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RESEARCH ARTICLE

Prevalence and Molecular Characterization of *Moniezia* Species in Ruminants Based on ITS1-5.8S rRNA from Van Province, Turkey

Ali Bilgin Yilmaz[1*], Erkan Azizoglu[2], Özdemir Adizel[3], Yaşar Göz[1], Burcak Aslan celik[4], Ozgur Yasar CELİK[5], Ozge Oktay Ayan[6], Aizhan Akhmetzhanova[7], Adnan Ayan[8], Muhammed Yasul[9], Assem Myrzhiyeva[10] and Ugur Uslu[11]

[1]Faculty of Health, Van Yuzuncu Yil University, Van, Türkiye; [2]Department of Plant and Animal Production, Colemerik Vocational School, Hakkari University, 30100, Hakkari, Türkiye; [3]Department of Biology, Faculty of Science, Van Yuzuncu Yıl University, 65080, Van, Türkiye; [4]Department of Parasitology, Faculty of Veterinary Medicine, Siirt University, Siirt, Türkiye; [5]Department of Internal Medicine, Faculty of Veterinary Medicine, Siirt University, Siirt, Türkiye; [6]Department of Parasitology, Faculty of Medicine, Van Yuzuncu Yil University, Van, Türkiye; [7]Department of Veterinary, Faculty of Agrarian, Shakarim State University of Semey, Kazakhstan; [8]Department of Parasitology, Faculty of Veterinary Medicine, Aksaray University, Aksaray, Türkiye; [9]Department of Parasitology, Faculty of Medicine, Van Yuzuncu Yil University, Van, Türkiye; [10]Veterinary Sciences, Kazakh National Agrarian Research University, Kazakhstan; [11]Department of Parasitology, Faculty of Medicine, University of Istanbul Medeniyet, Istanbul, Türkiye
*Corresponding author: alibilginyilmaz@yyu.edu.tr

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ABSTRACT

This study aimed to calculate the occurence of *Moniezia* species in cattle, sheep, and goats in Van province and to identify these species using morphological and molecular methods (ITS1-5.8S rRNA gene region analysis). Additionally, the study aimed to identify the genetic differences between *Moniezia expansa* and *Moniezia benedeni*. During the summer of 2022, intestinal contents were collected from 150 ruminants (50 cattle, 50 sheep, and 50 goats) slaughtered in slaughterhouses in Van province. The parasites were examined using Aceto-Carmine staining, and species identification was based on interproglottidal glands. Examination of the intestinal contents revealed that 2 out of 50 cattle (4%), 14 out of 50 sheep (28%), and 9 out of 50 goats (18%) were infected with *Moniezia*. Morphological and molecular analyses showed that the cattle samples were identified as *Moniezia benedeni*, goats samples as *Moniezia expansa* and those from sheep as 11 *Moniezia expansa* and 3 *Moniezia benedeni*. Following DNA extraction, the ITS1-5.8S rRNA gene region was amplified using PCR and subjected to sequence analysis. The relationship between species was examined by phylogenetic tree. This study confirms the prevalence of *Moniezia spp.* in Van/Türkiye by using the ITS1-5.8S rRNA gene.

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INTRODUCTION

Moniezia species, belonging to the family Anoplocephalidae within the order Cyclophyllidea, are cosmopolitan cestodes that inhabit the small intestinal tract of ruminants (Jyoti and Juyal, 2014; Diop *et al.*, 2015). Like all cestodes, *Moniezia* species have an indirect life cycle. Their definitive hosts are ruminants, and their intermediate hosts are oribatid mites (Denegri, 1993). Due to the high prevalence of oribatid mites in pastures during the summer, transmission primarily occurs in the summer months, and the parasite load in animals increases in late summer and fall (Ardeleanu *et al.*, 2007). Young ruminants are more susceptible to infection, as the prevalence of *Moniezia* is higher in younger animals compared to older ones. Although most *Moniezia* infections in ruminants are asymptomatic, a heavy parasite burden can lead to clinical signs such as weight loss, constipation, diarrhea, dysentery, and occasionally anemia. These symptoms are most commonly observed in young animals with poor nutritional status (Constable *et al.*, 2017).

However, *Moniezia* species are challenging to identify based solely on morphological characteristics, with considerable debate surrounding the validity of specific species or individual traits (Schmidt, 1986). Although at least 12 *Moniezia* species have been

described, morphological identification remains difficult due to the absence of interproglottidal glands and variations in rosette patterns (Diop *et al.*, 2015; Gao *et al.*, 2021). Given the limited or absent morphological features that can reliably distinguish between *Moniezia* species, the development of genetic markers is essential for accurate species identification and to provide a foundation for taxonomic research (Chilton *et al.*, 2007). Several studies have explored the evolutionary biology and population genetics of *Moniezia* species, utilizing NADH dehydrogenase subunit I (Nad1), Internal Transcribed Spacer 1 (ITS1), Cytochrome c oxidase subunit I (Cox1), 5.8S region of ribosomal RNA (5.8S rRNA), and Small subunit ribosomal ribonucleic acid (SSU rRNA) gene regions as molecular markers (Alberfkani *et.al*., 2022; Nagarajan *et.al*., 2023; Kumar and Kaur, 2023; Alshammari *et.al*., 2024). Molecular analysis of the ITS1 and 5.8S region of RNA has been found to be a highly useful marker for determining interspecific relationships among helminths (Ando *et al.*, 2006; Alberfkani *et al.*, 2022). The internal transcribed spacer 2 regions within the rRNA gene have been used as molecular markers for species identification due to the precise differences in sequences among closely related species and the minimal intra-species sequence variation. The 5.8S rRNA gene exhibits conserved sequences; therefore, the sequence of this gene has been utilized for phylogenetic analysis among distantly related organisms and for designing universal PCR primers.

The aim of this study was to diagnose *Moniezia* species from cattle, sheep and goats in Van province in eastern Turkey using traditional and molecular methods and to determine the prevalence of *Moniezia* species in these hosts.

MATERIALS AND METHODS

Study area-based sample collection: This study was conducted during the summer of 2022 in Van province (38°29'53"N, 43°22'22"E), located in the Eastern Anatolia region of Turkey. During the study period, the intestinal contents of 150 ruminants (50 sheep, 50 goats, and 50 cattle) slaughtered at different slaughterhouses were examined. The collected samples were transported to the laboratory. Washing of adult tapeworms was done with 0.85% normal saline solution and placed separately in sample containers containing 70% ethanol. The samples were kept at 4°C for subsequent DNA extraction.

Morphological identification: A portion of the parasites, specifically mature proglottids, was stained with asetocarmine (Loos-Frank, 2000) and mounted in Canada balsam. *Moniezia* species were identified based on the morphology of the inter-proglottidal glands (IPGs) using relevant literature (Schmidt, 1986). Another portion of each parasite was preserved in 70% ethanol for subsequent molecular analysis.

DNA extraction: Genomic DNA from each parasite sample was extracted using Genomic DNA extraction Kit provided by Fermentas (Germany) and subsequently stored at -20°C for PCR analysis. The ITS1 and 5.8S rRNA gene regions of the parasites from collected samples were amplified using primers given in Table 1 (Nguyen *et al.*, 2012).

Table 1. Nucleotide sequences of oligonucleotides used in the study.

Primer	Sequence	Nucleotide size (bp)
ITS1-F	5'-GCTGCTACCCGCATGATGTT -3'	
ITS1-R	5'-GGCAAGCCTATAGCCGCAAT-3'	692bp-718bp
ITS1-R1	5'-AGCAATAGTGCTTTAACGCGC -3'	

PCR amplification: In the PCR experiment, reaction volumes of 50µl were used. The reaction mixture contained 50pmol of each primer, 5µl of 10X PCR buffer [composition: 750mM Tris-HCl (pH 8.8), 200mM $(NH_4)_2SO_4$, 0.1% Tween 20], 200µM dNTPs, 2mM $MgCl_2$, 1.25U Taq DNA polymerase (Fermentas), 100ng of DNA template, and nuclease-free water. The PCR cycling conditions were as follows: initial denaturation at 94°C for 5min, followed by 35 cycles consisted of denaturation at 94°C for 1min, annealing at 54°C for 1min, and elongation at 72°C for 1min, with a final elongation step at 72°C for 10min. The amplified PCR products were separated on a 1.5% agarose gel by electrophoresis at 100 volts for 1 hour. The resulting bands were observed and photographed using a gel documentation system.

Sequence and phylogenetic analysis: To get the sequences, PCR products were sent to a private company (Medsantek, Istanbul, Turkey). Sequence chromatograms were checked and edited using BioEdit software (Hall, 1999). The final consensus sequences of isolates were compared with the isolates reported from various countries in the GenBank database. *Stilesia globipunctata* was used as the outgroup. The construction of phylogenetic tree was done by employing Maximu Liklihood Method using 1000 bootstrap replicates in MEGA 7.0 program (Kumar *et al.*, 2016).

RESULTS

As a result of this study, it was found that 4% (2/50) of cattle, 28% (14/50) of sheep, and 18% (9/50) of goats were infected with *Moniezia* species. Following morphological identification, the two samples detected in cattle were identified as *Moniezia benedeni*, while among the species detected in sheep, 11 samples were identified as *Moniezia expansa* and three samples as *Moniezia benedeni*. All nine samples from goats were identified as *Moniezia expansa*.

The adult form of the parasites, along with the vitelline gland, interproglottidal glands, and scolex in mature segments, were visualized using Aceto-Carmine staining (Fig. 1). In parallel with species identification through the Aceto-Carmine staining method, PCR and sequencing procedures were performed to molecularly distinguish between *M. expansa* and *M. benedeni.* The ITS 1F-ITS 1R primer pair produced PCR bands of 692 bp for *M. expansa* and 718 bp for *M. Benedeni*, based on nucleotide sequence results (Fig. 2).

Fig. 1: Morphological identification of *Moniezia* species. A- Mature *Moniezia spp*.; B- *Moniezia* spp. mature proglottid with degenerated Vitteline gland; C- Scolex; D- Mature proglottides of *Moniezia expansa* and IPGs; E- Mature proglottides of *Moniezia benedeni* and IPGs.

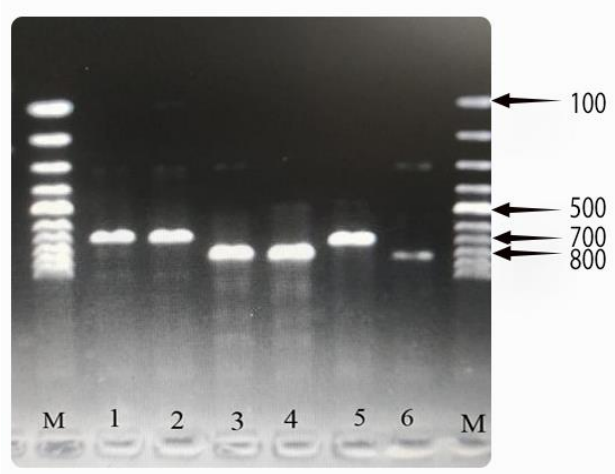

Fig. 2: PCR image showing amplification of ITS1–5.8S rRNA gene region of *Moniezia expansa* and *Moniezia benedeni*. M: 1000 bp marker; lanes 1, 2, and 5: *Moniezia expansa* (692 bp); lanes 3, 4, and 6: *Moniezia benedeni* (718 bp).

PCR was applied to a total of six samples, including two from cattle, two from goats, and two from sheep. Gene regions of the expected size were amplified from all six samples taken from cattle, goats, and sheep. Sequencing was performed on three positive PCR samples using forward and reverse primers. *M. expansa* isolates were registered in NCBI Gen Bank with accession numbers OR636090.1 and OR636119.1, while the *M. benedeni* isolate was registered with accession number OR644374.1.

Nearly complete sequences of the ITS1 gene region (682 bp and 718 bp) were obtained from *Moniezia* samples collected from cattle, sheep, and goats. The phylogenetic tree and genetic relationship analysis were constructed based on the ITS1 gene sequence, taking into account the similarity rates of *M. expansa* and *M. benedeni* isolates from the small intestines of cattle, sheep, and goats with global isolates obtained from NCBI GenBank (Fig. 3).

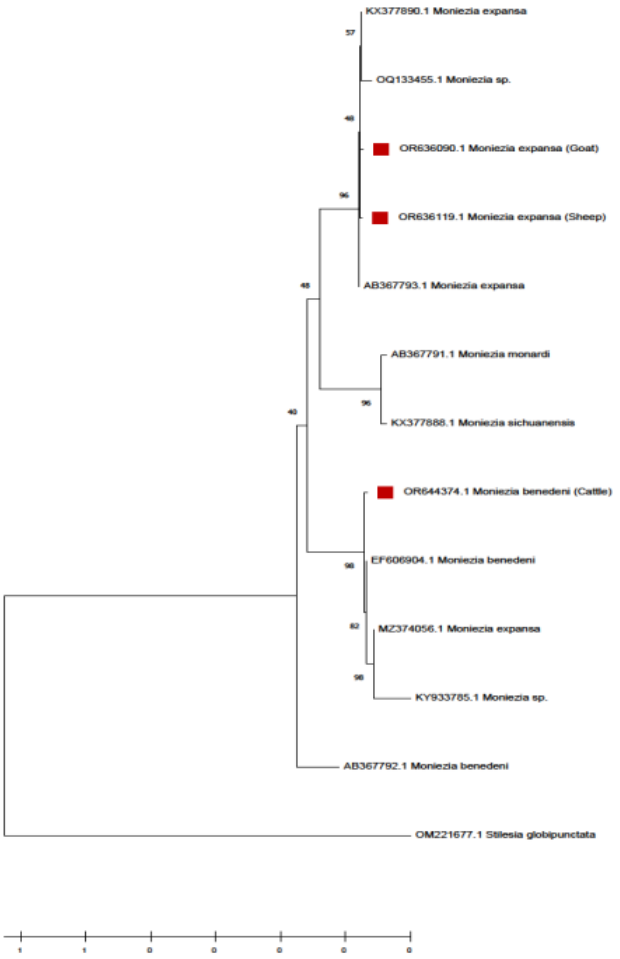

Fig. 3: Phylogenetic relationships of *Moniezia sp.* isolates based on the ITS1–5.8S rRNA gene region. The phylogenetic tree was constructed using the Maximum Likelihood method based on the ITS1–5.8S rRNA gene region. A bootstrap value of 1000 was selected for the analysis, and *Stilesia globipunctata* was used as the outgroup.

DISCUSSION

Moniezia expansa and *M. benedeni* are among the most common helminths found in ruminants, particularly in sheep, goats, cattle, zebu, buffalo, and camels in various countries around the world (Memmedov, 2009). In this study, the genetic diversity of *Moniezia spp*. collected from cattle, sheep, and goats in Van province was investigated.

Various studies on the detection and incidence of *Moniezia* species have been conducted worldwide. In a study conducted in Vietnam, prevalence rates of 20.6% in goats, 16.4% in sheep and 5.4% in cattle were reported. Of the 75 *Moniezia spp*. obtained from cattle, 9 were identified as *M. expansa* and 66 as *M. benedeni*. In goats, 138 were identified as *M. expansa* and 24 as *M. Benedeni from 162 Moniezia* species. Of the 150 *Moniezia* species collected from sheep, 132 were identified as *M. expansa* and 18 as *M. benedeni* (Nguyen *et al.*, 2012). In a study conducted in India, 2.39% of sheep were found to be infected with *M. expansa*, while *M. benedeni* was not detected in any of the sheep examined. Among goats, *M. expansa* and *M. benedeni* prevalence was 2.56% and 0.13%, respectively

(Dappawar *et al.*, 2018). In a study conducted on small ruminants in Pakistan, the overall infection rate was reported to be 27.2%, with higher infection rates observed the younger age group (<1 year; 32.9%) and in males (29.8%). Additionally, molecular and phylogenetic analysis based on partial sequencing of the cox1 gene revealed that these sequences were grouped within the *M. expansa* cluster. Among the Pakistani isolates, two distinct haplotypes were identified without any host tropism (Muqaddas *et al.*, 2024). In a study conducted by Hassanein *et al.* (2022) in Cairo, Egypt, the small intestines of 120 sheep were examined for cestode infections. The results revealed that *Moniezia expansa* was present with a prevalence of 10.8%. Analysis of the ITS-2 gene region identified different genotypes showing sequence identities ranging between 98.90% and 100%. The evolutionary differences between *Moniezia spp.* and other cestode genera were reported to range from 0.000 to 1.356. In the study conducted by Alberfkani *et al.* (2022), sequencing analysis using the cox1 gene revealed that 25 of the samples were *Moniezia expansa* and 7 were *Avitellina centripunctata*. Meanwhile, analysis using the ITS1 gene identified 20 *Moniezia expansa* and 12 *Moniezia spp*. The sequencing analysis confirmed the identity of these species with a homology rate ranging between 99.6% and 100%.

The prevalence rates reported in other studies are as follows: 94.10% in goats, 3.69% in cattle, and 1.10% in sheep in Malaysia (Zainalabidin *et al.*, 2021); 27.8% in sheep in Nakhchivan (Memmedov, 2009); 0.39% in cattle and 4.4% in sheep and goats in Ghana (Squire *et al.*, 2019); 18.22% in goats in Egypt (Hassan *et al.*, 2019); 25.34% and 15.48% in young and adult sheep, respectively, and 2.56% and 5.13% in goats in Spain (Rufino-Moya *et al.*, 2024); 2.11% in small ruminants in Bangladesh (Rahman *et al.*, 2017); and 2.11% in small ruminants in Ethiopia (Admasu and Nurlign, 2014). A study conducted in Vietnam using the 5.8S, ITS2, and cox1 gene regions revealed that *M. expansa* 5.8S-ITS2 sequences exhibited a high similarity (99.7%) to those from Japan and India, forming a distinct clade separate from *M. benedeni* identified in the study (Nguyen *et al.*, 2012; Ohtori *et al.*, 2015; Nagarajan *et.al*., 2023). In the case of cox1 sequences, *M. expansa* showed high similarity to Ethiopia sequences, as well as some Senegal and China sequences, clustering together into a common clade that was distinct from other clades observed in Senegal and China (Tam *et al.*, 2020).

As a result of this study, a prevalence rate of 4% was detected in cattle, and the identified *Moniezia* species was determined to be *M. benedeni*. These findings are consistent with previous studies (Nguyen *et al.*, 2012; Zainalabidin *et al.*, 2021). The prevalence rate in goats was found to be 18%, with the samples identified as *M. expansa*. These results align with studies conducted by other researchers (Nguyen *et al.*, 2012; Hassan *et al.*, 2019). In sheep, the study reported a prevalence rate of 28%, with 11 samples identified as *M. expansa* and three as *M. benedeni*. These findings are in agreement with the results of previous studies (Memmedov, 2009; Nguyen *et al.*, 2012). The differences observed among studies can be attributed to factors such as geographical conditions, climate, humidity levels, the age and education level of farmers, farm management practices, and the duration of animals' grazing periods on pastures (Zainalabidin *et al.*, 2021).

The morphological differentiation between *M. benedeni* and *M. expansa* has traditionally relied on the morphotype of the IPGs. However, rosette-type IPGs may be absent in certain regions or even throughout the entire strobila, while linear IPGs can be difficult to detect in poorly stained specimens, posing challenges for species-level morphological identification (Schmidt, 1986). Some studies have reported specimens lacking IPGs in goats and sheep from Ethiopia and Senegal (Diop *et al.*, 2015) or in goats, sheep, and cattle from central Vietnam (Nguyen *et al.*, 2012). In this study, no such defective specimens were encountered.

In this study, the *M. expansa* isolate obtained from goats (OR636090.1) showed a high similarity with samples from goats in China (KX377890.1, 99.58%), sheep in Japan (AB367793.1, 98.73%), and cattle in Iraq (ON454638.1, 98.51%). The *M. expansa* isolate obtained from sheep (OR636119.1) exhibited similarity with samples from goats in China (KX377890.1, 99.10%), sheep in Japan (AB367793.1, 98.36%), and cattle in Iraq (ON454637, 97.92%). Additionally, in this study, the *M. benedeni* isolate obtained from cattle (OR644374.1) was found to be similar to samples from cattle in Japan (LC628889.1, 99.82%), bison in Poland (EF606904.1, 99.18%), and zebu in India (OQ450348.1, 98.14%).

Based on the findings obtained from our study, we suggest that our strains may be derived from the ancestors of Polish and Chinese isolates and that factors such as intermediate host movements, import, and export processes may play a role in the worldwide distribution of these ancestors.

For the evolutionary analysis utilizing ITS1–5.8S rRNA nucleotide sequences, a maximum likelihood tree (Fig. 3) was generated with the highest log-likelihood value of -2339.828. This analysis incorporated 14 nucleotide sequences, comprising a total of 779 positions in the final dataset. The number of base substitutions per site between *M. benedeni* and *M. expansa* ranged from 0.15 to 0.01. The estimated transition/transversion bias (R) was 1.78, with 2,244 observed transition and transversion events.

When BLAST was performed on the sequence results of the isolates obtained from the study, it was observed that the overlap rates were low (88%, 92%). The low overlap rate was considered evidence of the presence of cryptic species in *M. expansa* and *M. benedeni*, as demonstrated in the study of Chilton *et al.* (2007) in Australia using multilocus enzyme electrophoresis. Multilocus enzyme electrophoresis is an important method for reliable species identification, as well as for demonstrating the importance of investigating genetic variation among geographical populations to reassess traditional taxonomy. In light of these data, a review of GenBank records shows that nucleotide data of three *Moniezia* species are available: *M. benedeni*, *M. expansa*, and *M. sichuanensis*. The low overlap rates suggest that there are *Moniezia* species other than these three. Considering that *M. expansa* and *M. benedeni* can parasitize the same hosts (Ba *et al.*, 1993) and that *M. expansa* is a common cestode found in ungulates in Europe, Asia, Africa, America, and Australia (Chilton *et al.*, 2007), this study highlights the importance of establishing genetic markers for the accurate identification of *Moniezia* species and forming the basis for taxonomic and population-based studies.

Conclusions: In conclusion, molecular markers are powerful tools used to distinguish individuals or isolates that resemble each other and have overlapping morphological features. This study is the first research on the molecular characterization of *M. expansa* and *M. benedeni* infecting sheep, cattle, and goats reared in Van/Turkey. In this study, genetic differences involving partial sequences of the molecular marker (ITS1–5.8S rRNA) were consistently observed between the two *Moniezia* species collected. Phylogenetic analysis revealed that these worms are clearly distinct species within the same genus. It has been demonstrated that there are differences between the cestodes identified as *M. benedeni* and *M. expansa*. At the same time, phylogenetic trees and morphological observations show that these species are genetically distinct. This study will directly or indirectly facilitate the future identification of these species.

Ethical statement: Ethics committee approval was obtained from the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (Date: 01/09/2021, Approval Number: 94880).

Conflict of interest: There is no conflict of interest.

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