Annals of Parasitology 2016, 62(1), 55–61
doi: 10.17420/ap6201.32

Original papers

Molecular and morphological characterization of *Parabronema skrjabini* of sheep and goats at three different geographical zones in Iran

Seyed Sajjad Hasheminasab, Fatemeh Jalousian, Behnam Meshgi

Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Corresponding Author: Seyed Sajjad Hasheminasab; e-mail: s.sajjad.hn@gmail.com

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 and morphological aspects of *Parabronema skrjabini* in sheep and goats in Iran. The worms were collected from these animal species from three different regions. An internal transcribed spacer 2 ribosomal DNA (ITS2-rDNA) fragment of *Parabronema skrjabini* was amplified by polymerase chain reaction (PCR) using a pair of specific primers (Para-Ir-R and Para-Ir-F). Morphological studies based on the body length, the frontal shield, spicules of male and egg dimensions were performed. ITS2-rDNA sequences were between 167 and 299bp in different isolates. ITS2 homology in different isolates was between 68 % and 77% compared with the sequence data in GenBank. Morphological results showed that the average length of male and female worms in sheep were 16.5mm and 36mm and in goats 16mm and 35.5mm, respectively. The average length of the small and large spicules in sheep were 657.5µm and 304.07µm and in goats 653.08µm and 302.66µm, respectively. To our knowledge, this is the first study in the world exploring the genetic diversity of *Parabronema* in sheep and goats. Add this sentence in discussion: the low ITS2-rDNA identity in different isolates from Iran as compared to the reference sequence in GenBank (68–77%) raise questions regarding the species identity of the parasites isolated in Iran.

Key words: *Parabronema skrjabini*, nematode, sheep, goat, PCR, Iran

Introduction

Parasitic diseases are a major constraint on the development of the livestock industry in developing countries, including Iran [1]. *Parabronema skrjabini* is one of the nematodes that inhabits the abomasum of ruminants and has a wide distribution in Africa, Asia and some Mediterranean countries. It has been reported in a number of studies from Mongolia [2], Kazakhstan [3], Saudi Arabia [4,5], Namibia [6], Turkey [7] and Iran [8]. The abomasum is one of the most important sites for nematode infection, and infection could be harmful to the health of infected animals causing economic losses due to reduced weight gain and other production loses [9–11]. Insufficient information is available about possible pathology caused by *P. skrjabini* infection and its morphomolecular dimensions have not been extensively studied. The introduced species in Iran is *P. skrjabini* which lives in the abomasum of small and large ruminants. Prevalence rates of between 1% and 5.43% in sheep [8,12], 0.8% in wild sheep [13], 28% in camel [14] and 1.72% in buffalo have been reported in Iran [15]. In order to differentiate between the abomasal nematodes, the ITS2-rDNA sequences were targeted as the best candidate for this purpose [16]. The ITS2 sequence of *Parabronema* has only been examined in one study in China in which samples were isolated from camels [17]. Therefore, the present phenotypic study was conducted to accurately identify the *P. skrjabini* infection in sheep and goats in Iran and to assess the level of intraspecies variation in this parasite using the ITS2-rDNA sequences. limited small number of studies have been published on the population

genetics of *P. skrjabini* in ruminants worldwide and particularly, on the South shores of the Mediterranean Sea. This work is the first study of ITS2-rDNA sequences of *P. skrjabini* from sheep and goats in the world, and its purpose was to investigate the molecular and morphological characteristics of *Parabronema* species collected from different areas of animal husbandry in Iran.

Materials and Methods

Table 1. The number of *Parabronema* sp. samples used in this study for morphology study

Parasite (code)	Host (number)	No. of isolates	Province in Iran
Parabronema sp. (PSS)	Sheep (17)	120	Sanandaj
Parabronema sp. (PSH)	Sheep (29)	230	Hamadan
Parabronema sp. (PSM)	Sheep (20)	180	Mashhad
Parabronema sp. (PGS)	Goat (30)	190	Sanandaj
Parabronema sp. (PGH)	Goat (38)	130	Hamadan
Parabronema sp. (PGM)	Goat (13)	210	Mashhad

Sample collection. Adult *Parabronema* sp. were recovered from the abomasa of naturally infected sheep and goats during abattoir inspections from 3 different geographical regions including Sanandaj, Hamadan and Mashhad provinces in the west and northeast of Iran, respectively (Table 1). The samples were labeled accordingly and preserved in 70% ethanol until used for the morphomolecular investigations.

Morphological studies. A total of 160 *Parabronema* isolates from sheep (40 male and 40 female) and goats (40 male and 40 female) were examined morphologically. The worms were mounted in phenol-alcohol on a glass slide and light pressure was applied to cause the worm body to lie flat without being damaged. The males were studied in order to determine body length, spicule length (right/left) and females to measure egg dimensions.

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. ITS2-rDNA was amplified from each isolate using the primer pair designed based on the rDNA genome sequence reported by Zhang et al. [17]. 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 carried out in a total volume of 100µL containing 1×PCR buffer, 100 mMol $MgCl_2$, 100 µM dNTP mix (Cinaclone, Iran), 20 pmol of each primer (Cinaclone Co.), 1U 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 45 s at 94°C (denaturing step), 45s at 59°C (annealing step), and 45 s 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 and directly sequenced.

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 sequence chromatograms were analyzed using the Geneious 5.1.6 software and compared to those deposited in GenBank (www.ncbi.nlm.nih.gov/) using the 'Basic Local Alignment Search Tool' (BLAST). All sequences for *P.skrjabini* were aligned and compared with one another and with those of the ITS2-rDNA of other spirurids available in GenBankTM. 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 the Neighbor joining and maximum parsimony methods using MEGA5 software [18]. Support values for internal nodes were estimated using a bootstrap resampling procedure with 1,000 replicates [19]. The sequences reported in this paper were deposited in GenBank with the following accession numbers: PSS1: KP670202.1, PSH-2: KP670201.1, PSS2: KP645202.1, PGM: KP645201.1, PSH1: KT318372.

Results

Morphology. The morphological results showed that the average length of males and female parasites in sheep was 16.5mm and 36mm and in

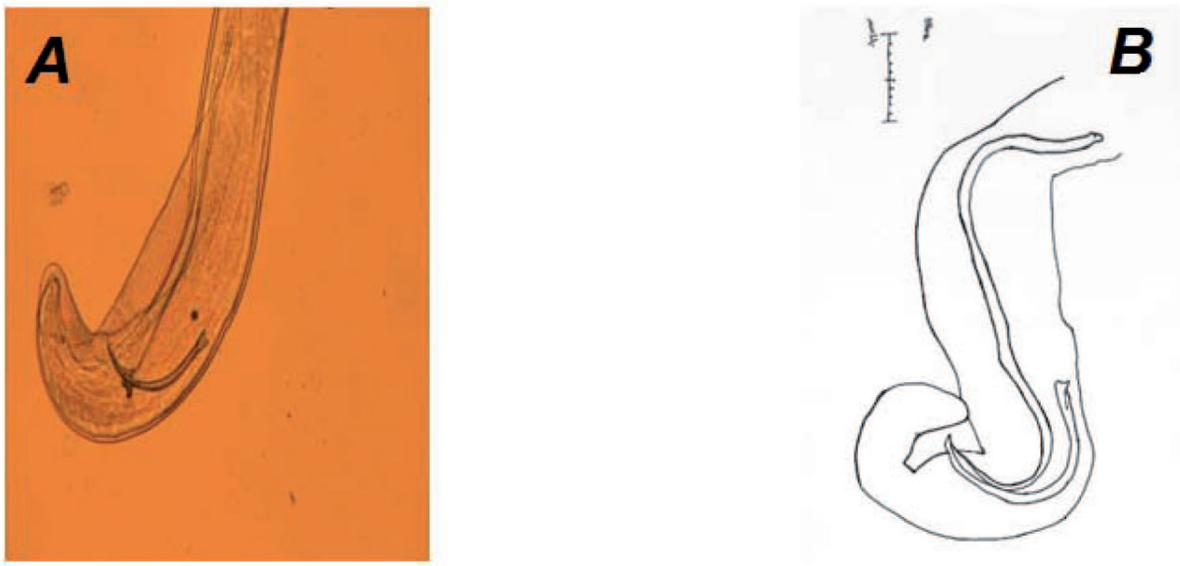

Fig. 1. Posterior extremity of *Parabronema* male (lateral view); **A**. Digital picture, **B**. Camera Lucida drawing

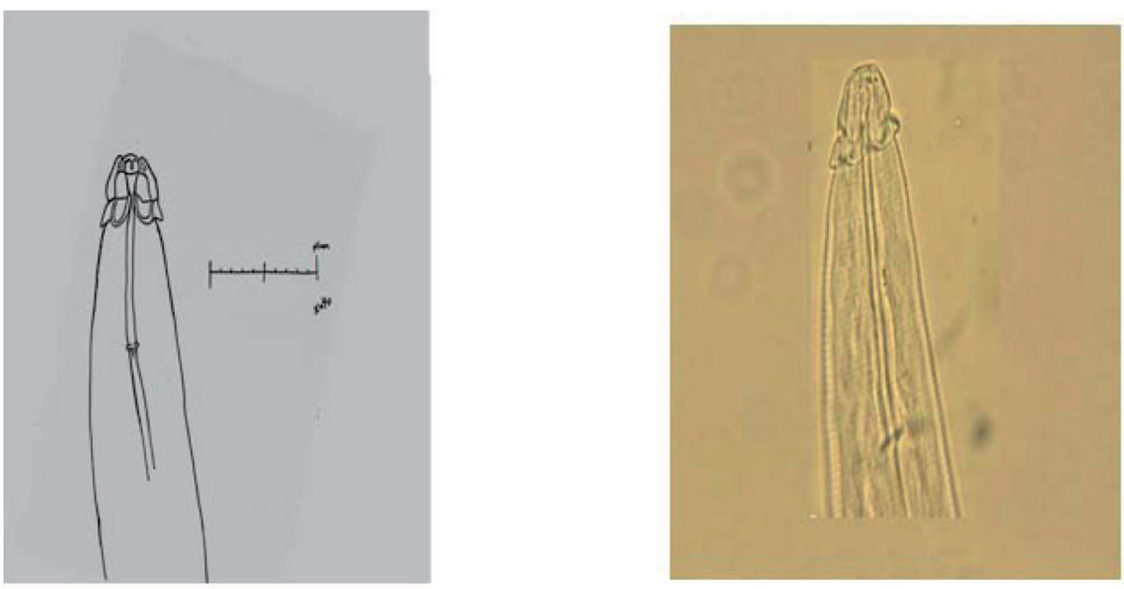

Fig. 2. Anterior portion of adult *Parabronema*; **A**. Camera Lucida drawing, **B**. Digital picture

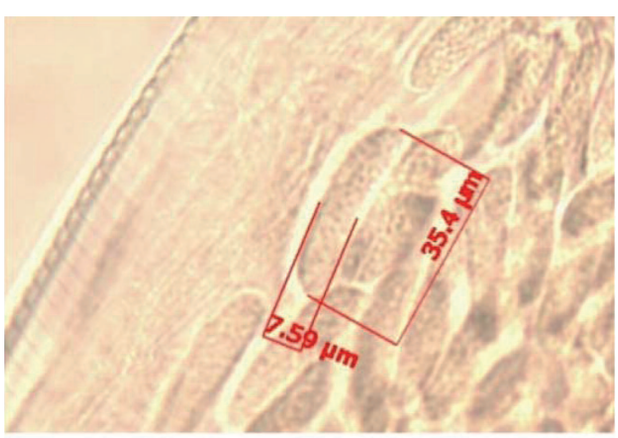

Fig. 3. Eggs *in utero*

goats 16mm and 35.5mm, respectively. The average size of the large spicules in ovine isolates was 657.1µm and of small spicules 304.1µm, and in caprine isolates 653.1µm and 302.7µm, respectively (Fig. 1). Also the average egg dimensions in sheep was 31×8.21µm and in goats 32.12×8µm (Fig. 3). These findings were true for all three sites without any significant differences.

Molecular analysis. The ITS2-rDNA for *P. skrjabini* of sheep and goat isolates were amplified and sequenced. PCR amplicons of 783bp in length for both sheep and goat isolates were obtained. ITS2-rDNA sequences were determined for 10 adult worms from sheep and goats (two worms for each

Table 2. Nucleotide composition of the ITS2-rDNA sequences of *P. skrjabini* from different geographical localities and hosts in Iran

Sequence Name	Nucleotide				GC(%)	Total
	T	C	A	G		
EU3755510*	87	39	110	45	29.9	281.0
PSH-1	58	18	69	22	24	167.0
PSH-2	106	36	107	44	27.3	293.0
PSS-1	97	45	108	49	31.4	299.0
PSS-2	106	33	107	44	26.6	290.0
PGM	87	32	84	39	29.3	242.0

*Sequence data has been deposited in GenBank

extraction) from the 3 different geographic regions. The sequences were compared with an available sequence in GenBank with accession number of EU3755510 (Fig. 4). ITS2-rDNA homology in different isolates was between 68% and 77%. Table 2 presents the comparison of all nucleotide sequences in the ITS2-rDNA of *P. skrjabini* from the different geographical regions with the reference ITS2 sequence from GenBank.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [20]. The tree with the highest log likelihood (-819.1970) is shown in Fig. 5, with bootstrap values for which the associated taxa clustered together shown next to the branches. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Joining 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 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 95 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [18].

Discussion

Parabronema skrjabini was originally described from Russian Turkestan and is known to occur in that region in cattle, camel, sheep and goats [21]. *P. skrjabini* is the species introduced in Iran, and has also been reported in Asia and Africa; Mongolia [2], Kazakhstan [3], Saudi Arabia [4,5], Namibia [6],

```
                10         20         30         40         50         60         70         80         90
EU375510        ATATCAAACA ACTCAATACA ACAGTAGCAA AAGAAGTATG TTTGAATTTA TATCATCAGG CGATAAAACG AAAAACATTT TAAATTTTAT
PGM             T---G-TTAT -ACA--C-AC T--T-GC-CC -G--G-G-CC ACCAC-AAA- C-GTT-TTTT TA--TTTTTT CCTCGGGGGA --TT--GA/A
PSH/1           ////////// ////////// ////////// ////////// ////////// ////////// ////////// ////////// //////////
PSH/2           TA--G-TTAT -ACA--C-AC T--T-GCACC -G--G-GGCA ACA-C-AAAC C-GTT-TTTT GA--TTTTTT CCTCGGGGGA -TT-A-ACGA
PSS/1           TA--G-TTAT -ACA--C-AC T--T-GCACC -GT-GCGGCA ACA-C-AAAG C-GT--GTTT GA--TT-TTT CCTCGGGGGA --T-A-ACGA
PSS/3           TA--G-TTTT -ACA--A-AC T--T-GCAGC CGT-G-GGG- ACCAC-AAA- CCGT--TTTT TA-ATTTTTT TTTCGGGGGG --TTA-ACGA

                100        110        120        130        140        150        160        170        180
EU375510        CCCCCGATAA GTTTGTGATA CCAGTATGAA AAATTTATTC CCGCCATAAT TATGGTGGGA AGAATTTCAT ACGGTGGATA TGTGTGCAAA
PGM             AATTTTT-TT T---TGT--T TA-A-CCCCT G-TAAGTA-G TATATCC--G ----AAAAAT TC---CC--- -TATAT-G-G ATA-AAAG--
PSH/1           ////////// ////////// ///A-CCCCT G-TAAGTA-G TATATCC--G ----AAAAAT TC---CC--- -TATAT-G-G ATA-AAAG--
PSH/2           AATTTTT-TT T---TGT--T TA-A-CCCCT G-TAAGTA-G TATATCC--G ----AAAAAT TC---CC--- -TATAT-G-G ATA-AAAG--
PSS/1           AATTTTT-TT T---TGTTGT TA-A-CCCCT G-GGAGGAAG TATATCC--G --A-AA-AA- TCGG-CC--- -TATAT-G-G ATACATTC-T
PSS/3           AATTTTT-TT T---TGT--T TA-A-CCCCT G-TAAGTAAG TATATCC--G --A-AAAAAT TC---CC--- -TATAT-G-G ATA-AAAG--

                190        200        210        220        230        240        250        260        270
EU375510        AACACAGGGA ATTGAGGGAA ATTCAAAAAT AATGCGAAAA TGAATTTCAT CAATTATTGT TCATGTGTAT ATGCATACAT TTTATCAAAC
PGM             TTTTTCATAT GAATGATAT- TG-GT----C GCAAT---TT GA--GAAAT- ---//////////////////////////////////////
PSH/1           TTTTTCATAT GAATGATAT- TG-GT----C GCAAT---TT AA--GAAAT- --CAA--AA- G--AAAA-GA --TTC-TTCA A--G-T-TTA
PSH/2           TTTTTCATAT GAATGATAT- TG-GT----C GCAAT---TT GA--GAAAT- --CAA--AA- G--AAAA-GA --TTC-TTCA A--G-T-TTA
PSS/1           TTTTTCATAT GAATGATAT- /G-GT----C GCAAA---/- AA-TGAA-T- --CAG-GAA- C--CAAA-GC --TTC-TTTC A--GCT----
PSS/3           TTTTTCATAT GAATGATAT- TG-GT----C GCAAT---TT GA--GAAAT- --CAA--AA- G--AAAA-GA --TTC-TTCA A--G-T-TTA

EU375510        TT
PGM             //
PSH/1           --
PSH/2           --
PSS/1           --
PSS/3           --
```

Fig. 4. Nucleotide comparison of the ITS2-rDNA sequences of *P. skrjabini* from different geographical localities and hosts in Iran using reference sequence with Accession no. EU375510 from GenBank. Oblique lines indicate gaps inserted for optimal alignment or end of the available sequence, whilst dashes nucleotides identity.

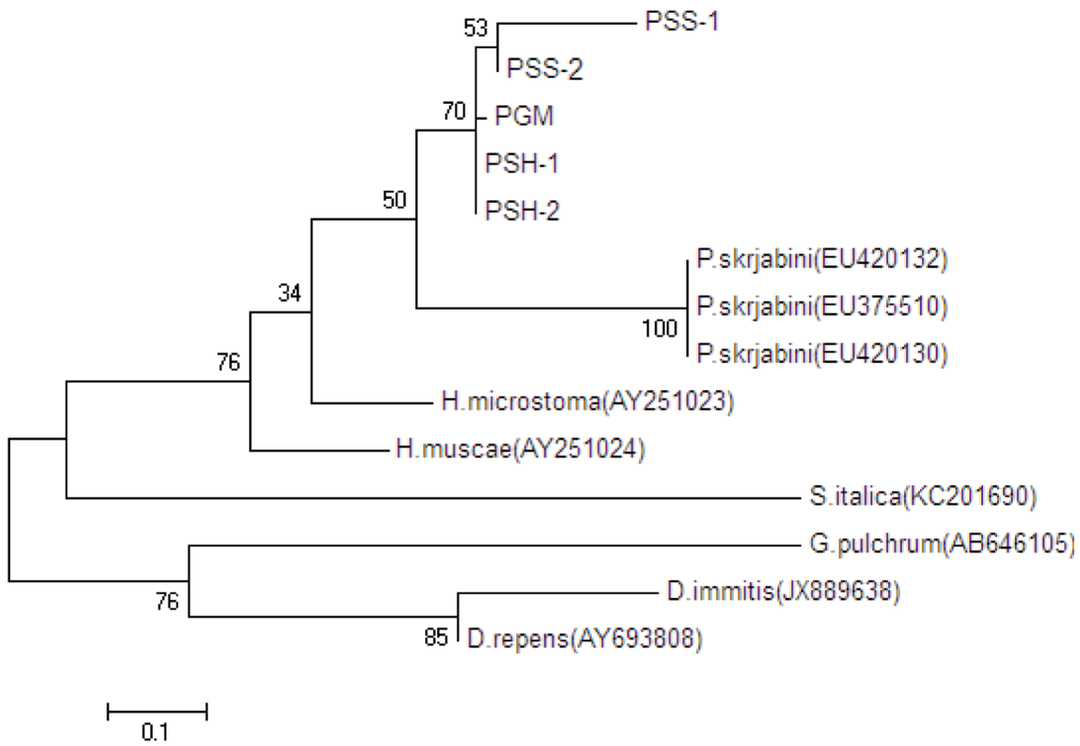

Fig. 5. Phylogenetic relationships of the *Parabronema* parasite compared with other spirurid parasites based on the ITS2-rDNA sequences (the phylogenetic analyses were conducted in Mega 5)

Turkey [7] and Iran [8]. Prevalence of between 1% and 5.43% in sheep [8, 12], 0.8% in wild sheep [13], 28% in camels [14] and 1.72% in buffalo have been reported in Iran [15]. *P. skrjabini* belongs to the order Spirurida, superfamily Spiruroidea, family Spiruridae and genus *Parabronema.* In this genus the anterior end of the worm has a large cuticular shield and cordons (horseshoe-like skin shields) that are a unique morphological feature in the *Parabronema* genus (Fig. 2). The posterior end of the male worm is curved. The genital pore of the female worm is located in the anterior 1/3 end. Larval eggs are oval in shape and are 35.4µm long and 7.59µm wide with a thick outer shell. In the present study, the morphological findings showed that the average length of male and female worms in sheep were respectively, 16.5mm and 36 mm and in goats 16mm and 35.5mm. The average length of the small and large spicules in sheep were, 657.5µm and 304.07µm and in goats 653.08µm and 302.66µm, respectively. Thus there were no significant differences between sheep and goat isolates based on morphological characteristics. This study is the first complete and comprehensive morphological survey on *Parabronema* in sheep and goats in the world. In the present study, the low ITS2-rDNA identity in different isolates from Iran as compared to the reference sequence in GenBank (68–77%) raise questions regarding the species identity of the parasites isolated in Iran. For this purpose, the ITS2-rDNA region was targeted by PCR as shown before [22]. The first and/or second internal transcribed spacers (ITS) of nuclear ribosomal DNA (ITS-1 and ITS-2, respectively) have proved ideal for this purpose [23,24], as a number of investigations have focused on the ITS regions for parasitic nematodes of the orders Strongylida and Ascaridida [25,26], species of *Mansonella* [27], *Dirofilaria* and *Dipetalonema* [28], *Thelazia* [29], etc. Traversa et al. [33] 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 sequence of *H. muscae* was similar in length to the ITS-2 of the spirurid *Onchocerca volvulus* [27]. The ITS2-rDNA region has been used to identify the species *Haemonchus* [30], to infer the relationship of species within the genus *Nematodirus* [31] and to

investigate *Taenia* species [32]. Characterization of the ITS-2 sequences of *Habronema* revealed sequence lengths and G+C contents of 296 bp and 29.5% for *H. microstoma*, and of 334 bp and 35.9% for *H. muscae*, respectively, for its two species [33]. Molecular studies on the genus *Parabronema* have been mainly based on rDNA genes available in Genbank. Studies of ITS1 and ITS2 gene sequences of *P. skrjabini* in camels have shown 30.2–60.1% similarity with other nematodes 17]. In this paper, ITS2 sequence length differed from 167 to 299bp. ITS2 sequence identity of different isolates from Iran was between 68% to 77% compared with the sequences in Genbank. Phylogenetic tree analysis (Fig. 5) of *P. skrjabini* isolates with other spirurida (like *Setaria*, *Gongylonema*, *Dirofilaria* and *Habronema*) and a *P. skrjabini* reference sequence from Genbank showed that although all of them may have a common ancestor, are grouped on different branches; even the *P. skrjabini* species are distributed on several branches. The sequences in this study were more similar to Habronema spp. There was a significant difference between the isolates in this study and sequences in Genbank. The goat sequence from Mashhad (PGM) was more analogous with sequences of Hamadan isolates (PSH1 and PSH2) from sheep. The low ITS2-rDNA identity in different isolates from Iran as compared to the reference sequence in GenBank (68–77%) raise questions regarding the species identity of the parasites isolated in Iran. Very limited number of 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-2 sequences of *P. skrjabini* from sheep and goats in Iran. The results show genetic diversity which opens 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.

References

[1] Hasheminasab S.S., Moradi P., Talvar H.M., Wright I., Darbandi M.S. 2015. *Buxtonella* spp. like infection in cattle in Sanandaj province, Iran. *Annals of Parasitology* 61**:** 247-251.

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

[3] 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.

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

[5] 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.

[6] 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.

[7] Yukari B.A. 2005. Seasonal activity of gastro-intestinal nematodes in goats in Burdur region, Turkey. *Turk J Vet Anim Sci* 29**:** 441-448.

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

[9] 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.

[10] 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.

[11] 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.

[12] 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.

[13] 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.

[14] 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.

[15] Eslami A., Zamani Herglani Y. 1989. Abattoir investigation on the helminth infections of buffalo in Iran. *Journal of Veterinary Faculty, University of Tehran* 44**:** 25-34.

[16] 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.

[17] 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* [J]. *Chinese Journal of Animal and Veterinary Sciences* 5**:** 026.

[18] 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.

[19] Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution***:** 783-791.

[20] 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.

[21] 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.

[22] Blouin M.S. 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *International Journal for Parasitology* 32**:** 527-531.

[23] 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.

[24] Hasheminasab S.S. 2015. Molecular characterization of the first internal transcribed spacer of rDNA of *Parabronema skrjabini* for the first time in sheep. *Annals of Parasitology* 61**:** 241-246.

[25] 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.

[26] 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.

[27] 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.

[28] Mar P-H., Yang I-C., Chang G-N., Fei AC-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.

[29] 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.

[30] Stevenson L.A., Chilton N.B., Gasser R.B. 1995. Differentiation of *Haemonchus placei* from *H. contortus* (Nematoda: Trichostrongylidae) by the ribosomal DNA second internal transcribed spacer. *International Journal for Parasitology* 25**:** 483-488.

[31] Audebert F., Durette-Desset M-C., Chilton N.B. 2000. Internal transcribed spacer rDNA can be used to infer the phylogenetic relationships of species within the genus *Nematodirus* (Nematoda: Molineoidea). *International Journal for Parasitology* 30**:** 187-191.

[32] Gasser R.B., Zhu X. McManus D.P. 1999. NADH dehydrogenase subunit 1 and cytochrome c oxidase subunit I sequences compared for members of the genus *Taenia* (Cestoda). *International Journal for Parasitology* 29**:** 1965-1970.

[33] Traversa D., Giangaspero A., Iorio R., Otranto D., Paoletti B., Gasser R. 2004. Semi-nested PCR for the specific detection of *Habronema microstoma* or *Habronema muscae* DNA in horse faeces. *Parasitology* 129**:** 733-739.

Received 3 January 2016
Accepted 4 March 2016