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Molecular identification of *Paramphistomidae* obtained from ruminants in Van province

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Abstract: This study was aimed to identify *Paramphistomum* species from infected ruminants (sheep and cattle) by molecular methods between March 2018 and September 2018 at Van municipality slaughterhouse. In the research, the rDNA ITS-2 gene region of adult Paramphistomums was amplified by PCR method. Amplicons 399 bp long were viewed in agarose gel. As a result of bidirectional sequence analysis made from PCR amplicons, *Paramphistomum leydeni* and *Calicophoron daubneyi* species were identifyed. Amplicon sequences were compared by BLAST with reference sequences from Genbank. Phylogenetic tree was created with the Neighbor-Joining method by using the MEGA 7 program. Genotypes obtained from isolates were compared for exact or closest similarities. In conclusion, *C. daubneyi*, which was previously detected by morphological methods in Turkey, in this study, it was identified for the first time by using molecular methods. Also in this study, P. leydeni was reported for the first time in Turkey.
Keywords: Molecular identification, *Paramphistomum* spp., PCR, Van.

Van ilindeki ruminatlardan elde edilen *Paramphistomidae*'lerin moleküler olarak belirlenmesi

Özet: Bu çalışmada, Mart 2018-Eylül 2018 tarihleri arasında Van belediyesi mezbahasında enfekte ruminantlardan (koyun ve sığır) elde edilen *Paramphistomum* türlerinin moleküler yöntem kullanılarak belirlenmesi amaçlanmıştır. Araştırmada, erişkin *Paramphistomum*'ların rDNA ITS-2 gen bölgesi PCR yöntemi ile çoğaltıldı. Agaroz jelde, 399 bp uzunluğunda amplikonlar görüntülendi. PCR amplikonlarından yapılan çift yönlü sekans analizi sonucunda *Paramphistomum leydeni* ve *Calicophoron daubneyi* türleri belirlendi. Amplicon dizileri, BLAST ile Genbank'taki referans dizilerle karşılaştırıldı. MEGA 7 programı kullanılarak Neighbor-Joining yöntemi ile filogenetik ağaç oluşturuldu. İzolatlardan elde edilen genotipler tam yada en yakın benzerlikleri karşılaştırıldı. Sonuç olarak, Türkiye'de daha önce morfolojik yöntemlerle tespit edilen *C. daubneyi*, bu çalışmada moleküler yöntemler kullanılarak ilk kez tespit edildi. Ayrıca bu çalışmada, Türkiye'de ilk kez *P. leydeni* bildirildi.
Anahtar sözcükler: Moleküler belirleme, *Paramphistomum* spp., PCR, Van.

Introduction

Paramphistomosis or amphistomiasis is known as a disease caused by parasites classified in *Paramphistomidae* and infects various domestic and wild ruminant species. The adult form of the parasite resides in the rumen and reticulum and the young form resides in the small intestine. While small numbers of adult parasites do not cause serious problems in the hosts, larger numbers of parasites damage the rumen tissue. Clinical symptoms might not be present despite the damage and loss in the rumen papillae caused by the adult parasites. Young and immature paramphistomes, resident in the first part of the duodenum and ileum, cause severe hemorrhage and necrosis in this region. Although mature flukes of paramphistomum species are not very pathogenic, immature flukes are trematodes that can cause serious pathological disorders, loss of yield, clinical symptoms and adversely affect animal health. (2, 3, 5, 20, 26).

Species of the genus *Paramphistomum* live in the stomach (rumen-reticulum) and rarely in the bile ducts of the ruminants. They are observed in countries with tropical and subtropical climates. Several studies reported two sub-genera such as *Paramphistomum* and *Explanatum*, based on the ratio between the mouth sucker and posterior sucker sizes (26).

Although the disease is identified worldwide, it is known to be more common in tropical and subtropical regions, especially in Africa, Asia, Austria and Western

Europe. The prevalence of *Paramphistomum* species varies between regions depending on various variables such as host, intermediate host, active species and meteorological and environmental factors. Therefore, different prevalence rates have been reported in studies conducted in various countries. The most common type in Turkey is *Paramphistomum cervi*, followed by *Paramphistomum ichikawai* and *Calicophoron daubneyi* (10, 15, 20).

The traditional method for identifying these economically significant helminths is based on common morphological features. Furthermore, the traditional diagnostic method is challenging and error-prone, particularly for soft-bodied parasites such as trematodes, which are morphologically similar but genetically diverse in terms of resistance and sensitivity. It could eliminate such challenges in identifying these structural parasites through the use of ultra-structural observations (9, 18, 24).

Histomorphological identification of parasites was conducted with the examination of "median-sagittal" sections that pass through the center of muscular organs such as pharynx, genital hole and acetabulum. However, these morphological differences are highly challenging and have several disadvantages. Molecular methods, especially those used in phylogenetic studies, were proposed as an alternative method for the differentiation of the species. ITS-2 gene region sequence could be used as a specific marker for this purpose. For instance, polymerase chain reaction (PCR) could facilitate the diagnosis through studying highly preserved molecular markers such as ITS-2 rDNA (11, 21, 22).

Given that *Paramphistomum* species could be morphologically similar, molecular biological techniques could be helpful in the classification and identification of these species. Itagaki et al. (8) and Rinaldi et al. (17) characterized several *Paramphistomum* genera and species by molecular methods and by focusing on the ITS-2 gene region that characterized the species (19).

This study aimed to determine the molecular characterization of paramphistomoid parasites infecting cattle and sheep using molecular methods in the province of Van in Turkey.

Materials and Methods

In this study, were examined the rumen and reticulum of sheep and cattle slaughtered by regular visits to the Van Municipality Slaughterhouse 4 times a month between March 2018 and September 2018. A total of 2600 sheep and 1335 cattle were examined after slaughtering, 169 sheep and 195 cattle were identified as infected. An average of 30-40 adult flukes were collected from the rumen and/or reticulum of naturally infected ruminants and placed in labeled containers.

The collected adult flukes were washed with PBS (Phosphate-buffer saline) and placed in 70% ethanol. The samples were kept at -20°C until the tests were conducted at the Department of Parasitology, the Faculty of Veterinary Medicine, Van Yüzüncü Yıl University, Turkey.

Molecular analysis: Paramphistomes, which were initially preserved with 20 ml of ethanol, were washed 5 times with PBS for genomic DNA isolation. Subsequently, paramphistomes were thoroughly crushed in a homogenizer (Brand: Qiagen Model: Tissue LyserII). The tissues, crushed based on the kit procedure, were transferred to Eppendorf tubes. The DNA purification was conducted manually based on the general guidelines provided by the Thermo Scientific GeneJET Genomic DNA Purification Kit (Catalog number: K0722).

Paramphistomum spp. were identified using ITS-2 F 5′-AGAACATCGACATCTTGAAC-3' (1) and R 5′TATGCTTAAATTCAGCGGGT-3′ (13) primers by PCR. The total volume of 25 µl PCR was prepared with 2.5 µl 10X PCR buffer, 1 µl 25mM $MgCl_2$, 0.5 µl 25mM dNTP, 0.5 µl from each primer, 5U/µl 0.2 µlTaq DNA polymerase, 5 µl genomic DNA and 14.8 µl dH_2O. The cycling conditions used were 94°C for 5 min (initial denaturation), then 94°C for 1 min (denaturation), 50°C for 35 s (annealing), 72°C for 1 min (extension) for 30 cycles, and a final extension at 72°C for 10 min. (Brand: BIO-RAD, Model: Power pac Basic). The PCR product was run on 2% agarose gel at 100 volts for 1 hour (Electrophoresis: BİO-RAD, Model: Power pac Basic). The samples were imaged under UV light (Figure 1).

Sequence analysis and phylogenetic analysis: 25µl of those PCR products that were run on agarose gel and exhibited a DNA sequence placed into PCR tubes and 2 µl primer (10 pmol) was used for each sample. The tubes were tagged and sent to Medsantek (Istanbul, Turkey) for sequence analysis. The amplicons purified at Medsantek were duplex sequence analysis with an Applied Biosystems 377 DNA Sequencer device. Phylogenetic tree was created via the neighbor joining method and the Bootstrap test (100 repetitions). The evolutionary closeness between the obtained *P. leydeni* and the sequences of *C. daubneyi* isolates was created an UPGMA dendrogram using the MEGA 7 program and the neighbor joining model (Figure 2). The phylogenetic tree obtained by comparing the sequences of the amplified ITS-2 region with KP201674, AY790883, LN610457, KP201674, KX668976, KX668943, KX668944, MH558675, KJ995529,HM209064, KF564869 and KC503920 are presented in Figure 2. *Schistoma haematobium* was used as the outer group (KT354669).

Results

A product size of 399 bp was obtained from the PCR products ran on electrophoresis (Figure 1). As a result of sequence analysis of amplicons obtained from adult parasites; *Paramphistomum leydeni* was identified from 5 sheep and 3 cattle, and the *Calicophoron daubneyi* was identified from 2 cattle.

ITS-2 sequences for all isolates were successfully amplified and the sequences produced were registered to GenBank. Access numbers of GenBank registered isolates are shown in Figure 2. Depending on the upper class of *Paramphistomatidae* in Dendogram; Evolutionary proximity between *P. leydeni*, *C. daubneyi*, *P. cervi*, *Explanatum explanatum*, *Cotylophoron* spp. and the *C. clavula* species, that are supported by a high boostrap value 100% in the *C. daubneyi* and 96% in the *P. leydeni*. It was observed that the *P. leydeni* and *C. daubneyi* isolates obtained from cattle and sheep showed 100% similarity in terms of nucleotide sequences among themselves. In the comparative nucleotide sequences of the ITS-2 gene region of the our isolates, no genetic differences were observed between *P. leydeni* 8 isolates and *C. daubneyi* 2 isolates (Figure 3).

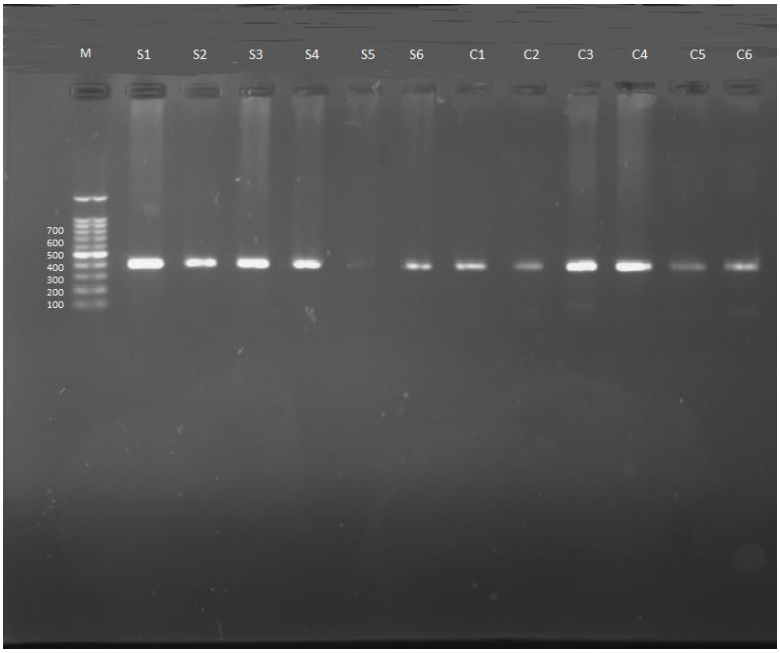

Figure 1. PCR amplification of rDNA ITS2 gene region in *Paramphistomum* species found in sheep and cattle (Amplicon length 399 bp).
S (1-2-3-4-6): PCR amplicons of adult parasites from sheep.
C (1-2-3-4-5): PCR amplicons of adult parasites from cattle.

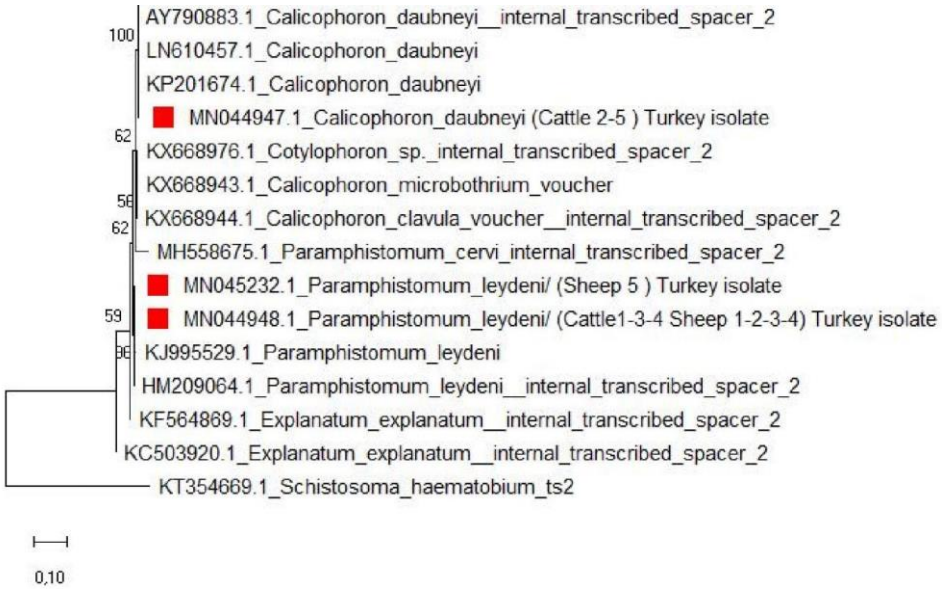

Figure 2. Phylogenetic relationship of the sequences obtained in the study and the sequences obtained from GenBank.
The tree was constructed by the neighbor -joining analysis (NJ) with genetic distance of 0.10.

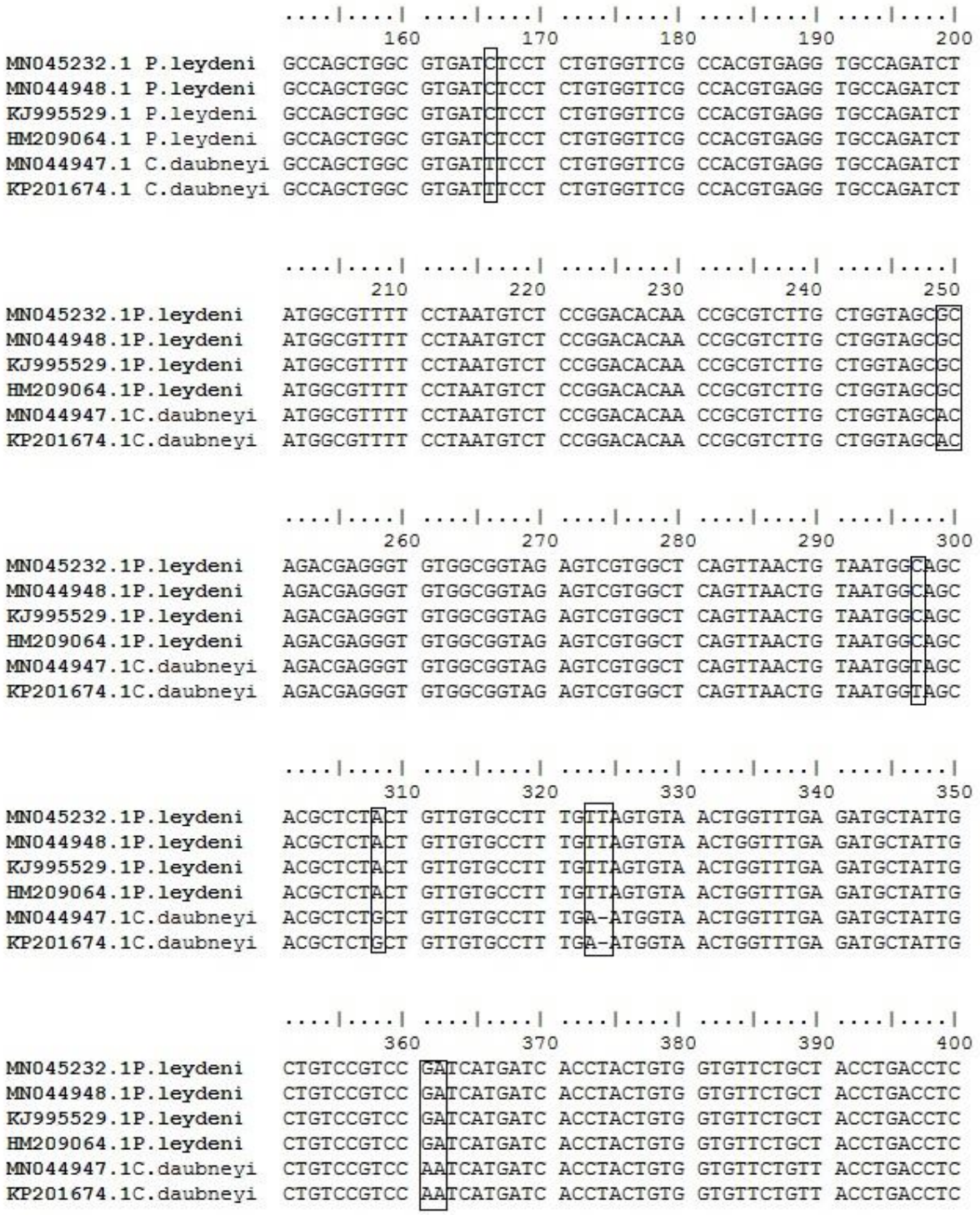

Figure 3. Comparative nucleotide sequences of the ITS-2 gene region of the isolates.

Discussion and Conclusion

Paramphistomoid worms are difficult to identify because most have thick robust bodies in which the internal organs are hard to see. Specific identification becomes more difficult, as the immature flukes responsible for the disease are not sexually mature. Therefore, the researcher can make a dubious diagnosis to identify the few mature flukes that can be found in the animal's rumen. Because of such problems, better alternative approaches need to be developed to identify the species in this group. A variety of molecular tools now complemented traditional diagnostic techniques in parasitology to help in resolving the vexing taxonomic issues associated with describing new species or strains

based on phenotypic characteristics and/or epidemiological observations in particular endemic areas. PCR-based techniques that provide rDNA ITS-2 sequences have proven to be a reliable tool to identify digenean species and restore phylogenetic relationships. In addition, ITS-2 has been found to be a useful marker for species identification of parampistomes (4, 6, 8, 9, 12, 16, 17, 25, 27, 28).

A variety of molecular methods are used in epidemiological parasitology studies in certain endemic regions to assist in solving complex taxonomic problems associated with conventional diagnostic assays or the identification of new species and strains based on the origin of phenotypic features (25). In this study, DNA analyzes of *Paramphistomum* spp. collected from ruminants (cattle and sheep) in Van province was performed, studied by PCR (polymerase chain reaction) method, and the rDNA ITS-2 gene region was characterized. As a result of sequence analysis of amplicons, *Calicophoron daubneyi* from 2 cattle, *Paramphistomum leydeni* from 3 cattle and 5 sheep were identified. The presence of *C. daubneyi* was previously reported in Turkey by morphological diagnosis method, and its presence was reported using molecular methods in this study.

In the molecular study on paramphistomum eggs in Ireland; *C. daubneyi* and *P. leydeni* have been reported (14). In this study, the *C. daubneyi* sequences matched with sequences isolated from Ireland (14), Algeria (27) and southern Italy (17).

Some researchers have investigated possible intra-specific variations of ITS-2, due to the fact that length differences were not observed in PCR products of *C. daubneyi* samples. The study also did not see any intra-specific variations between the samples. In the study, confirmed by the ITS-2 sequence analyses, which indicated 100% homologies. The finding of the present study suggests that ITS-2 can serve as an effective genetic marker for the molecular identification of paramphistomes, as already demonstrated for other parasitic helminths (17).

Paramhistomum parasites collected from a reindeer in Croatia reported *P.cervi* and *P. leydeni* as a result of sequence analysis conducted in the rDNA ITS-2 gene region by molecular methods (23). Sanabria et al. (19) *Paramphistomum leydeni* was identified molecular using 5.8S and 28S partial ITS-2 rDNA sequences. Ichikawa et al. (7) studied fluke parasites cattle and water buffalo from Myanmar for morphological and molecular characterization using ITS-2, partial 5.8S and 28S rDNA sequences. ITS-2 sequences of *Explanatum explanatum* specimens showed similarity and variability at 7 nucleotide sites compared to *Paramphistomum leydeni*. In this study, unlike previously described species as macroscopically in Turkey that, *P. leydeni* was reported for the first time using molecular methods. The *P. leydeni* sequences in this study matched those isolated from cattle from Uruguay (unpublished), cattle from Argentina (19).

In conclusion, paramphistomiasis in ruminants is still a widespread parasitic infection that leads to economic losses in the Van region of Turkey. Molecular methods, which provide accurate results, have the potential to contribute to the taxonomic identification and epidemiological studies of paramphistomiasis. The results of the present study confirm that ITS-2 is a useful molecular marker for species identification of paramphistomes and can be used to determine the relationship of samples within the different taxa of *Paramphistomoidea*. The use of the ITS-2 rDNA gene region for the identification of *Paramphistomum* species was carried out for the first time in Turkey by PCR method, and a species not previously reported in Turkey was identified with this study. Since there are few studies focusing on the taxonomic and morphological description of *Paramphistomum* in Turkey, the importance of a careful review of previous findings on paramphistomum infections was emphasized. We believe that this study will be an important resource for interspecies variation studies of paramphistomas and will provide an exemplary study for comparative ribosomal genomic and systematic studies of digeneans in Turkey.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors have no conflict of interest.

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