

Preliminary evidence for the occurrence of β -tubulin isotype 3 polymorphisms in *Fasciola hepatica* isolates in cattle and sheep in Turkey

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Summary

Fasciolosis caused by *Fasciola hepatica* is a common parasitic disease of livestock especially sheep and cattle. In this study molecular characterization of β -tubulin isotype 3 gene in *Fasciola hepatica* isolates from cattle and sheep in Turkey was carried out. For this purpose a total of 80 adult *Fasciola hepatica* isolates were collected from 20 sheep and 20 cattle in Kayseri and Erzurum provinces. PCR-RFLP was performed on β -tubulin isotype 3 gene and *MbolI* revealed two fragments of approximately 350 bp and 390 bp, whereas *HphI* enzyme yielded 210, 340 and 540 bp bands, *HindIII* yielded 380 and 450 bp bands in all samples. A total of 80 isolates were tested by SSCP and all of them presented the same band profiles. Six samples (4 sheep and 2 cattle) were randomly selected and DNA sequence of a 935 bp coding fragment of β -tubulin isotype 3 was performed. Sheep samples were more polymorphic than the cattle. This β -tubulin isotype 3 gene polymorphism of *F. hepatica* isolates from sheep and cattle of two distinct geographical areas of Turkey have been investigated for the first time.

Keywords: *Fasciola hepatica*; β -tubulin 3; Polymorphism; Sheep; Cattle; Turkey

Introduction

The liver fluke, *Fasciola hepatica*, is a worldwide distributed trematode parasite of great veterinary importance, which causes significant economic losses (feed conversion efficiency, poor carcass conformation, malnutrition, decreased fertility and milk yield) in livestock (Khoramian *et al.*, 2014). It is a food-borne trematode zoonosis and is classified as neglected tropical disease (WHO, 2015). Fasciolosis is an asymptomatic infection in cattle and causes more serious pathological implications in sheep. Acute fasciolosis in sheep occurs seasonally and manifests itself with a relatively swollen and painful abdomen; and sudden death occurs within 2 – 6 weeks after infection. Advanced chronic infection is fatal in sheep, this rate is less in cattle (Mas-Coma *et al.*, 2005). Survival is longer in cases of significant liver damage (7 – 10 weeks

in subacute disease, however, death can occur due to hemorrhage and anemia. Although chronic fasciolosis can be seen during all seasons of the year, it occurs primarily in autumn and winter. Anemia, unthriftiness, submandibular edema and decreased milk production are the most important symptoms. The benzimidazole derivative triclabendazole is a highly specific drug for the treatment of fasciolosis (Fairweather & Boray, 1999). Microtubules are highly conserved among species, the polymers of α and β -tubulin, in heterodimer form. The *Fasciola hepatica* genome codes for at least five α and six β -tubulin isotypes (Ryan *et al.*, 2008). β -tubulin proteins have been characterised from a variety of helminth species. To date, however, there is no data about the polymorphism in the β -tubulin gene from isolates in Turkey.

In the present study we investigated the possible polymorphism in the 935 bp fragment of β -tubulin isotype 3 gene of *F. hepatica*

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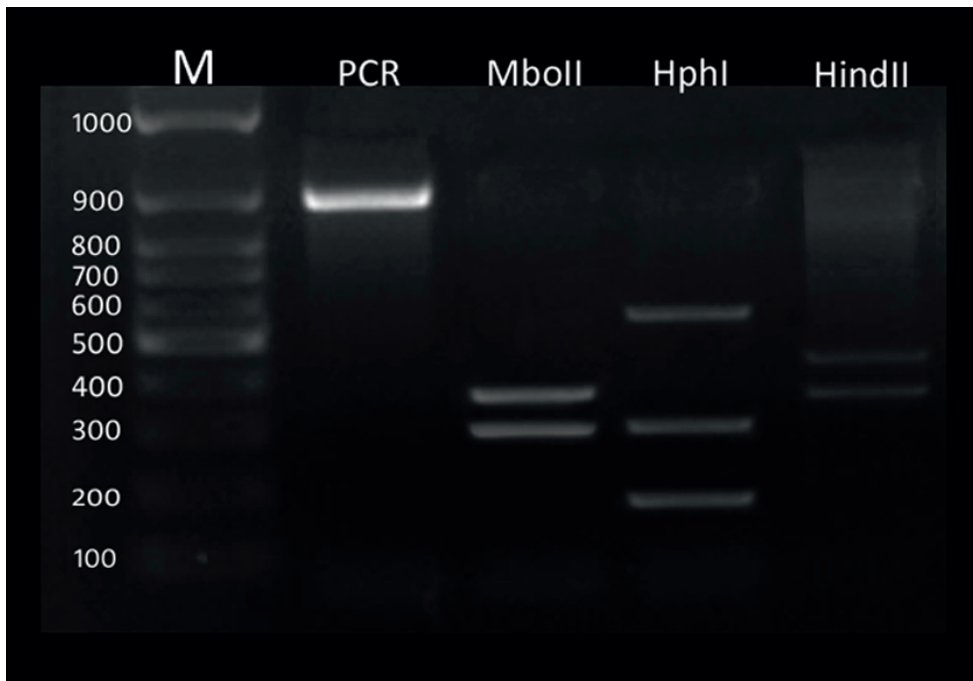


Fig. 1. PCR and PCR-RFLP results of *Fasciola hepatica* β -tubulin gene. M: Marker (100 bp); PCR: β -tubulin gene (935 bp); MboII: Restriction with *MboII* (350 and 390 bp); HphI: Restriction with *HphI* (210, 340 and 540 bp); HindII: Restriction with *HindII* (380 and 450 bp).

which has been collected from sheep and cattle isolates from different geographical areas of Turkey.

Materials and Methods

Sample Collection

The study defines an isolate as a single adult *F. hepatica* from each separate liver. A total of 80 isolates from livestock (sheep n=20, cattle n=20) originating from Erzurum and Kayseri provinces were collected over a period of 2 years (January 2014-December 2015) during random visits to licensed slaughterhouses in Turkey. The isolates were preserved in 70 % ethanol and were transferred to the laboratory for genomic DNA isolation.

Genomic DNA Isolation

Total genomic DNA (gDNA) was isolated from the adult parasite tissue using the GeneAll[®] Tissue Kit (Korea) according to the manufacturer's instructions. Prior to gDNA isolation, the anterior part of each adult parasite was dissected and ethanol residue was removed by at least five times repeated wash in 600 μ l 1 M PBS (pH=7,4). gDNA quality was evaluated by optical density ratio (260/280 nm), using a NanoDrop 8000 Spectrophotometer (Thermo Scientific). gDNA isolated samples were used for further PCR and sequence analysis.

Polymerase Chain Reaction (PCR)

A fragment (935 bp) of β -tubulin isotype 3 gene was amplified using the primers β -tub295f (5'-AAYAAATGGGCYAARGGNCA-

TA-3') and β -tub1230r (5'-TCRGTRAAYTCCATYTCRTCCAT-3') previously reported (Ryan *et al.*, 2008). We used the following conditions: initial denaturation step of 10 min at 95 °C and 40 cycles of 1 min at 94 °C (denaturation), 1 min at 56 °C (primer annealing), 1 min at 72 °C (extension) followed by a final extension step of 10 min at 72 °C. Amplicons were electrophoretically visualized on an ethidium bromide stained 1.4 % agarose gel under UV.

Restriction Fragment Length Polymorphism (PCR-RFLP)

The restriction enzymes used for this procedure were determined using previously published sequences (HM535826, HM535800 and HM535806) in PubMed. NEBCutter V2.0 program was used to determine which enzymes cut the sequence and *MboII* (5 U/ μ l, 300U Thermo Scientific), *HphI* (5 U/ μ l, 300U Thermo Scientific) and *HindII* (5 U/ μ l, 300U Thermo Scientific) were used in this

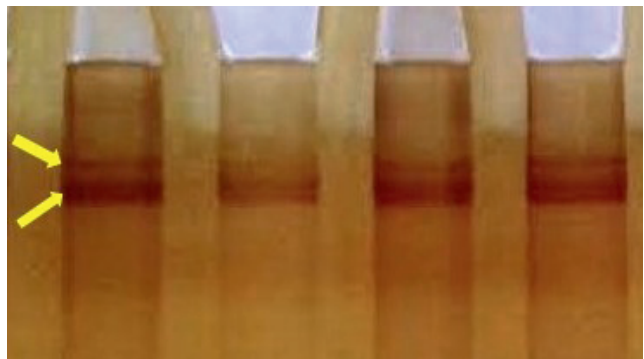


Fig. 2. SSCP band profiles of some selected samples.

study. PCR products were digested during 3 hours with restriction enzymes at 37 °C using buffers recommended by the manufacturer (Thermo Scientific) in a final 18,7 µl volume reaction mix, containing 10 µl of PCR product, 1 µl from each enzyme (*MbolI*, *HphI* and *HindIII*), 2 µl restriction buffer, 0.2 µl BSA and 5.5 µl distilled water. The restriction fragments were separated on 3 % agarose

gel, stained with ethidium bromide and photographed (Vilber Lourmat Quantum-ST5-1100/26MX).

Single Stranded Conformation Polymorphism (SSCP)

The method was performed according to Simsek *et al.* (2012). Eight microliters of each PCR product were mixed with 12 µl of

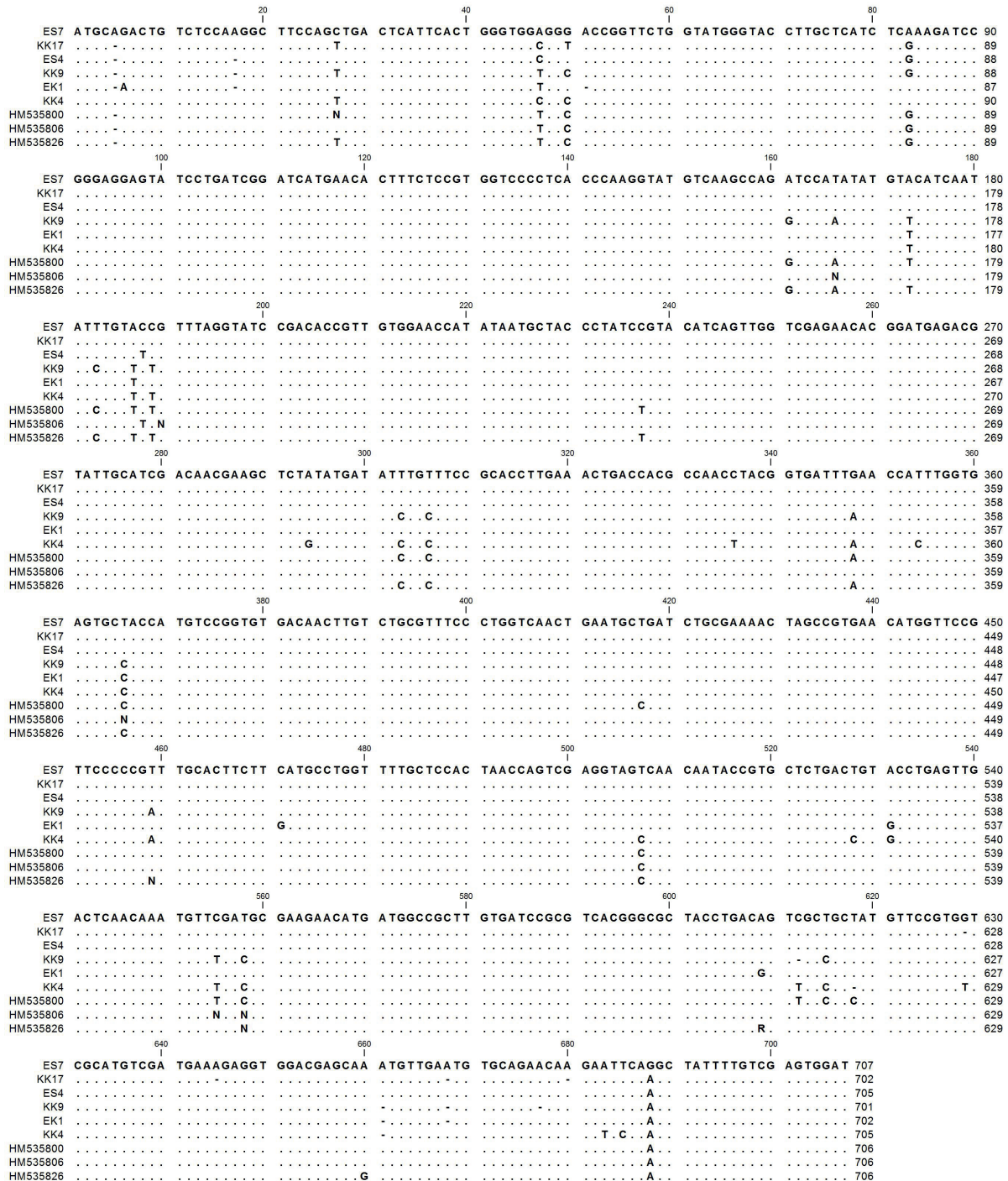


Fig. 3. Nucleotide sequence alignment of samples analyzed in this study using with the reference sequences (HM535800, HM535806 and HM535826).

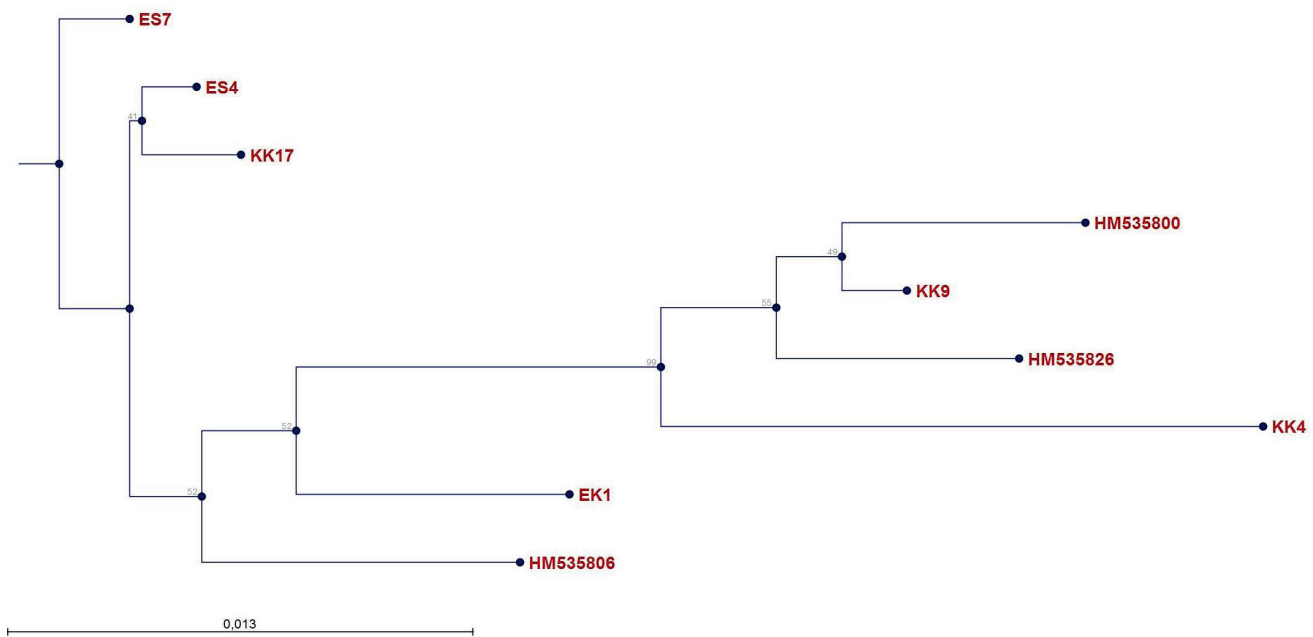


Fig. 4. Phylogenetic tree view of the aligned nucleotide sequences using with the reference sequences (HM535800, HM535806 and HM535826).

denaturing buffer (10 mM NaOH, 95 % formamide, 0.05 % bromophenol blue and 0.05 % xylene cyanole). Following the denaturation at 95 °C for 10 min and subsequent snap-cooling on a freeze block (-20 °C), 10 µl of individual samples were loaded into the gel. The electrophoresis was carried out in 12 % acrylamide-bisacrylamide (49:1) non-denaturing gels containing 10 % glycerol, at 200 V constant current during 3 h at room temperature. The band profiles were displayed by silver staining.

DNA Sequence Analysis

The *F. hepatica* partial β -tubulin gene sequences were obtained automatically. Nucleotide sequence analysis was undertaken by BLAST algorithms and databases from the NCBI (<http://www.ncbi.nlm.nih.gov>). The following accession numbers were given to sequences: KU523368, KU523369, KU523370, KU523371, KU523372 and KU523373.

Ethical Approval and/or Informed Consent

For this study formal consent is not required (Firat University, Local Ethics Committee of Animal Experiments, Decision no: 2014/73).

Results

A 935 bp fragment of β -tubulin isotype 3 gene was amplified through PCR in 80 *F. hepatica* isolates (Fig. 1). The molecular characterization of *F. hepatica* was carried out on the basis of PCR-RFLP and SSCP analysis of β -tubulin isotype 3 gene and sequence analysis as well. Three restriction enzymes (*MbolI*, *HphI* and *HindIII*) were used to differentiate the β -tubulin isotype

3 gene of adult flukes. Different band patterns were observed in PCR-RFLP after enzyme digestion. The PCR-RFLP profile obtained from *MbolI* revealed two fragments of approximately 350 bp and 390 bp (Fig. 1), whereas *HphI* enzyme yielded 210, 340 and 540 bp bands (Fig. 1), and *HindIII* yielded 380 and 450 bp bands (Fig. 1) in all samples. A total of 80 isolates were tested by SSCP and all of them presented the same band profile (Fig. 2). Six samples (n=4 from sheep in Kayseri, n=2 from cattle in Erzurum) were randomly selected and sequenced. Four of those samples were isolated from sheep in Kayseri (KK4, Accession no: KU523371; KK9, Accession no: KU523372; KK17, Accession no: KU523373) and Erzurum (EK1, Accession no: KU523368) while the remaining two samples were isolated from cattle in Erzurum (ES4, Accession no: KU523369 and ES7 Accession no: KU523370). Multiple sequence alignment was performed including the previously published sequences (HM535800, HM535806 and HM535826) used for alignment and the results are shown in Fig. 3. According to alignment results, much more polymorphic sites were seen in sheep isolates (KK4, KK9 ve KK17) than the cattle. It has been illustrated in a genetic tree as well (Fig. 4). Amino acid comparisons of the samples were performed and mutations at different ratios at the sequence were observed (Fig. 5). The β -tubulin 3 gene sequences described in the current study were compared to those described previously (HM535800, HM535806 and HM535826). Tyrosin amino acid was detected in all sequenced and control sequences in 200th position.

Discussion

Molecular studies on the genetic diversity of *Fasciola* species using

		200	
ES4	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
ES7	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
EK1	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
KK17	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
KK4	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
KK9	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
HM535806	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
HM535826	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
HM535800	VS DTVVEPYNATLSVHQLVENTDETYCIDNEAL	Y	DICFRTLKLTTPTYGDLNHLVSATM 225
ES4	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF		286
ES7	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF		286
EK1	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF	L	286
KK17	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF		286
KK4	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF		286
KK9	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF		286
HM535806	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF	X	286
HM535826	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF		286
HM535800	SGVTTCLRFPGQLNADLRKLVANMVPFRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMF		286
ES4	DAKNMMAACDPRHGRYLTVA		306
ES7	DAKNMMAACDPRHGRYLTVA		306
EK1	DAKNMMAACDPRHGRYLTVA		306
KK17	DAKNMMAACDPRHGRYLTVA		306
KK4	DAKNMMAACDPRHGRYLTVA		306
KK9	DAKNMMAACDPRHGRYLTVP		306
HM535806	X	DAKNMMAACDPRHGRYLTVA	306
HM535826	X	DAKNMMAACDPRHGRYLTVA	306
HM535800	DAKNMMAACDPRHGRYLTVA		306

Fig. 5. Alignment of the partial β -tubulin isotype 3 amino acid sequence with other relevant sequences (HM535800, HM535806 and HM535826). Alignment has been started from 99. position of the reference sequences. Amino acid residues that are conserved in at least 2 proteins are indicated in white back ground. Rectangle on the alignment indicate Tyr amino acid in the 200th position.

mt-DNA have confirmed genetically varied populations (Elliot *et al.*, 2014). Level of genetic diversity of *F. hepatica* suggests that it may have the potential to adapt to selection pressure within the environment with clonal reproduction in snails allowing rapid dispersal of resistant populations (Prugnotte *et al.*, 2005). Microtubules are the major promoters of cytoskeleton and are widespread in all eukaryotic cells. They accompany some functions including cell division and intracellular transport mechanisms (Nogales *et al.*, 1998). It has been previously established that the adult flukes express at least six tubulin isotypes designated as *F. hepatica* β -tub1 to β -tub6 (Ryan *et al.*, 2008). The gene coding β -tub isotype 3 was chosen in the current study. The total length of the gene is 1335 bp. Selected primers were used for amplification of region 935 bp in length. This is a region encoding 242 amino acids of the protein sequence.

Cwiklinski *et al.* (2015) highlighted tubulins exhibit the highest log fold-changes in expression throughout *F. hepatica* development. They identified the full complement of five α -tubulin and six β -tubulin isotypes and found duplication of β -tubulin isotype 3. We

investigated levels of polymorphism among individual *F. hepatica* isolates collected from sheep and cattle by partial sequencing of the β -tubulin isotype 3. It has been detected more nucleotide polymorphism in sheep isolates than cattle. This may be due to the more common prevalence of sheep fasciolosis than the cattle in Turkey.

In our study, DNA sequences of 6 randomly selected samples were obtained from samples amplified by β -tubulin isotype 3 gene PCR of 80 *F. hepatica* isolates from Erzurum and Kayseri provinces. Sequence analysis resulted in amino acid comparisons of the samples, we observed the Tyrosin (Y) in the 200th position in all sequenced samples. Ryan *et al.* (2008) also identified Tyrosine at position 200 of the β -tubulin isotypes 1 – 3, Phenylalanine (F) in isotype 4 and 6 in two samples and Leucine in isotype 5 in one. Teofanova *et al.* (2011) examined mutation and polymorphism in the β -tubulin 3 gene in 204 *F. hepatica* isolates obtained from Greece (n = 143), Poland (n = 48) and Bulgaria (n = 13) using sequence analysis. As a result, two major lineages for the 28S rDNA gene based on the highly polymorphic 105th nucleotide position

have been found. The basic lineages were observed within Greek, Bulgarian, and Polish *F. hepatica* populations but the distribution of additional haplotypes differed between the populations from the three countries. For the β -tubulin isotype 3 gene multiple polymorphic sites were revealed but no explicit clades. The SNPs were spread unequally in all studied geographical regions with an evident distinction between the Greek and Polish specimens. In our study, a few polymorphic points were identified in the sequenced sheep and cattle samples. These results indicate that phylogenetic analysis based on partial sequences of β -tubulin isotype 3 belonging to *F. hepatica* may support the polymorphism and allow understanding of the variation on the β -tubulin gene. Our findings suggest that the genetic diversity of *F. hepatica* β -tubulin isotype 3 of sheep and cattle isolates still continues. However for the better understanding of the sequence variability it needs to more lengths partial sequences.

In conclusion, this is the first report of a β -tubulin isotype 3 characterization in Turkey. The results of the current study contributed to understanding of the genetic structure and heterogeneity of *F. hepatica* β -tubulin isotype 3 in Turkey. However it needs further studies by using more sequenced samples.

Conflict of Interest

The authors fully declare that there is no financial or other potential conflict of interest.

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References

CWIKLINSKI, K., DALTON, J.P., DUFRESNE, P.J., LA COURSE, J., WILLIAMS D.J., HODGKINSON, J., PATERSON, S. (2015): The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation

to the host environment and the capacity for rapid evolution. *Genome Biol.*, 3(16):71. doi: 10.1186/s13059-015-0632-2

ELLIOTT, T., MULLER, A., BROCKWELL, Y., MURPHY, N., GRILLO, V., TOET, H.M., ANDERSON, G., SANGSTER, N., SPITHILL, T.W. (2014): Evidence for high genetic diversity of NAD1 and COX1 mitochondrial haplotypes among triclabendazole resistant and susceptible populations and field isolates of *Fasciola hepatica* (liver fluke) in Australia. *Vet. Parasitol.*, 200(1-2): 90-6. DOI: 10.1016/j.vetpar.2013.11.019

FAIRWEATHER, I., BORAY, J.C. (1999): Fasciolicides: efficacy, actions, resistance and its management. *Vet. J.*, 158: 81 – 112

KHORAMIAN, H., ARBABI, M., OSQOI, M.M., DELAVARI, M., HOOSHYAR, H., ASGARI, M. (2014): Prevalence of ruminants fascioliasis and their economic effects in Kashan, center of Iran. *Asian Pacific J. Trop. Biomed.*, 4: 918 – 922. DOI: 10.12980/APJTB.4.2014AP-JTB-2014-0157

MAS-COMA, S., BARGUES, M.D., VALERO, M.A. (2005): Fascioliasis and other plant-borne trematode zoonoses. *Int. J. Parasitol.*, 35: 1255 – 78. DOI: 10.1016/j.ijpara.2005.07.010

NOGALES, E., WOLF, S.G., DOWNING, K.H. (1998): Structure of the alpha beta tubulin dimer by electron crystallography. *Nature*, 391: 199 – 203

PRUGNOLLE, F., LIU, H., DE MEEUS, T., BALLOUX, F. (2005): Population genetics of complex life-cycle parasites: an illustration with trematodes. *Int. J. Parasitol.* 35: 255 – 263

RYAN, L.A., HOEY, E., TRUDGETT, A., FAIRWEATHER, I., FUCHS, M., ROBINSON, M.W., CHAMBERS, E., TIMSON, D., RYAN, E., FETWELL, T., IVENS, A., BENTLEY, G., JOHNSTON, D. (2008): *Fasciola hepatica* expresses multiple a- and b-tubulin isotypes. *Mol. Biochem. Parasitol.*, 159: 73 – 78. DOI: 10.1016/j.molbiopara.2008.02.001

SIMSEK, S., OZCETIN, C., BALKAYA, I. (2012): Detection of polymorphism in AgB1 gene from sheep, cattle and human isolates of *Echinococcus granulosus* by SSCP. *Vet. Parasitol.*, 184: 352 – 355. DOI: 10.1016/j.vetpar.2011.09.002

TEOFANOVA, D., KANTZOURA, V., WALKER, S., RADOSLAVOV, G., HRISTOV, P., THEODOROPOULOS, G., BANKOV, I., TRUDGETT, A. (2011): Genetic diversity of liver flukes (*Fasciola hepatica*) from Eastern Europe. *Infect. Gen. Evol.*, 11: 109 – 115. DOI: 10.1016/j.meegid.2010.10.002

WHO (2015): Neglected tropical diseases list. Internet Access: http://www.who.int/neglected_diseases/diseases/en/