers 1-28 of the target DNA, M – molecular weight marker (Fermentas) (100-10000 n.p., 100-1000 to step 100

n.p.; step 1000-10000 to step 1000 n.p.), K^- – negative control.

As it can be seen from Figure 2, in the PCR products of DNA samples with sequence numbers 6, 8, 9, 14, 18, 20, and 21 there are no specific bands or they have low intensity. This indicates either a low concentration of DNA or inhibition of the reaction. PCR products of remaining images were used to determine the nucleotide sequence. Processed nucleotide sequences were genotyped with the use of BLAST international database NCBI.

Taking into account the literature data[25-29], indicating the presence of some mistakes in international banks GeneBank of nucleotide sequences (http://www.ncbi.nlm.nih.gov/), additionally we conducted further construction of phylogenetic trees from nucleotide sequences deposited in NCBI. For each species at least three sequences of displays in order to minimize errors were selected. As an example there is a phylogenetic tree constructed for Trichophyton spp. (Figure 3).

The data in Figure 3 indicate that the nucleotide sequence identified from cultures of 144 and 146 are located on the same clade with nucleotide sequences of 4 species: *Trichophyton rubrum*, *T. kanei*, *T. fischeri*, *T. raubitscheii*. The same result was obtained using a marker SSU. Similar work has been done for RPB1 and RPB2 markers, however, with their help it was not possible to type fungi. Summary results of research are shown in Table 2.

This is due to the highly conserved ribosomal region of these species that does not allow for the identification of the species in its use.

However, markers RPB1 and RPB2 allowed differentiating these cultures to *T. rubrum*. In contrast, the fourth culture, studied by analyzing

Table 1. Concentration of DNA isolated from microscopic fungi

#	Numbers of strains	Concentration (ng/ml)	Purity of DNA	#	Numbers of strains	Concentration (ng/ml)	Purity of DNA
1	1	3887,26	2,06	28	129	456,46	2,04
2	2	2610,55	2,14	29	134	4,20	1,76
3	3	1701,64	1,99	30	144	237,36	2,10
4	4	174,93	2,07	31	146	405,22	2,12
5	5	458,72	2,10	32	149	515,95	2,09
6	5.1	1666,48	2,13	33	152	1417,77	2,12
7	5.2	131,03	2,03	34	156	6,65	1,97
8	6	1652,50	2,15	35	156 ïð.	4144,56	2,08
9	7	9,30	2,06	36	179	3638,88	2,08
10	8	72,99	1,74	37	181	1808,65	2,11
11	9	206,77	2,10	38	182	4932,87	1,82
12	H11	128,52	2.00	39	183	1143,98	2,09
13	12	121,86	1,95	40	198	4217,12	2,03
14	13	151,91	2,10	41	208.1	1903,53	2,12
15	14	47,23	1,96	42	213	1280,71	2,09
16	16ê	18,40	2,12	43	214	338,42	2,12
17	16Ñ	4,75	2,07	44	230	153,93	1,98
18	41	721,92	2,15	45	266	5082,93	1,73
19	42	231,91	2,06	46	268	3794,11	2,11
20	79	794,6	2,15	47	298	5465,25	1,07
21	86	258,30	2,05	48	303	1503,96	2,01
22	112	2,25	2,12	49	328	1594,43	2,05
23	113.1	148,75	2,04	50	376	35,15	1,84
24	117	232,28	2,10	51	384	44,85	1,98
25	123.2	172,84	2,07	52	627	165,15	2,08
26	123.3	1691,59	2,12	53	1748	1029,85	2,09
27	128	28,07	1,90	54	16010	269,92	2,08

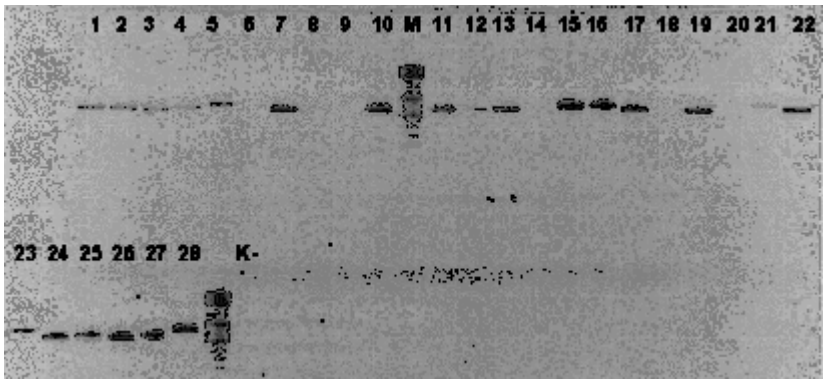

Fig. 2. PCR Electrophoregram products of ITS region.

Fig. 3. Phylogenetic tree constructed on the basis of nucleotide sequence analysis of ITS region *Trichophyton* spp

method of ITS and SSU, belongs to *T. verrucosum*, but using markers RPB1 and RPB2 has a maximum nucleotide sequence with different kinds of *Trichophyton* spp., due to the lack of international databases of nucleotide sequences of *T. verrucosum* markers RPB1 and RPB2.

In the case of confirmation of identification by morphological methods we will be recharged international database with specified markers.

Thus, we have carried out work on the genetic typing of pathogens dermatomycoses isolated from pathological material. Studies indicate widespread classical pathogens of dermatomycoses. Along with typical activators where fungi are dermatomycetes it was identified several atypical pathogens including *Ch. globosum*, *Ch. nigricolor*, *Alternaria alternate*, *Asp. versicolor*, *Phoma macrostoma* and others. Our studies confirm the data shown in the writings of employees of mycological Reference Laboratory (USA) and other authors[10, 30, 31].

According to the study phenotype and genotype characteristics selected dermatomycetes, humans' and animals' pathogens, circulating in Kazakhstan are not only classic dermatomycetes, but also fungi and yeast.

Table 2. Results of genotyping cultures using two markers

#	Name and number of strains	Results of genetic identification of regions	
		ITS	SSU
1	*Trichophyton verrucosum* #1	*T. verrucosum*	*T. verrucosum*
2	*Trichophyton mentagrophytes* #5	*T. interdigitale*	*T. interdigitale*
3	*Aspergillus sp.* #6	*Asp. versicolor*	*Asp. versicolor*
4	*Trichophyton verrucosum* #4	*T. verrucosum*	*T. verrucosum*
5	*Chaetomium globosum #12*	*Ch. globosum*	*Ch. globosum*
6	*Microsporum canis #13*	*M. canis*	*M. canis*
7	*Stemphylium sp. #5.2*	*Alternaria alternata*	*A. alternata*
8	*Trichophyton tonsurans #86*	*T. tonsurans*	*T. tonsurans*
9	*Chaetomium globosum #117*	*Ch. globosum*	*Ch. globosum*
10	*Ch. nigricolor #123.2*	*Ch. nigricolor*	*Ch. nigricolor*
11	*Ch. nigricolor #123.3*	*Ch. nigricolor*	*Ch. nigricolor*
12	*Ch. iranianum #128*	*Ch. iranianum*	*Ch. iranianum*
13	*Ch. globosum #129*	*Ch. globosum*	*Ch. globosum*
14	*Ch. globosum #113*	*Ch. globosum*	*Ch. globosum*
15	*Trichophyton rubrum #144*	*T. rubrum /* *T. kanei /* *T. fischeri /* *T. raubitscheii*	*T. rubrum /* *T. kanei /* *T. fischeri /* *T. raubitscheii*
16	*Trichophyton rubrum #146*	*T. rubrum /* *T. kanei /* *T. fischeri /* *T. raubitscheii*	*T. rubrum /* *T. kanei /* *T. fischeri /* *T. raubitscheii*
17	*Eurotium sp. #230*	*Eurotium sp.*	*Eurotium sp.*
18	*Aphanocladium aranearum #627*	*Aphanocladium aranearum*	*Aphanocladium aranearum*
19	*Penicillium dipodomyicola #1748*	*Penicillium dipodomyicola*	*Penicillium dipodomyicola*
20	*Arthroderma vanbreuseghemii #16010*	*Arthroderma vanbreuseghemii*	*Arthroderma vanbreuseghemii*
21	*Phoma sp. #Í11*	*Ph. macrostoma*	*Ph. macrostoma*

Comparison of the phenotypic and genotypic data characteristics showed that these results do not match 100%. By typing results using primers for primer pair ITS4 and ITS5 it is seen that this primer pair identifies dermatomycetes to genus and species. By typing results using primers SSU after a search in the database, information is received with the name of all genetically related genera and related species of fungi, whose sequences are similar to each other. The findings of our studies are consistent with the work of scientists who reported a lack of correctness of the primers used when working with dermatomycetes[32-35].

CONCLUSION

1. Isolation of DNA extraction using a buffer containing Tris-HCl, NaCl, EDTA, SDS protocol described O.E. Amer at all. (2003), modified by us it was allowed to obtain samples with concentrations above 10 ng/ml in 91% of cases.
2. Conducting a PCR amplification and sequencing using primers ITS4 and ITS5 primers at an annealing temperature of 52 °C allowed to obtain nucleotide sequence ITS region extending to 580 n.p. chromatograms with good quality, which genotyped using BLAST international database NCBI.
3. If the strain was phenotypically identified and requires only confirmation of agent's name, it is possible to use primers ITS. If sequence-typing is used to determine the degree of kinship in building cultures of the phylogenetic tree, this can be offered primer SSU.

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