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Original papers

Molecular characterization of the first internal transcribed spacer of rDNA of *Parabronema skrjabini* for the first time in sheep

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ABSTRACT. *Parabronema skrjabini* is a spirurid nematode of the family Habronematidae that lives in the abomasum of ruminants such as sheep and goats. The purpose of this study was to investigate the molecular aspects of *Parabronema skrjabini* in sheep. The worms were collected from sheep in Sanandaj (west of Iran). The first internal transcribed spacer (ITS) of ribosomal DNA (rDNA) nucleotide fragments of *Parabronema skrjabini* were amplified by polymerase chain reaction (PCR) using two pairs of specific primers (Para-Ir-R and Para-Ir-F). ITS1 homology in the sequence of this study was 69% compared with the sequence data in GenBank. To our knowledge, this is the first study in the world exploring the genetic diversity of *P. skrjabini* in sheep based on ITS1.

Key words: *Parabronema skrjabini*, PCR, Sanandaj, Iran

Introduction

Parabronema skrjabini is one of the nematodes that occurs in the abomasum of ruminants and has a wide distribution in Africa, Asia and some Mediterranean countries. It has been reported in Mongolia [1], Kazakhstan [2], Saudi Arabia [3,4], Namibia [5], Turkey [6] and Iran [7]. The abomasum is one of the most important sites for nematode infection, which could be harmful to the health of infected animals and causes economic losses due to reduced weight gain and other production losses [8–10]. Insufficient information is available about possible pathology caused by *P. skrjabini* infection and its molecular dimensions have not been extensively studied. The species introduced in Iran is *P. skrjabini* which lives in the abomasum of small and large ruminants. Prevalence of between 1% and 5.43% in sheep [7,11], 0.8% in wild sheep [12], 28% in camels [13] and 1.72% in buffalo [14] has been reported. A comprehensive study of molecular differences between isolates of *P. skrjabini* have not been carried out. In order to differentiate the abomasal nematodes, evaluation of the ITS1 sequences was selected as the best candidate [15]. A range of studies has demonstrated that polymerase chain reaction (PCR)-based approaches can be used for the species specific identification of parasitic nematodes (from different orders), irrespective of developmental stage [16]. *P. skrjabini* and *Habronema* are located in the same family of Habronematidae. PCR-based assays have been developed for the diagnosis of skin parasitic diseases [17–19] and molecular approaches have been shown to be useful for the specific identification and differentiation of *Habronema microstoma* and *Habronema muscae* [20,21]. The ITS1 sequence of *Parabronema* has only been examined in one study in China in which samples were isolated from camels [22]. The purpose of this study was to investigate the molecular characteristics of *Parabronema* species which were collected from animal husbandry in Iran. Therefore, the present study was conducted to accurately identify *P. skrjabini* infection in sheep and to assess the level of intraspecific variation in this parasite using internal transcribed spacer 1 (ITS1) sequences. Very limited number of studies have been published on population genetics of *P. skrjabini* in ruminants worldwide. To our

knowledge, this is the first study in Iran exploring the genetic diversity of *P. skrjabini* in domestic ruminants. These results can be used to monitor *P. skrjabini* resistance to anthelmintic drugs in a context of new gastro-intestinal parasite control approaches, integrating more ecological tools such as farming management and medicinal plants leading to a less frequent but more effective use of anthelmintic drugs, integrated in a sustainable agriculture program.

Material and Methods

Sample collection. Adult *Parabronema* sp. were recovered from the abomasa of naturally infected sheep during abattoir inspection from Sanandaj provinces in the west of Iran. The samples were labeled and preserved in 70% ethanol until used for molecular examinations.

DNA extraction and PCR. DNA extraction from worms was performed using a DNA extraction kit (MBST, City, Iran) according to the manufacturer's instructions. Extracted DNA was stored at -20°C until further processing. ITS1-rDNA was amplified from each isolate using the primer pairs that were designed based on the rDNA genome sequence reported by Zhang et al. [22]. The forward primer was Para-Ir-F: 5'-GTA GGT GAA CCT GCG GAA GG-3' and reverse primer was Para-Ir-R: 5'-CTG AGC TGA GGT CAA CGA AT-3'. The PCR mixture was in a total volume of 100µL containing 1×PCR buffer, 100 mM $MgCl_2$, 100 µM dNTP mix (Cinaclone, Iran), 20 µM of each primer (Cinaclone Co.), 5 units/µL Taq DNA polymerase (Cinaclone) and 1µl of template DNA (100 ng DNA) in an automated thermocycler. The PCR was performed using the following protocol: 5 min. incubation at 94°C to denature the double stranded DNA, 33 cycles of 45s at 94°C (denaturing step), 45s at 59°C (annealing step), and 45s at 72°C (extension step). Finally, the PCR was completed with an additional extension step for 5 min. at 72°C. Samples without genomic DNA were used as negative controls. The PCR products were analyzed on 1% agarose gels in 0.5×TBE buffer and visualized using Sybersafe staining (Cinaclon, Iran) and a UV transilluminator. The PCR product was purified using a quick PCR product purification kit (MBST, Iran) according to the manufacturer's recommendations.

Sequence analysis and phylogeny. Genomic DNA sequencing based on Sanger's method was performed in both directions for each of the 5 PCR products by the Kawsar Biotech Co. Iran. The sequences were analyzed using the compared to those registered in GenBank (www.ncbi.nlm.nih.gov/) using the 'Basic Local Alignment Search Tool' (BLAST). ITS1 sequence for *P. skrjabini* was aligned and compared with one another and with those of the ITS1 of other spirurids available in GenBank. DNA sequences of closely related species were also downloaded and used in the phylogenetic analysis. Multiple sequence alignments were made with the Clustal W. Phylogenetic analyses were performed based on neighbor joining and maximum parsimony methods using MEGA6 software [23]. Support values for internal nodes were estimated using a bootstrap resampling procedure with 1,000 replicates [24]. The sequences reported in this paper were deposited in GenBank with accession number of KT339317.

Results

The ITS1-rDNA for *P. skrjabini* of sheep isolates were amplified and sequenced. ITS1 sequences were determined for adult worms of sheep isolates from Sanandaj. The sequencing

Table 1. The composition of nucleotides in the ITS1 sequences of *P. skrjabini* in sheep from Iran compared with the ITS1 sequence from GenBank

Sequence Name	Nucleotide				GC(%)	Total
	T	C	A	G		
P. skrjabini	28.89	22.4	32.5	16.2	38.6	308
EU375510	30.2	16.8	33.9	19.1	35.9	298
EU420130	30.2	16.8	33.9	19.1	35.9	298
EU420131	29.7	17.6	33.4	19.3	36.9	296

results were compared with available sequences in GenBank (EU375510). ITS1 homology in our isolate with sequences in Genbank was 69% (Fig.1). Table 1 lists the composition of nucleotides in the ITS1 sequences of *P. skrjabini* in sheep from Iran compared with the ITS1 sequence from GenBank. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [25]. The tree with the highest log likelihood (-786.6283) is shown (Fig. 2). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 115 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [23].

Discussion

P. skrjabini was originally described from Russian Turkestan and is known to occur in that region in cattle, camel, sheep and goats [26]. It has also been reported in Mongolia [1], Kazakhstan [2], Saudi Arabia [3,4], Namibia [5], Turkey [6] and Iran [7]. The introduced species in Iran is *Parabronema skrjabini,* which lives in the abomasum of small and large ruminants. There is no exact molecular study or assessment of its prevalence in Iran. A satisfactory method for molecular analysis is using the ribosomal gene complex (rDNA). A range of studies has demonstrated that polymerase chain

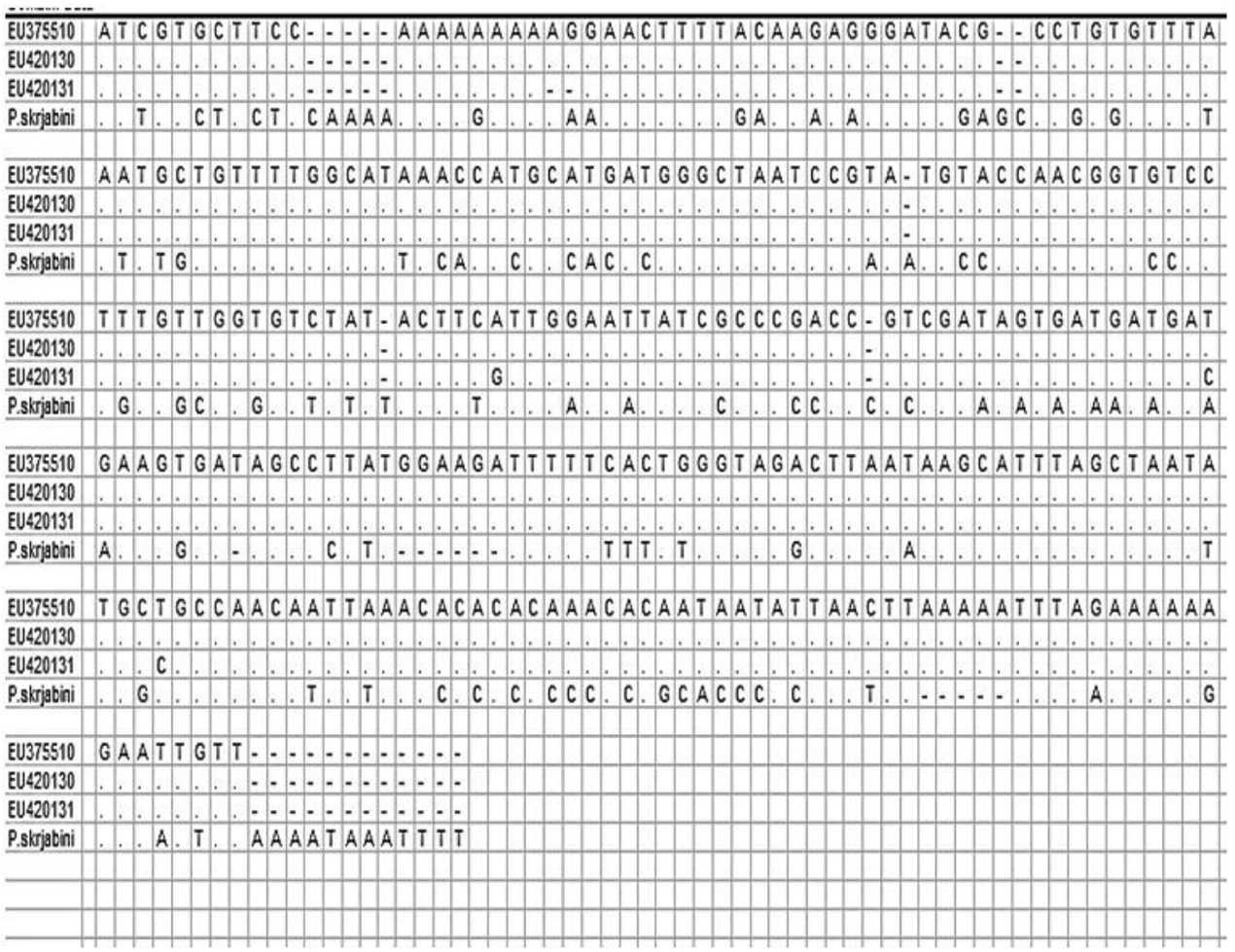

Fig. 1. ITS1 locus alignment of ovine isolates of *P. skrjabini* in the present study and recorded data in GenBank (Accession no. EU375510) using MEGA6. Dots indicate base identical to EU375510 and horizontal lines indicate gaps inserted for optimal alignment or end of the available sequence.

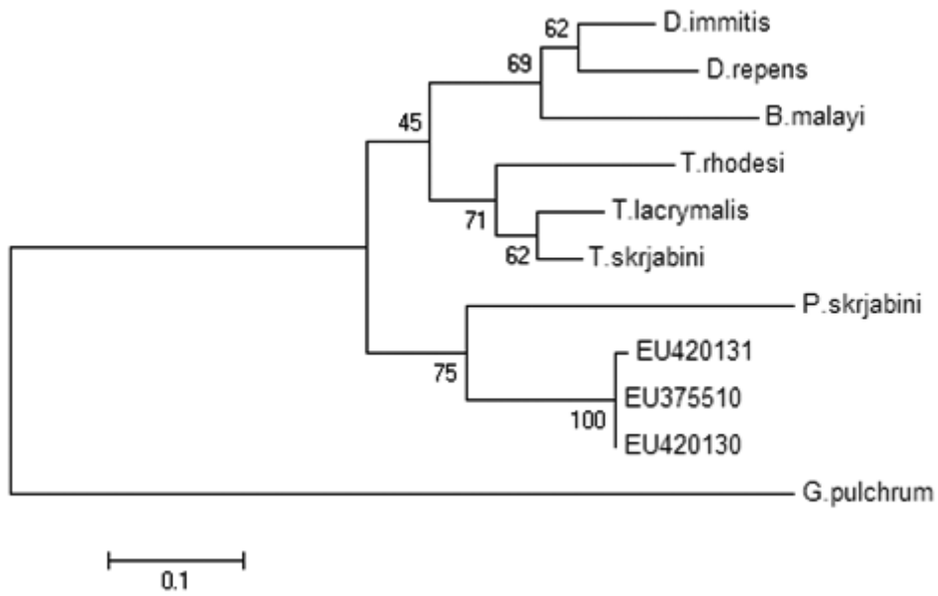

Fig. 2. ITS1 locus alignment of ovine isolates of *P. skrjabini* in the present study and recorded data in GenBank (Accession no. EU375510) using MEGA6. Dots indicate base identical to EU375510 and horizontal lines indicate gaps inserted for optimal alignment or end of the available sequence.

reaction (PCR) can be used for the species-specific identification of parasitic nematodes based on different ribosomal and mitochondrial gene markers [16]. The first and/or second internal transcribed spacers (ITS) of nuclear ribosomal DNA (ITS-1 and ITS-2, respectively) have been indicated for this purpose [27], as a number of investigations have focused on the ITS regions for parasitic nematodes of the orders Strongylida and Ascaridida [28,29], species of *Mansonella* [19], *Dirofilaria*, *Dipetalonema* [30], *Thelazia* [31], etc. *P. skrjabini* and *Habronema* are located in the same family of Habronematidae. Traversa et al. [20] submitted a specific approach for PCR-based identification of *H. microstoma* and *H. muscae* by characterization of the ITS-2 (including 5.8S and 28S ribosomal RNA (rRNA) flanking regions) ribosomal DNA from feces, skin, and muscid fly samples. The molecular characterization revealed that despite the lengths of the ITS-2 of *H. microstoma* and *H. muscae* differing significantly, a number of conserved domains were observed in the ITS-2 sequences of both species; however, the sequences of *H. muscae* were similar in length to the ITS-2 of the spirurid *Onchocerca volvulus* [19]. ITS1 has been used to determine the abomasal parasites in many studies. Molecular studies on the genus *Parabronema* have been mainly on rDNA genes that are available in GenBank. rDNA sequences with accession number of EU375510 and EU420131 were presented in GenBank. They represent ITS1 and ITS2 genes of *P. skrjabini* in camels. They have investigated three *P. skrjabini* regions; the full size ITS of 837bp, ITS2, 18s and 5.8s of 372bp and finally ITS1, 28s and 5.8s of 484bp in length. The homology of the ITS1 was between 48.8% and 61.7%. As previously mentioned, *P. skrjabini* is located in the Spirurida. The ITS region has been used to study spirurid parasites as well. For example ITS1 has been used to study three *Thelazia* species [31]. The aim of their study was to characterize the first internal transcribed spacer (ITS1) ribosomal DNA sequences of these 5 species. The length of the ITS1 sequences ranged from 357 bp (*T. lacrymalis*) to 905 bp (*T. callipaeda*). Interspecific differences ranged from 35 to 77%. Polymorphic sites were detected in each species. Intraspecific variation varied from 0.3 to 2.5%. The characteristics of the ITS1 of *Thelazia* spp. show similarities to those of other Spirurida. In this study the sequencing results were compared with sequences available in GenBank. ITS1 homology from the isolate in the present study was 69% compared with the sequences in GenBank (Fig. 1). Phylogenetic tree analysis depicted in Fig. 2 in relation to different isolates of *P. skrjabini* with other Spirurida (like *Brugia*, *Gongylonema* and *Dirofilaria*) and *P. skrjabini* in GenBank indicated that the Iranian

isolates are from a different lineage to those from camels. Moreover, the other spiruridae used are more distantly related. There was a significant difference between the isolates in this study and sequences in GenBank. The employment of molecular methods does not impoverish the field of systematics, as has been affirmed, but should be treated as part of the data for analyses of the relationships among taxa [32]. Attempts are now being made to integrate morphological and molecular analyses of nematodes, an approach which should provide a more effective means of characterizing nematodes [33,34]. Very limited studies have been published on population genetics of *P. skrjabini* in ruminants in the world and particularly, on the south shore of the Mediterranean Sea. This work is the first study of ITS-1 sequences of *P. skrjabini* from sheep and goats in Iran. Our results show a genetic diversity. These results can be used to monitor *P. skrjabini* resistance to anthelmintic drugs in a context of new gastro-intestinal parasite control approaches integrating more ecological tools such as farming management and medicinal plants leading to a less frequent but more effective use of anthelmintic drugs, integrated into a sustainable agriculture program. Nevertheless, the results obtained herein for ITS1 of *P. skrjabini* open fascinating avenues for future studies investigating in depth the phylogenetic relationships of spirurid nematodes and, in particular, those existing among species ranked within the Thelazioidea and Habronematoidea superfamilies. Both the 5.8S ribosomal RNA gene and ITS1 are either conservative (5.8S ribosomal RNA gene) or divergent to be used for meaningful phylogenetic inference of Habronematoidea within Spirurida [20].

Conclusions

The results reported here provide information on the rDNA of *P. skrjabini* and suggest that the taxonomical and phylogenetic relationships of the *Parabronema* genus within the Spirurida should be investigated more in depth. A better comprehension of the evolution of *P. skrjabini* would be of importance in investigating some still poorly known aspects of ecology and biology of these nematodes (Spirurida), especially regarding their population genetic makeup and relationships with the arthropods acting as intermediate hosts.

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References

[1] Sharkhuu T. 2001. Helminths of goats in Mongolia. *Veterinary Parasitology* 101: 161-169.

[2] Morgan E., Torgerson P., Shaikenov B., Usenbayev A., Moore A., Medley G., Milner-Gulland E. 2006. Agricultural restructuring and gastrointestinal parasitism in domestic ruminants on the rangelands of Kazakhstan. *Veterinary Parasitology* 139: 180-191.

[3] El-Azazy O. 1990. Absence of hypobiosis in abomasal nematodes of sheep and goats in Egypt. *Veterinary Parasitology* 37: 55-60.

[4] Magzoub M., Omer O., Haroun E., Mahmoud O. 2000. Effect of season on gastrointestinal nematode infection in Saudi Arabian camels (*Camelus dromedarius*). *Journal of Camel Practice and Research* 7: 107-108.

[5] Krecek R., Boomker J., Penzhorn B., Scheepers L. 1990. Internal parasites of giraffes (*Giraffa camelopardalis angolensis*) from Etosha National Park, Namibia. *Journal of Wildlife Diseases* 26: 395-397.

[6] Yukari B.A. 2005. Seasonal activity of gastro-intestinal nematodes in goats in Burdur region, Turkey. *Turkish Journal of Veterinary and Animal Science* 29: 441-448.

[7] Eslami A., Nabavi L. 1976. Species of gastro-intestinal nematodes of sheep from Iran. *Bulletin de la Société de Pathologie Exotique* 69: 92-95.

[8] McKenna P. 2010. Update on the prevalence of anthelmintic resistance in gastrointestinal nematodes of sheep in New Zealand. *New Zealand Veterinary Journal* 58: 172-173.

[9] Sissay M.M., Uggla A., Waller P.J. 2007. Prevalence and seasonal incidence of nematode parasites and fluke infections of sheep and goats in eastern Ethiopia. *Tropical Animal Health and Production* 39: 521-531.

[10] Uriarte J., Llorente M., Valderrábano J. 2003. Seasonal changes of gastrointestinal nematode burden in sheep under an intensive grazing system. *Veterinary Parasitology* 118: 79-92.

[11] Nabavi R., Eslami A., Shokrani H., Bokaie S., Shayan P., Saadati D. 2011. Study on the prevalence, intensity and seasonal dynamics of abomasal helminths in sheep from different climatic zones of Iran. *World Applied Sciences Journal* 12: 441-445.

[12] Eslami A., Meydani M., Maleki S., Zargarzadeh A. 1979. Gastrointestinal nematodes of wild sheep (*Ovis orientalis*) from Iran. *Journal of Wildlife Diseases* 15:

263-265.
[13] Mirzayans A., Halim R. 1979. Parasitic infection of *Camelus dromedarius* from Iran. *Bulletin de la Société de Pathologie Exotique et de Ses Filiales* 73:442-445.
[14] Eslami A., Zamani Herglani Y. 1989. Abattoir investigation on the helminth infections of buffalo in Iran. *Journal of the Veterinary Faculty, University of Tehran* 44: 25-34.
[15] Zarlenga D., Gasbarre L., Boyd P., Leighton E., Lichtenfels J. 1998. Identification and semi-quantitation of *Ostertagia ostertagi* eggs by enzymatic amplification of ITS-1 sequences. *Veterinary Parasitology* 77: 245-257.
[16] Blouin M.S. 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal for Parasitology* 32: 527-531.
[17] Fischer P., Büttner D., Bamuhiiga J., Williams S.A. 1998. Detection of the filarial parasite *Mansonella streptocerca* in skin biopsies by a nested polymerase chain reaction-based assay. *The American Journal of Tropical Medicine and Hygiene* 58: 816-820.
[18] Toé L., Boatin B.A., Adjami A., Back C., Merriweather A., Unnasch T.R. 1998. Detection of *Onchocerca volvulus* infection by O-150 polymerase chain reaction analysis of skin scratches. *Journal of Infectious Diseases* 178: 282-285.
[19] Morales-Hojas R., Post R.J., Shelley A.J., Maia-Herzog M., Coscarón S., Cheke R.A. 2001. Characterisation of nuclear ribosomal DNA sequences from *Onchocerca volvulus* and *Mansonella ozzardi* (Nematoda: Filarioidea) and development of a PCR-based method for their detection in skin biopsies. *International Journal for Parasitology* 31: 169-177.
[20] Traversa D., Giangaspero A., Galli P., Paoletti B., Otranto D., Gasser R.B. 2004. Specific identification of *Habronema microstoma* and *Habronema muscae* (Spirurida, Habronematidae) by PCR using markers in ribosomal DNA. *Molecular and Cellular Probes* 18: 215-221.
[21] Iorio R., Šlapeta J., Otranto D., Paoletti B., Giangaspero A., Traversa D. 2009. Phylogenetic relationships of *Habronema microstoma* and *Habronema muscae* (Spirurida: Habronematidae) within the order Spirurida inferred using mitochondrial cytochrome c oxidase subunit 1 (cox1) gene analysis. *Parasitology Research* 104: 979-984.
[22] Zhang X.D., Yang X.Y., Yang L.R., Li L.C., Zuo H.T., Na R.H., Zhao Z.G, Wang J.J. 2009. Cloning and sequence analysis of the rDNA-ITS of *Parabronema skrjabini*. *Chinese Journal of Animal and Veterinary Sciences* 5: 026.
[23] Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: molecular Evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.
[24] Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.
[25] Tamura K., Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10: 512-526.
[26] Sarwar M. 1954. On the transference of *Parabronema skrjabini* Rassowska, 1924 to *Squamanema* Thiel, 1925 and its occurrence in sheep and goats in Indo-Pakistan. *Journal of Helminthology* 28: 143-150.
[27] Gasser R.B., Newton S.E. 2000. Genomic and genetic research on bursate nematodes: significance, implications and prospects. *International Journal for Parasitology* 30: 509-534.
[28] Hung G.C., Gasser R., Beveridge I., Chilton N. 1999. Species-specific amplification by PCR of ribosomal DNA from some equine strongyles. *Parasitology* 119: 69-80.
[29] Zhu X., Gasser R., Chilton N., Jacobs D. 2001. Molecular approaches for studying ascaridoid nematodes with zoonotic potential, with an emphasis on *Toxocara* species. *Journal of Helminthology* 75: 101-108.
[30] Mar P.H., Yang I.C., Chang G.N., Fei A.C.Y. 2002. Specific polymerase chain reaction for differential diagnosis of *Dirofilaria immitis* and *Dipetalonema reconditum* using primers derived from internal transcribed spacer region 2 (ITS2). *Veterinary Parasitology* 106: 243-252.
[31] Otranto D., Tarsitano E., Traversa D., Giangaspero A., De Luca F., Puccini V. 2001. Differentiation among three species of bovine *Thelazia* (Nematoda: Thelaziidae) by polymerase chain reaction–restriction fragment length polymorphism of the first internal transcribed spacer ITS-1 (rDNA). *International Journal for Parasitology* 31: 1693-1698.
[32] Blaxter M.L., De Ley P., Garey J.R., Liu L.X., Scheldeman P., Vierstraete A., Vanfleteren J.R., Mackey L.Y., Dorris M., Frisse L.M. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature* 392: 71-75.
[33] Evans K. 1995. Invited paper: closing the gap between molecular biologists and traditional nematologists. *Nematologica* 41: 385-394.
[34] Thomas W.K., Vida J., Frisse L.M., Mundo M., Baldwin J.G. 1997. DNA sequences from formalin-fixed nematodes: Integrating molecular and morphological approaches to taxonomy. *Journal of Nematology* 29:250.

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