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RESEARCH ARTICLE

Isolation and Molecular Characterization of Bovine *Trichophyton verrucosum* Strains Based on Sequence Analysis of Internal Transcribed Spacer Region (ITS1) and Microsatellite Loci

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ABSTRACT

The aim of this study was to isolate *Trichophyton verrucosum* from cattle and investigate the genetic diversity of the isolated strains by sequencing ITS1 region and by fragment length analysis of microsatellite loci. In this study, 84 hair and skin scraping samples from cattle with dermatophytosis were investigated by direct microscopy and culture methods. Furthermore, DNA was isolated from the colonies and sequence of ITS1 region was determined by direct sequencing. In addition, primers were designed for amplification of 11 loci showing simple tandem repeats (CHFD, SRT, HP1, HP2, HP3, HP4 TVMS1, TVMS2, TVMS3, TVMS4 and TVMS5) and PCR products of each locus were examined by fragment length analysis. Out of 84 samples 25 (29.7%) *T. verrucosum* strains were isolated. Sequence analysis of ITS1 region revealed that all strains have the same sequence. The observed fragment lengths of the microsatellite loci CHFD, SRT, HP1, HP2, HP3, HP4 TVMS1, TVMS2, TVMS3, TVMS4 and TVMS5 were 136 bp, 170 bp 178 bp, 423 bp, 356 bp, 225 bp, 232 bp, 201 bp, 270bp, 173 bp, respectively. None of the microsatellite loci examined showed fragment length variation among the samples included into the study. The results suggested that dermatophyte species could be distinguished based on the sequence of ITS1 region, while absence of sequence variation at ITS1 region did not allow distinguishing between *T. verrucosum* strains. On the other hand, no fragment length variation of the microsatellite loci among the *T. verrucosum* strains examined was observed.

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INTRODUCTION

Dermatophytes cause various infections of skin, hair and nails defined as dermatophytosis, in humans and animals. The most common agent isolated from dermatophytosis cases in cattle is *Trichophyton verrucosum* (Papini *et al*., 2009). *Trichophyton verrucosum* in cattle causes skin lesions defined as ringworm characterized by local hair loss and desquamation with pruritis. Trichophytosis causes significant economic losses in the livestock sector due to the damage in the skin integrity, and is a zoonotic disease threatening human health (Klous *et al*., 2016).

Direct microscopy and culture methods are mostly used in the routine identification of dermatophytes. Various methods have been used to determine the subtypes of dermatophyte species but still none of them can be determined as a gold standard. Molecular genetics methods such as restriction fragment length polymerphism (RFLP) of mitochondrial DNA, sequencing internal transcribed spacer (ITS) regions of DNA encoding ribosomal RNA (rRNA) (Rezaei-Matehkolaei *et al*., 2013; Elavarashi *et al*., 2013), random amplification of polymorphic DNA (RAPD) (Liu *et al*., 1997), have provided significant benefits in distinguishing dermatophyte species and strains. Additionally, the multi locus enzyme electrophoresis, allozyme comparisons, electrophoretic karyotyping, polymerase chain reaction-enzyme immunoassay, DNA sequence analysis, Southern hybridization and pulsed-field gel electrophoresis analysis techniques have also been used in diagnosis of fungal species (Howard, 2002).

Molecular studies such as DNA homology have demonstrated close relationship between dermatophyte

species (Cafarchia *et al*., 2013; Neji *et al*., 2016). Genes encoding ribosomal RNAs in fungi are located in a region close to each other and they are transcribed as a single RNA molecule. The regions between 18S, 5,8S and 28S rRNA genes are called internal transcribed sequence (ITS) and spliced out during RNA maturation. The region between the 18S and 5,8S rRNA sequences is defined as ITS1 while the part between 5,8S and 28S region is called ITS2. Sequences of the ITS regions in dermatophytes consist of both conserved and variable portions that have been shown to be useful in determining the relationship between different genus or species/ strains (Li *et al*., 2008; Rezaei-Matehkolaei *et al*., 2013). Specific universal primers have been used for amplifying by PCR and sequencing the ITS region (Hsiao *et al*., 2005).

Determining the origin of an infection is crucial for monitoring the distribution of the disesase agents in epidemiological studies. However molecular genetics techniques such as sequencing ITS region have shown little genetic variation among *T. verrucosum* strains (Neji *et al*., 2016). However, microsatellites or sequence tagged sites showing tandem repeats of short sequence motives might be a polymorhpic marker alternative in genetical characterizing the strains of different dermatophytosis agents.

Microsatellites show a high polymorphism due to a mutation mechanism defined as slippage (Moore, 1983). Due to their high polymorphism, microsatellite loci were widely used to investigate genetic structure of populations (Bozkaya and Gürler, 2011), chromosome and quantitative trait loci (QTL) mapping, (Lv *et al*., 2016) and for forensic purposes such as identity or parentage testing (Harrison *et al*., 2014). Several variable microsatellite markers have been defined in different dermatophytosis agents (Ohst *et al*., 2004; Kaszubiak *et al*., 2004; Cano *et al*., 2005; Pasquetti *et al*., 2013). Ohst *et al*. (2004) have reported that certain microsatellite regions in *T. rubrum* may be variable in repeat number and are useful for revealing genotypic and the geographical differences among strains of this species. Kaszubiak *et al*. (2004) have found a polymorphic microsatellite locus having an $(AC)_{11}$ repeat motive and suitable to be used for distinguishing in *Microsporum audouinii* strains. Microsatellite regions showing variations in repeat number have been defined by several research groups in *Microsporum canis* (Cano *et al*., 2005; Pasquetti *et al*., 2013).

However, to our knowledge there is no report on the variability of microstaellite loci in *T. verrucosum* species. The aim of this project was to isolate *T. verrucosum* strains from cattle with dermatophytosis in Sanliurfa and to characterize them based on the Internal Transcribed Spacer (ITS) sequences as well as on the fragment length differences of microsatellite markers.

MATERIALS AND METHODS

Collection of the samples: Skin scraping samples were collected from 84 cows showing dermatophytosis symptoms in Sanliurfa province of Turkey. Skin scraping samples were taken from the margin of the lesions by using a sterile scalpel blade into petri dishes, after the affected area was cleaned with cotton wool soaked in 70% ethanol.

Direct microscopy and culture: A small portion of the samples was suspended in 15% potassium hydroxide solution and examined with a light microscope for presence and shapes of the spores and hyphea. The samples were inoculated onto Dermatophyte Test Medium (DTM) and Sabouraud Dextrose Agar (SDA) (Oxoid S.p.A) containing cycloheximide (500 mg/ml), chloramphenicol (50 mg/ml), thiamine (4 mg/l) and inositol (100 mg/l) and incubated at 37°C for 4 weeks. The colonies were examined macroscopically and microscopically by considering their shape, profile, texture, surface appearance and colour. A small fragment of the colonies was examined with a light microscope by staining with Lactophenol Aniline Blue (Oxoid S.p.A.). Hyphae and conidia structures were evaluated for identifying the agents at genus and species level.

Isolation of DNA: Fungal DNA was isolated from the colonies grown on agar as reported by Liu *et al*. (2000). Small amount of the colonies was incubated in 500µl lysis solution (400mM Tris-HCl 60mM EDTA, 150mM NaCl, 1% sodium dodecyl sulfate, [pH 8.0]) for 10 minutes at room temperature. Proteins were precipitated by adding 150µl 3M potassium acetate solution. Precipitation of DNA was performed by adding equal volume of isopropanol. The DNA pellet was washed with 70% ethanol and dissolved in 100µl nuclease free water.

Sequencing of the ITS region: Amplification of the ITS region by PCR was performed according to the procedure reported by Hsiao *et al*. (2005). The mixtures of PCR consisted of 25pmol each primer, (ITS-1: 5′-TCCGTAG GTGAACCTGCGG-3′ and ITS-4: 5′-TCCTCCGCTTA TTGATATGC-3′), 2 U of Taq-polymerase (Fermentas, Vilnius, Lithuania), 0.2mM dNTP mixture, 5µl 10X reaction buffer and 1µl genomic DNA. The thermal conditions applied were an initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 minute and at 72°C for 1 minute. The PCR products were electrophoretically separated on 1.5% agarose gel.

The PCR products were bidirectionally sequenced by using a Big Dye Cycle Sequencing kit v 3.1 of Applied Biosystems on an ABI PRISM 3130XL automated sequencer (Applied Biosystems). The primer pair used for PCR amplification above was also used for sequencing.

The base sequences were aligned by using Bioedit Alignment Sequence Editor (Hall, 1999). The detected sequences were compared with similar sequences obtained from the GenBank using web-based BLAST program (http://blast.ncbi.nlm.nih.gov). Phylogenetic relationships between sequences were assessed by using Maximum Likelihood method (Tamura and Nei, 1993) included in MEGA 5 program (Tamura *et al*., 2011).

Fragment length analysis of microsatellite loci: Primers were designed by using Primer 3 (www.ncbi.nlm.nih.gov) program for 11 loci showing 8-14 repeat numbers of different sequence motives (Table 1). The mixtures of PCR consisted of 10pmol each primers, 2U of Taq polymerase (Fermentas, Vilnius, Lithuania), 2mM $MgCl_2$, 0.2mM each dNTP, 2.5µl reaction buffer (10X) and 1µl genomic DNA. A touchdown PCR protocol was applied for annealing temperature beginning from 60°C and

decreasing 1°C at each step to 50°C. The PCR products were separated on a capillary electrophoresis system (Applied Biosystems, 3130xl Genetic Analyzer, Foster City CA). Peak Scanner Software v1.0 (Applied Biosystems) was used for identifying the peaks and sizing the fragments.

RESULTS

Direct microscopic examination with 15% KOH revealed that 23 of 84 samples (27.3%) were positive for *T. verrucosum* showing parallel chains of large, round translucent, ectothrix arthrospores near or attached to partially lysed hairs. By culturing of these samples on DTM and Sabouraud Dextrose Agar (SDA) (Oxoid S.p.A) a total of 25 (29.7%) *T. verrucosum* strains were isolated.

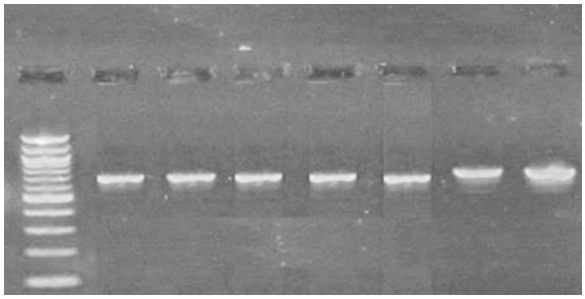

Fig. 1: ITS gel image of PCR products of some *T. verrucosum* strains isolated from the cattles with dermatophytosis in this study.

By sequencing the PCR products (Fig. 1) obtained by amplification of the ITS region the sequence of a 622bp fragment was obtained (Fig. 2). Alignment of the sequences obtained from different samples showed that all sequences were identical. Comparison of the ITS sequence detected in this study with other ITS sequences by BLAST screening revealed a 100% similarity to the sequences from other *T. verrucosum* strains submitted to GenBank (Fig. 3). Phylogenetic analysis showed that sequences from *T. verrucosum* strains along with the sequence found in the present study were clustered together on the same branch (Fig. 3). On the other hand ITS sequences of certain strains of *Trichophyton violaceum*, *Trichophyton mentagrophytes*, *Trichophyton erinacei* and *Arthroderma benheimia* were found to be more similar to each other than to that of *T. verrucosum*. The most distant group to *T. verrucosum* was found to be *Trichophyton concentricum*.

All the microsatellite loci except for TVMS1 were successfully amplified by PCR. The observed fragment lengths of CHFD, SRT, HP1, HP2, HP3, HP4, TVMS2, TVMS3, TVMS4 and TVMS5 loci were found to be the same as expected fragment lengths and 136 bp, 170 bp 178 bp, 423 bp, 356 bp, 225 bp, 232 bp, 201 bp, 270 bp, 173 bp, respectively. (Fig. 4). None of the loci examined showed fragment length variation among the samples included in this study.

DISCUSSION

Trichophyton verrucosum is the most frequently isolated fungal pathogen of cattle which has been reported to be the natural reservoirs of these agents (Khosravi and Mahmoudi 2003; Papini *et al*., 2009; Yildirim *et al*., 2010; Hameed *et al*., 2017). In a study conducted in Italy, *T. verrucosum* was isolated from 87.7% of 294 cattle raised in 20 different farms (Papini *et al*., 2009). Khosravi and Mahmoudi (2003) isolated *T. verrucosum* from 85% of the cattle with skin lesions, while they identified 15% of the isolated agents as *T. mentagrophytes* in Iran. On the other hand, Yildirim *et al*. (2010) were able to isolate *T. verrucosum* from 44% of 50 cattle showing dermatophytosis lesions. The lower isolation rate (29.7%) achieved in our study might be attributed to different sanitary conditions in the farms, where the samples were obtained and to seasonal differences.

Several studies have shown that ITS1 sequences are species specific and useful for identification of dermatophytosis agents (Li *et al*., 2008; Garcia Garces *et al*., 2016). In accordance with the literature we observed that the sequences of ITS region from *T. verrucosum* were different from those obtained from other dermatophytosis agents. However, we observed that certain *Arthroderma benheimia* sequences were more similar to those of

Table 1: Information of the microsatellite loci included in to the study for the detection of genetic variability among *T. verrucosum* strains

Locus		Primer sequence (5'-3')	Repeat motive	EPL	Label	Accession number
CHFD	F-	CTGCAGCAGCCTCGGGATGC	$(GA)_{14}$	136	FAM	XM_003019101.1
	R-	GAGTTGCTCGCCACGCTGGA				
SRT	F-	TGCGCAGATCGTCAGATGGGG	$(GA)_{12}$	170	FAM	XM_003022163.1
	R-	GCTGTGCTTGAAGTGCTTCGGC				
HP1	F-	TGGGAGGAACCCATGAGCACCA	$(GA)_{13}$	178	FAM	XM_003024802.1
	R-	TCAGCCAAACCGCCGCTCAG				
HP2	F-	GGCCGTTGGAACCAAGTGCG	$(GA)_{12}$	423	FAM	NW_003315234.1
	R-	TGGCATCAACCAGAGGCGAGT				
HP3	F-	TTGTGTCTCCCCTCCCGTTA	$(CA)_9$	356	HEX	NW_003315167.1
	R-	GTTGCATGTCCACCCAACTT				
HP4	F-	GCATCCGCATCCTCAACCGCA	$(ACAG)_8$	225	FAM	XM_003025501.1
	R-	TCCGCCATTTACCGTTCTTGGTTGC				
TVMS1	F-	CGTACGGCACGAGTCTACTAC	$(CA)_{10}$	86	FAM	ACYE01000021.1
	R	ATAGGAAGAGATGCACCCCG				
TVMS2	F-	TGCTAGACGGAGTGAAGGGA	$(GT)_{11}$	232	FAM	ACYE01000098.1
	R-	ATTTAGCAGGGCGGCTTTTG				
TVMS3	F-	CAGCTGTTGATGTGTAGTAAAAGA	$(GT)_{13}$	201	FAM	ACYE01000336.1
	R-	ATTCTACAGCCTGTGGGGTTG				
TVMS4	F-	ACCTGTCAACCTGAAAGGCTC	$(CA)_{13}$	270	FAM	ACYE01000080.1
	R-	CCTTCAACAGCAGGGAGGAG				
TVMS5	F-	AAGGTAACAGTCGGCTCACG	$(GT)_{12}$	173	FAM	ACYE01000219.1
	R-	CACCTTATATCCTTGACGGCCA				

(EPL**:** Expected product length in base pairs).

GGCTGGCCCCCCACGATAGGGATCAGCGTTCCATCAGGGGTGTGCAGATGTGCGCCGGCCTTACGCCCCATTCTTGTCTACCT
TACTCGGTTGCCTCGGCGGGCCGCGCTCTCCCCGGAGAGTCGTCCGGCGAGCCTCTTCGGGGGCTTTAGCTGGATCGCGCCCG
CCGGAGGACAGACATCAAAAAATCTTGAAGAGCTGTCAGTCTGAGCGTTAGCAAGCAAAATCAGTTAAAACTTTCAACAACG
GATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAA
TCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAACCCCTCAAGCTCGGCTTGTG
TGATGGACGACCGTCCGGCCCCCTCTTTCGGGGGCGGGACGCGCCCGAAAAGCAGTGGCCAGGCCGCGATTCCGGCTTCCTG
GGCGAATGGGCAATCAAACCAGCGCCCTCAGGACCGGCCGCTCTGGCCTTCCCCCAAATCTCTCTGAGATTTTTTTCAGGTTG
ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCA¶

Fig. 2: The sequence detected among *T. verrucosum* strains isolated from the cattles with dermatophytosis in this study.

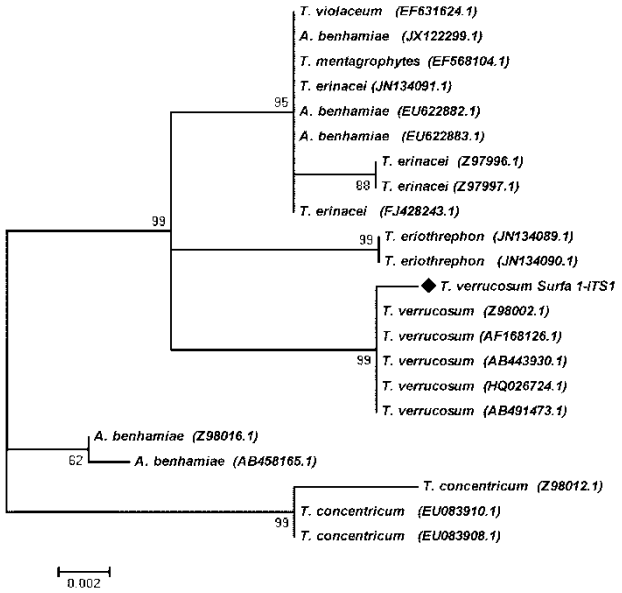

Fig. 3: Dendrogram showing the phylogenetic relationships among the strains of certain dermatophytosis agents based on the sequence of ITS region (Bootstrapping by 1000 replicates). The sequence of the samples included into this study was indicated by filled diamond.

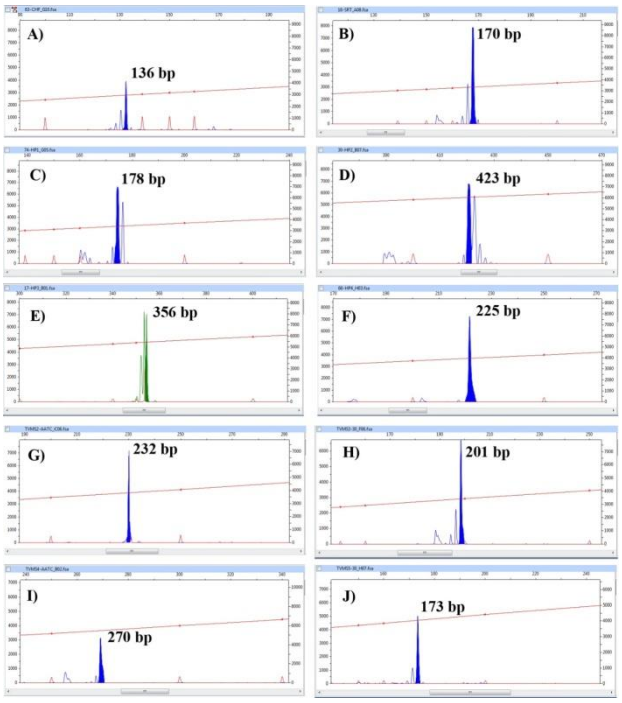

Fig. 4: Electropherograms of the examined loci of microsatellites. A) CHFD, B) SRT, C) HP1, D) HP2, E) HP3, F) HP4, G) TVMS2, H) TVMS3, I) TVMS4 and J) TVMS5. Measured fragment lengths in bp were shown on the peaks.

Trichophyton mentagrophytes, *Trichophyton concentricum* and *Trichophyton violaceum* than to those of other sequences from these species (Fig. 3). These results suggested that different dermatophyte species could not be distinguished merely based on ITS1 sequences.

By sequencing the ITS region of the strains used in the present study we did not observe any variation in the sequence of this region. However, Neji *et al*. (2016) reported two different types of *T. verrucosum* strains isolated from human patients with tinea corporis, tinea capitis or sycois. The incompatibility between the results of Neji *et al*. (2016) and those of the present study might be explained by the difference in the hosts from which the strains isolated or the sampling from a small area in the present study.

Due to their high variation microsatellites have been widely used in eukaryotic organisms (Bozkaya *et al*., 2011; Pasquetti *et al*., 2013; Harrison *et al*., 2014). Microsatellite markers have also been investigated in different dermatophytosis agents. Kaszubiak *et al*. (2004) have found a polymorphic microsatellite locus having an $(AC)_{11}$ repeat motive which is suitable for distinguishing in *Microsporum audouinii* strains. Ohst *et al*. (2004) have reported that microsatellites are useful genetic markers for genotyping *T. rubrum* strains and determining their geographical distribution. Graser *et al*. (2007) have detected polymorphic microsatellite loci in *T. rubrum* causing dermatophytosis in humans and shown that these microsatellite loci can be used for distinguishing *T. rubrum* strains. Microsatellite regions showing variations in repeat number have been defined by several research groups in *Microsporum canis* (Cano *et al*., 2005; Pasquetti *et al*., 2013).

Several studies showed that loci having higher repeat numbers have a higher mutation rate, thus a higher polymorphism (Brinkmann *et al*., 1998). Therefore, DNA regions carrying 8 to 14 repeats of different sequence motives were selected for genotyping *T. verrucosum* strains in our study. However, the selected loci did not show any polymorphism among the *T. verrucosum* isolates with respect to fragment length. The reason of this observation might be that the included loci were subjected to selection due to their functional significance. Another explanation could be a low variation among the isolated strains. The reason of this lower variation might be due to sampling from a small area so that all strains isolated were clones of the same strain. In accordance with the findings of this study, Yu *et al*. (2004) investigated genetic diversity of *Microsporum canis* strains isolated from 14 children with tinea capitis in a school by using RAPD technique and ITS sequences and they found no differences between strains. Pasquetti *et al*. (2013) isolated 26 *M.*

canis strains that infected different species such as humans, cats and dogs from 13 different countries and they found a total of 22 different genotypes for eight microsatellite loci.

Conclusions: The results indicated that although *T. verrucosum* strains isolated in this study showed no variation. *T. verrucosum* could be distinguished from other dermatophyte species based on the nucleotide sequence of ITS1 region. On the other hand, no fragment length variation of the microsatellite loci among the examined *T. verrucosum* strains was observed. Because the samples used in the present study were collected from a small area, further studies using larger sample size from wider geographical areas might reveal the diversity in *T. verrucosum* strains with respect to fragment lengths of the microsatellite loci examined in the present study.

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