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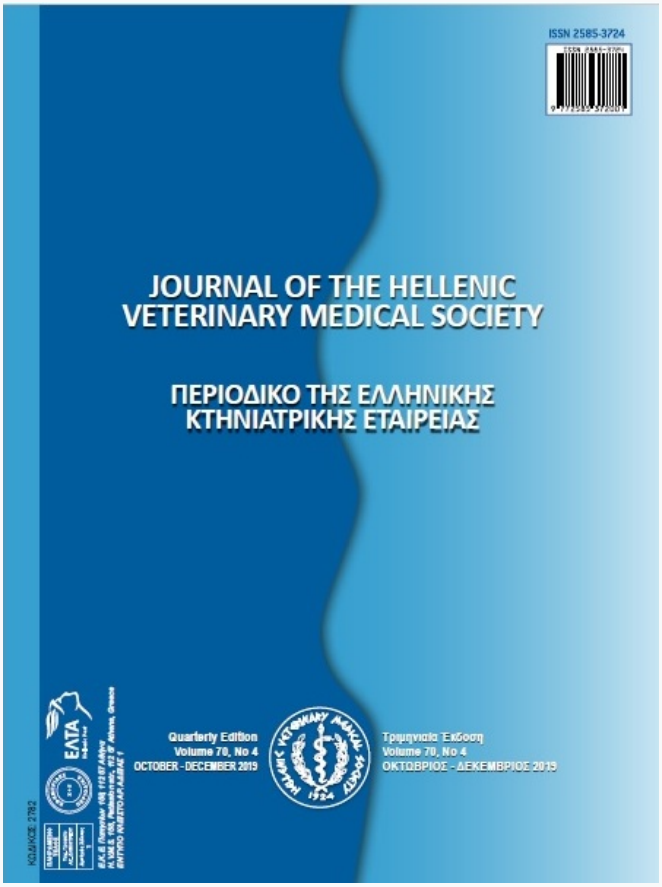

Ultrastructural and molecular characteristics of Setaria species based on sequence analysis of genomic and mitochondrial gene markers in cattle (Bos taurus) and buffaloes (Bubalus bubalis) from Iran

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Research article
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Ultrastructural and molecular characteristics of *Setaria* species based on sequence analysis of genomic and mitochondrial gene markers in cattle (*Bos taurus*) and buffaloes (*Bubalus bubalis*) from Iran

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ABSTRACT. The aim of the present study was to investigate the ultrastructural characteristics and genetic diversity of *Setaria* parasites from cattle (n=696) and buffalo (n= 522) from Khuzestan province of Iran and to compare them with available data from other countries/regions by sequences analysis of the 12S rDNA and the mitochondrial cytochrome C oxidase subunit I (*cox1*) genes. Based on SEM (Scanning Electron Micrographs) and light microscopy, all the isolated worms were identified as *Setaria labiatopapillosa*. Our results showed that 12.3% of cattle were infected with *Setaria* spp., while no infection was found in buffaloes. The maximal prevalence was observed in cattle younger than one year old. The prevalence rate was not influenced by the season of the year or gender. Comparison of the obtained sequences from *Setaria* with sequences of *Setaria* spp. from GenBank confirmed that all samples belong to the species *S. labiatopapillosa*. The phylogenetic tree constructed using *cox1* and 12S rDNA genes of several other filarial nematodes showed that the Khuzestan isolates share a common branch with *S. labiatopapillosa* from other regions. Intra-specific variation was observed in 12S rDNA but not in *cox1*. In conclusion, our results indicating that *S. labiatopapillosa* is the main species involved in the spread of setarial infection in south-west of Iran and the identified worms corresponded mostly to worms that reported previously throughout other continents.

Keywords: *Setaria species*; Ultrastructural; phylogenetic; Iran.

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INTRODUCTION

Filariasis in man and animals is considered as a major health hazard with important medical, veterinary, and economic consequences, affecting millions of people and animals globally (World Health Organization, 2007). *Setaria* species (Family: Onchocercidae and Subfamily: Setariinae) are filarial nematodes that mainly inhabit the peritoneal cavity of ungulates and rodents. *S. digitata, S. marshalli, S. cervi* and *S. labiatopapillosa* are the main species that have been reported from cattle and buffaloes (Anderson, 2000; Islam et al., 1992). Several species of mosquitoes, including *Aedes*, *Culex*, *Armigeres*, *Anopheles* and *Haematobia* fly are vectors for *Setaria* spp. (Anderson 2000; Cancrini et al., 1997). Furthermore, there are some reports about the congenital transmission of *S. digitata* and *S. marshalli* in cattle (Fujii et al., 1995; Wee et al., 1996; Anderson, 2000).

Setariosis is a polyorganic parasitic disease. The migration of adult worms to the bladder, heart, lung, mesenteric lymph nodes, liver, spinal cord and eyes have been reported. Adult worms are not pathogenic in the peritoneal cavity, although they may cause a mild fibrinous peritonitis in definitive hosts (Golovko and Shchetinsky, 2005; Sundar and D'Souza, 2015). Ectopic migration of microfilariae in the central nervous system of nonspecific hosts (sheep, horses, goats and man) and sometimes even in the specific host can cause cerebrospinal nematodiasis or cerebrospinal setariosis that is recognized with clinical signs such as ataxia, lack of motor coordination and paralysis (Tung et al., 2003; Mahmoud et al., 2004). Additionally, in some areas of the world such as Iran and Romania, human infection with larval and adult stages of *Setaria* spp. have also been reported (Talu et al., 2012; Nabie et al., 2017).

Several methods have been used for epidemiological studies and discrimination of species diversity. Traditionally, these worms are identified based on morphological features including cuticular ring, dorsal, ventral and lateral lips, lateral appendages, mouth opening, terminal knob in female worms and spicules and patterns of cloacal papillae in male worms (Shoho and Uni, 1977). Conventionally, the light microscope is used to identify the various species of *Setaria* spp. but, additional studies are needed to evaluate and confirm their morphological variations. SEM (Scanning Electron Micrographs) is a powerful tool with high quality that may facilitate the identification of the *Setaria* spp. morphological variations (Almeida et al., 1991; Ronghang and Roy, 2013; Kumar and Kumar, 2016). So far, no study has been carried out to determine the morphological differences between *Setaria* species in Iran.

Genetic diversity of the parasite spp. may produce different phenotypes which can be associated with host-parasite interaction (Brunner and Eizaguirre, 2016; Viney and Diaz, 2012). Although a few light microscopic studies have been reported on the prevalence of *Setaria* in the cattle and buffaloes particularly from north of Iran (Dawoodi, 2014; Bazargani et al., 2008; Khedri et al., 2014; Nabie et al., 2017), there is no information about the prevalence of setariosis in the southern Iran and ultrastructural and molecular characteristics of parasites have not been studied so far. Thus, the purpose of this study was to determine the prevalence, ultrastructural and phylogenic characteristics of *Setaria* spp. in cattle and buffaloes in Khuzestan province of Iran.

MATERIALS AND METHODS

Sampling

During the period from November 2014 to October 2016, a total of 696 cattle (437 male and 259 female) and 522 buffaloes (341 male and 181 female) in different age groups (Table 2) and examined for detection of infection with *Setaria* spp. The slaughterhouse of Ahvaz is a centre for receiving animals from different areas of Khuzestan province. After slaughtering the animals, blood samples were taken from each one, and the abdominal cavity was carefully examined for the presence of *Setaria* spp. The worms were collected and transferred to containers containing phosphate buffered saline (pH = 7.4). Season of sampling, the number of isolated worms, gender and age of each animal were recorded. Some of the specimens were fixed in 70% ethanol for light microscopic examination and DNA extraction. The Modified Knott's technique was used to detect microfilariae in the blood as described by Watanabe et al. (2004), and the numbers and length size of microfilariae were recorded.

Morphological examination

After the relaxation of the worm samples in hot water and transparency within lacto phenol, they were mounted using the glycerine-gelatine solution and examined under light microscope.

The methods of Ronghang and Roy (2013) and Kumar and Kumar (2016) with some modification were used for ultrastructural study of samples by

SEM. Briefly, worms were washed in PBS, and the anterior and posterior ends of the worms were cut and fixed in 5% glutaraldehyde (Sigma, USA) for 24h at 4 ˚C. Some samples were post fixed in 1% osmium tetroxide (Sigma, USA) for 4h at 4 ˚C. Then the dehydration process of all samples was performed with a gradient series of ethanol at 4 ˚C and the samples were dried using Tetramethylsilane (Sigma, USA). Finally, specimens were mounted on aluminium stubs, coated with a thin layer of gold, and examined with Leo 1455 VP SEM (Carl-Zeiss, Germany) at 18-23 KV. *Setaria* species were determined according to diagnostic keys (Shoho and Uni, 1977).

PCR Amplification

Template DNA from whole blood samples and adult *Setaria* worms were extracted using Genomic DNA extraction Kit (SinaClon Bioscience, Iran) according to manufacturer's instruction. In order to identify microfilariae in blood samples that were negative based on modified Knott's test (97 cattle and 30 buffaloes), PCR reaction was performed on tandemly repeated DNA using M2F and M2R primers as described by Wijesundera et al. (1999). For molecular characterization and phylogenetic analysis of *Setaria* spp., mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and 12S rDNA genes were amplified by PCR according to methods that previously described by Yatawara et al. (2007) and Casiraghi et al. (2001). Characteristics of primers that were used in the present study are shown in table 1.

Table 1. PCR conditions and primer sequences for tandemly repeated DNA, *cox1* and 12Sr DNA.

Target gene	Organism	Primer sequence (5′→3′)	Annealing temperature (°C)	Product size (bp)
tandemly repeated DNA	Microfilariae	M2F:CCGACATCAAGTTCATG M2R: GATTCAAGAACATGGTG	48	Ladder like
cox1	Adult worm	COX1intF: TGATTGGTGGTTTTGGTAA COX1intR: ATAAGTACGAGTATCAATATC	54	680
12S rDNA	Adult worm	12SF:GTTCCAGAATAATCGGCTA 12SR:ATT GACGGATG (AG) TTTGTACC	54	450

All PCR reactions were performed in a 20 µl volume containing: 10 µl of Taq DNA polymerase master mix Red (Amplicon, Denmark, MgCl2: 1.5 mM), 0.5 µl of each primer (10 µM) (Macrogen, South Korea) and 3 µl of DNA template (~100 ng) and 6 µl of DNase free water. PCR cycling included an initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, specific annealing for 45s, and extension at 72 º C for 1 min. Then a final extension at 72ºC for 5 min was performed as well. PCR reactions included a negative control, consisting of the reaction mix and 3 µl of DNase free water instead of template DNA. A positive control consisting of DNA sample isolated from intact worm was used for PCR reaction on blood samples. PCR products were electrophoresed on 1.5% agarose (SinaClon Bioscience, Iran) in Tris-acetate-EDTA (TAE) buffer, stained with Green SafeStain (SinaClon Bioscience, Iran) and visualized under ultraviolet light.

Phylogenetic analyses

PCR products of *cox1* and 12s rDNA were purified using Gel recovery kit (Vivantis, Malaysia) and sequenced on both strands using Big Dye Terminator V.3.1 Cycle Sequencing kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Sequences were aligned together using ClustalW (Larkin et al., 2007) software to determine the consensus sequences. Consensus 12S rDNA and cox1 sequences were subjected to BLASTn analysis (http://blast.ncbi.nlm.nih.gov) and compared to all nucleotide sequences of *Setaria* species available in the current databases. Sequence identities (in %) were calculated by pairwise comparisons. Subsequently, the consensus sequences were aligned with a selected subset of closely related sequences of the genus *Setaria*. Phylogenetic relationships were inferred based on analyses employing the Neighbor-Joining (NJ) method using MEGA7. The topological sta-

bility of the tree was evaluated by 1000 bootstrap replications.

Statistical analysis

The findings of this study were analyzed using SPSS software (version 21). The associations between age, gender, season and infection were analyzed by Chi-square test (X^2-test). The level of significance was at 5%.

RESULTS

Prevalence

As shown in table 2, 12.3% (95% CI: 9.9-14.8%) of cattle were infected with *S. labiatopapillosa*, while no infection was found in buffaloes. The prevalence rate of infection with adult worm, microfilariae and the both were 9.77% (68 out of 696), 8.76% (61 out of 696) and 6.17% (43 out of 696), respectively. Out of the 437 male cattle screened, 56 (12.8 %) were infected, while the prevalence rate of infection in females was 11.5% (30 out of 259). There was no statistically significant difference in infection between genders (P= 0.633). The prevalence of infection in cattle younger than one year, one to three years and more than three years was 19.18% (40 out of 228), 9.79% (28 out of 286) and 9.89% (18 out of 182), respectively. There were significant differences between age groups and infection (P= 0.015). The prevalence rate of infection in spring, summer, autumn and winter was 16.57%, 12.19%, 9.45% and 11%, respectively. The prevalence of infection with *Setaria* in spring (16.57%) was greater than other seasons, but there was no statistically significant difference between different seasons (P=0.224) (table 3).

Table 2. Prevalence of *Setaria* infection in cattle and buffaloes.

Animals	Gender		Total	Age		
	Male	Female		<1	1 - <3	3 - >3
Cattle examined	437	259	696	228	286	182
Infected Cattle (%)	56 (12.8)	30 (11.5)	86 (12.3)	40 (17.5)	28 (9.7)	18 (9.8)
Buffalos examined	341	181	522	133	247	142
Infected buffaloes (%)	0	0	0	0	0	0

Table 3. Prevalence of *Setaria* infection in cattle based on gender, season and age.

Variables	Parameters	No. examined	No. positive (%)	*P*-value
Gender	Male	437	56 (12.8)	0.633
	Female	259	30 (11.5)	
Season	Spring	175	29 (16.57)	0.224
	Summer	164	20 (12.19)	
	Autumn	148	14 (9.45)	
	Winter	209	23 (11)	
Age	<1	228	40 (19.18)[a]	0.015
	1 - <3	286	28 (9.79)	
	3 - >3	182	18 (9.89)	
Total		696	86 (12.3)	

* Figures in columns with different characters are statistically significant.

The first stage microfilariae were isolated from 61 out of 696 (8.76%) blood samples of cattle by the modified Knott's test. PCR amplification of tandemly repeated DNA of *Setaria* genome in negative blood samples (negative on modified Knott's test) revealed that 47.42% (46 out of 97) of cattle blood samples were positive and ladder-like pattern was seen in gel electrophoresis (Fig. 3A) while no filarial DNA was detected in blood samples of buffaloes. This suggests that Knott's test (8.76%) in comparison to PCR technique (47.42%) cannot indicate up to thirty percent (38.6%) of the negative blood samples.

Morphology

A total of 101 worms (4 male and 97 female) were isolated from examined cattle. The average number of recovered worms in each animal was 1.36 (range: 1-15). All isolated worms were identified as *S. labiatopapillosa* based on light microscopy and SEM. The average lengths of male and female worms were 4.9 and 9.6 cm, respectively. At the anterior end of female and male worms, cuticular peribuccal ring existed with dorsal and ventral lips with notched elevation, lateral lips and elliptical shaped mouth opening (Fig. 1A, 1B and 2A). A pair of amphids with cuticular mosaic appearance was located in the lateral sides of the peribuccal ring (Fig 1C and 1D). Genital pore in female worms was situated on the ventro-lateral side (Fig 1A and 1B). Lateral appendages were seen at posterior end in both sexes (Fig 1E and 1G). A knob was detected at the end of the tail in female worms with blunt papilla and transverse lines at the base (Fig. 1E and 1F). Male *S. labiatopapillosa* had coiled tail with three pair of pre-cloacal, one pair ad-cloacal and four pair of post-cloacal papilla. Furthermore, ventral bands and a single blade-like spicule emerging from the cloaca were seen at the posterior end of male worms (Fig. 1G and 1H).

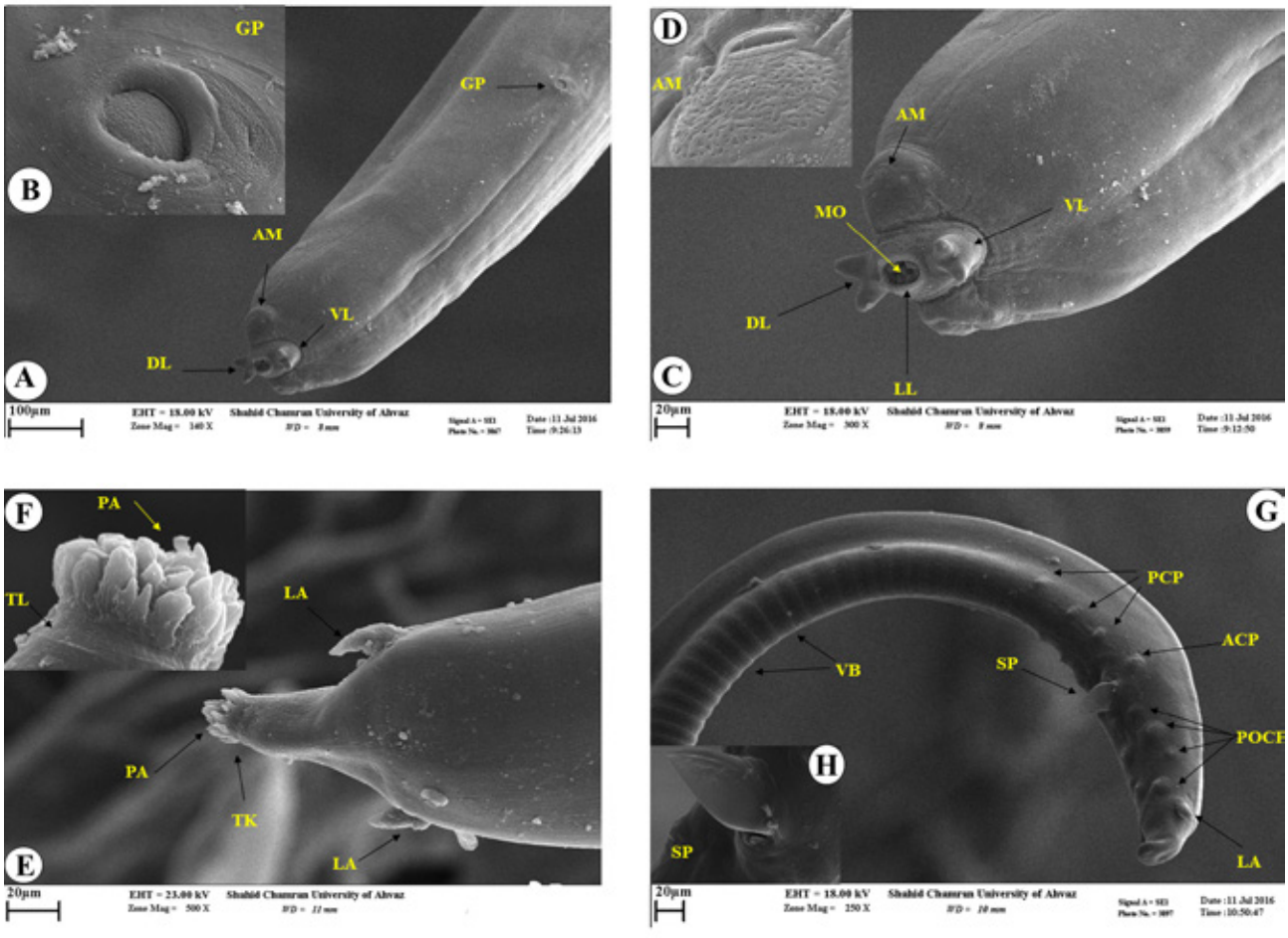


Fig. 1. Morphological characteristics of *S. labiatopaoillosa* studied by SEM. A: Anterior end of Female worm (140X). B: Genital pore (3000X). C: Anterior end of Female worm (300X). D: Amphid (3000 X). E: Posterior end of Female worm (500X). F: Blunt papilla and transverse lines at the tail end in female worm (3000X). G: Posterior end of male worm (250X). H: Spicule (3000X). ACP: Ad-Cloacal Papilla, AM: Amphid, DL: Dorsal Lip, GP: Genital pore, LA: Lateral Appendages, LL: Lateral Lips, MO: Mouth Opening, PA: Papilla, PCP: Pre Cloacal Papilla, POCP: Post-Cloacal Papilla, SP: Spicule, TK: Terminal Knob, TL: Transverse Lines, VB: Ventral Bands and VL: Ventral Lip.

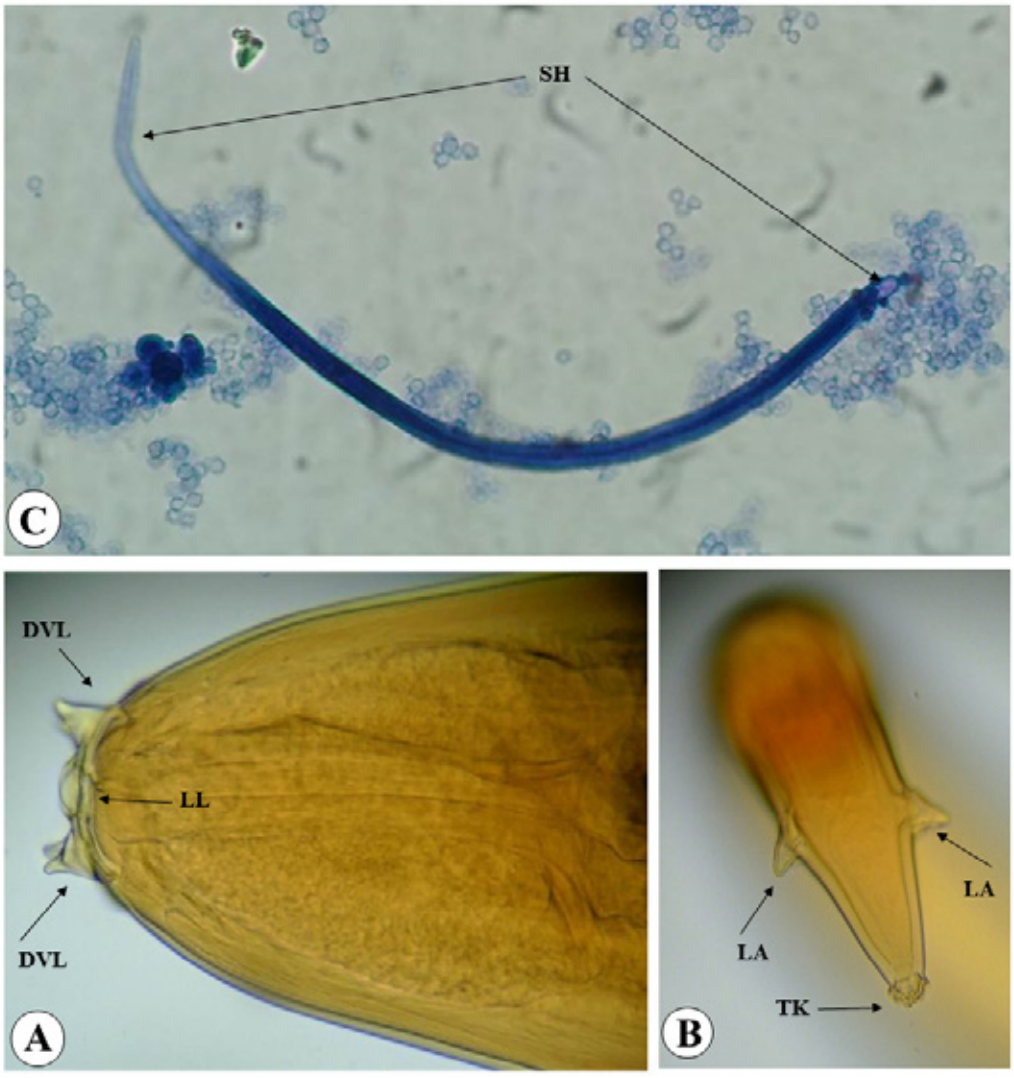


Fig. 2. Light Microscopic Images of adult female *S. labiatopaoillosa* and microfilariae. A: Anterior end of Female. B: Posterior end of Female worm. C: Sheathed first stage microfilaria in blood of infected cattle. DVL: Dorsal or Ventral Lips. LA: Lateral Appendages, LL: Lateral Lips, SH: Sheath and TK: Terminal Knob.

The average length of sheathed first stage microfilariae was 304.6 μm (Fig. 2C). The average number of microfilaria in blood samples of infected cattle was 537.25 ±74.89 /ml (range: 45-2520/ml).

Molecular characteristics of *cox1* and 12S rDNA genes

PCR amplification of the *cox1* and 12S rDNA genes of *S. labiatopapillosa* produced 680 and 450 base pairs fragments, respectively (Fig. 3B). The DNA sequences of *cox1* (6 variable sites) and 12S rDNA (53 variable sites) obtained in the present study shared 82-99% identities with other *Setaria* species (Table 4 and 5). DNA sequences obtained in the present study showed the highest identity (>98%) to *cox1* and 12S rDNA of *S. labiatopapillosa*, while the lowest similarity was found to sequences from *S. equina*.

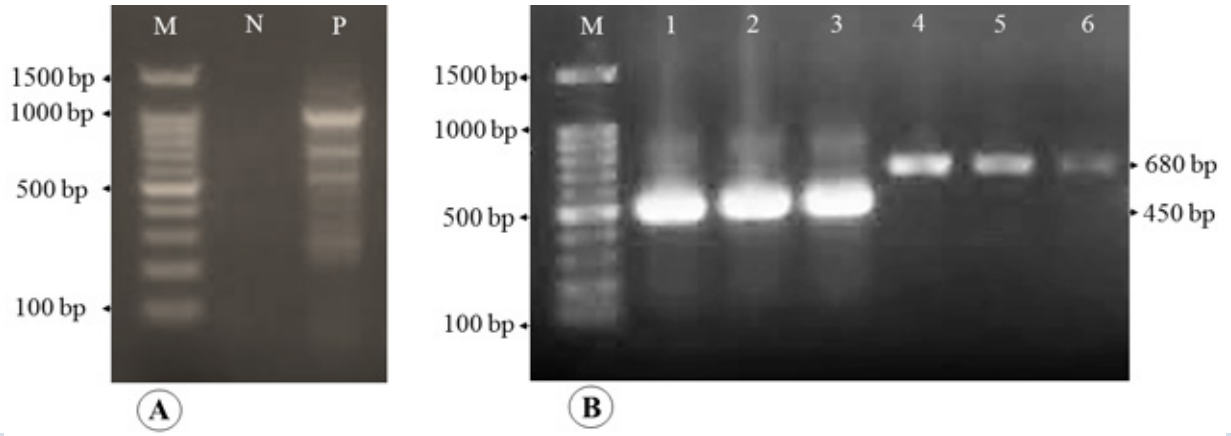


Fig. 3. A: PCR amplification of tandemly repeated DNA isolated from blood of cattle infected with *S. labiatopaoillosa* microfilariae (N: negative control, P: positive sample). B: The PCR products of 450 bp and 680 respectively, for *cox1* (1-3) and 12s rDNA (4-6) obtained from adult worm. Lane M is a 100 bp ladder.

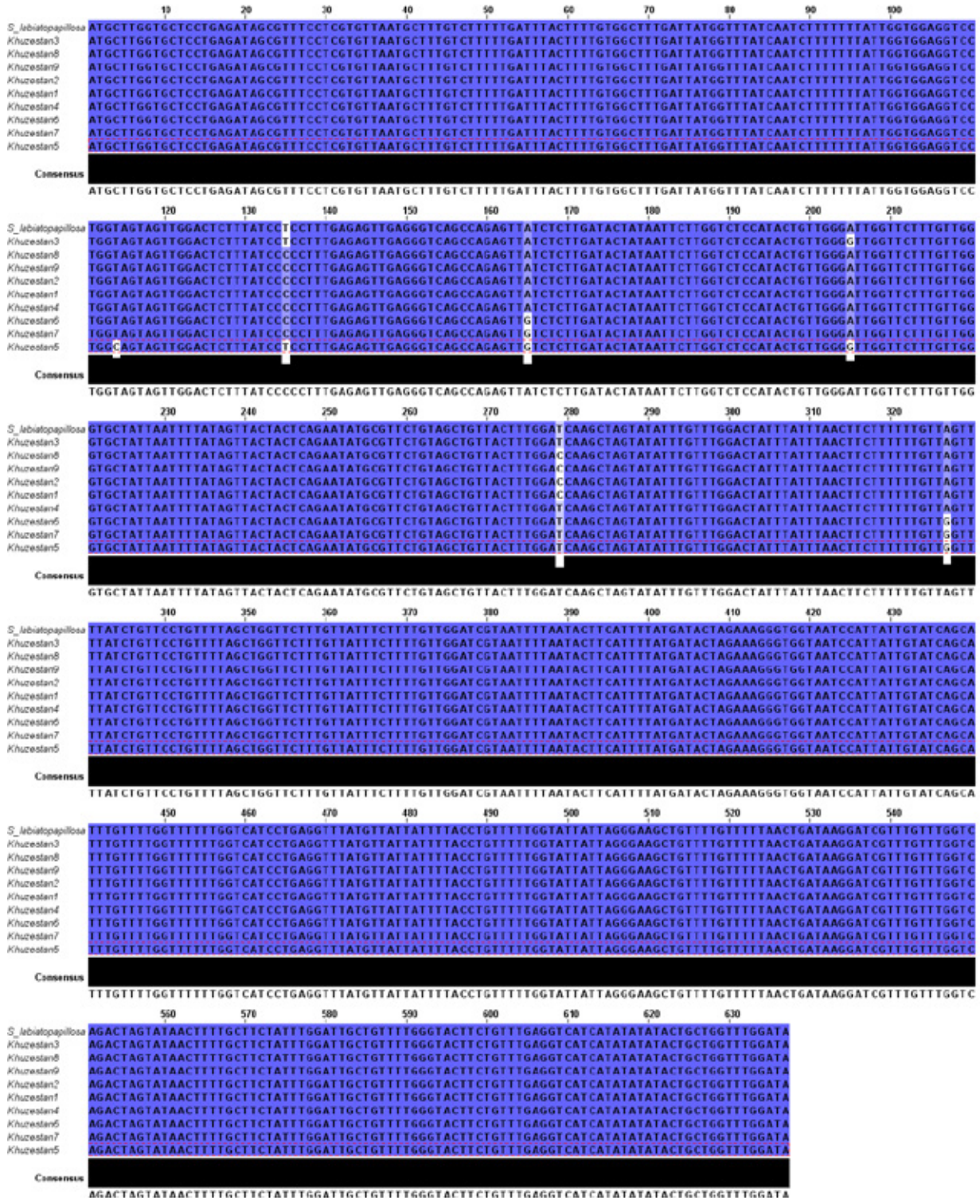
Table 4. Similarity values of *cox1* gene of *S. labiatopapillosa* obtained from cattle of Khuzestan province and those from other filarial species and related nematodes. Data was analysed using nBLAST tool.

Species	Accession No.	Maximal identity (%)
Setaria labiatopapillosa	AJ544872.1	99
Setaria digitata	EF174425.1	91
Setaria cervi	JF800924.1	91
Setaria tundra	KF692106.1	91
Dirofilaria repens	KX265048.1	90
Setaria equina	AJ544873.1	90
Wuchereria bancrofti	JQ316200.1	90
Brugia timori	AP017686.1	89
Onchocerca ochengi	KX181289.1	89
Dipetalonema evansi	KR184805.1	88
Brugia pahangi	AP017680.1	88

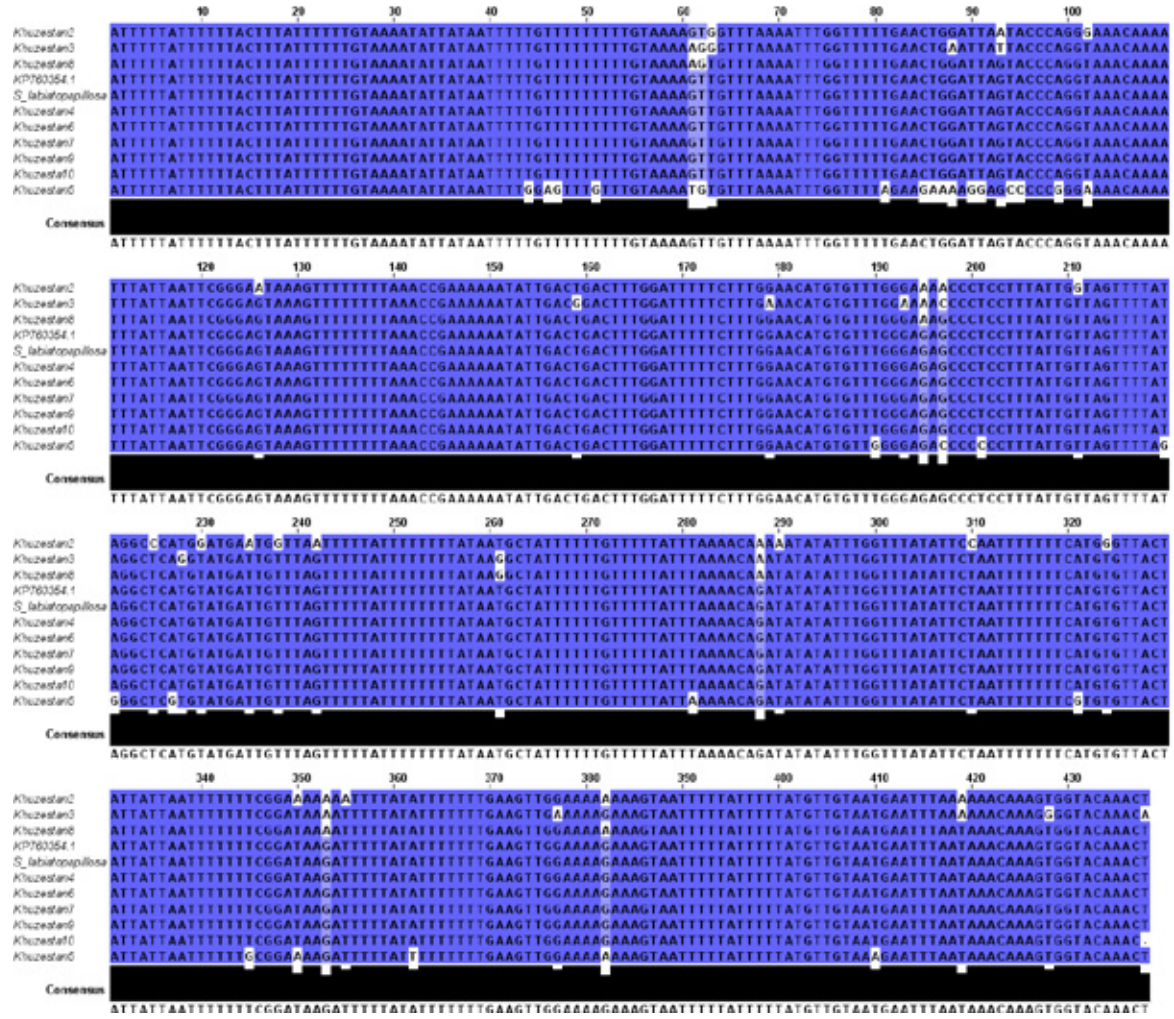
Table 5. Similarity values of 12S rDNA gene of *S. labiatopapillosa* obtained from cattle of Khuzestan province and those from other filarial species and related nematodes. Data was analysed using nBLAST tool.

Species	Accession number	Maximal identity (%)
Setaria labiatopapillosa	KP760354.1	98
Setaria digitata	EF179382.1	89
Setaria tundra	AM779828.1	87
Setaria equina	KU291446.1	82
Piratuba scaffi	AM779831.1	82
Cercopithifilaria tumidicervicata	AM779788.1	82
Litomosoides brasiliensis	KP760336.1	81
Thelazia gulosa	AJ544857.1	80
Thelazia callipaeda	LK984781.1	77

Sequences of *cox1* and 12S rDNA (supplementary files 1 and 2) from all isolates had more than 99% similarity to each other and to *S. labiatopapillosa* sequences that have been previously deposited in GenBank (12S rDNA: KP760354.1, *cox1*: AJ544872.1). 12S rDNA sequences were deposited in GenBank under the accession numbers MF589577, MF589578, MF589579 and MF589580. *Cox1* sequences were deposited in GenBank under the accession numbers MF589581, MF589582, MF589583, MF589584 and MF589585.



Supplementary 1. Multiple sequence alignment of the *cox1* sequences of *S. labiatopaoillosa* obtained from cattle of Khuzestan province and Reference GeneBank sequence (Accession NO: AJ544872.1) using ClustalW alignment tool. Different nucleotides in each position are shown with no colour while identical amino acid residues are colored.



Supplementary 2. Multiple sequence alignment of the 12S rDNA sequences of *S. labiatopaoillosa* obtained from cattle of Khuzestan province and Reference GeneBank sequence (Accession NO: KP760354.1) using ClustalW alignment tool. Different nucleotides in each position are shown with no colour while identical amino acid residues are colored.

According to the phylogenetic tree of *cox1* (Fig 4), the Khuzestan isolates were clearly grouped with *S. labiatopapillosa* in one node that had high bootstrap values for NJ (0.98). However, sequence analysis showed slight variability within the Khuzestan isolates, which was reflected in their topology in the phylogenetic tree. Accordingly, the Khuzestan isolates were grouped in two clusters. Cluster I (1, 2, 3, 4, 8 and 9 isolates) were more closely related with *S. labiatopapillosa* when compared with cluster II (5, 6, 7 isolates). Due to the lower bootstrap values (0.62), other *Setaria* species including *S. digitata*, *S.cervi*, *S.tundra* and *S. equina* were grouped in separate clades based on *cox1* sequence.

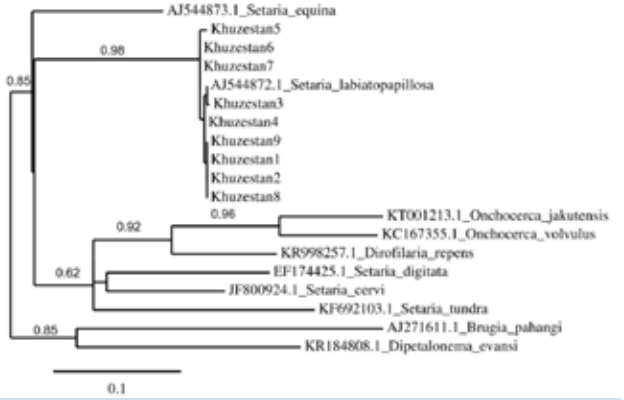


Fig. 4. Neighbor-Joining phylogenetic relationship of 9 isolates of *S. labiatopapillosa* from cattle of Ahvaz, Iran. The analysis was based on *cox1* gene sequences (680 bp). Percentage bootstrap support (more than 50%) from 1000 replicate samples is indicated at the right of the supported node. Accession numbers for sequences obtained from GenBank are shown, followed by different Filarioidea species. The scale bar indicates distance.

As shown in Fig 5 based on 12s rDNA sequences of Khuzestan isolates and the other 10 filarial nematodes downloaded from GenBank, all Khuzestan isolates with *S. labiatopapillosa* were grouped in one clade. Other *Setaria* spp. including *S. cervi*, *S. digitata*, *S. tundra* and *S. equina* formed a sister clade well separated from the one consisting of *S. labiatopapillosa* and Khuzestan isolates. Based on 12s rDNA sequence Khuzestan isolates divided into two subclades. Isolates 1, 4, 6, 7, 9 and 10 showed the highest relationship with *S. labiatopapillosa*, while isolates 2, 3 and 8 had the highest genetic variation in relation to *S. labiatopapillosa*.

Our results have also shown that intra-specific variation was observed in *cox1* but not in 12S rDNA.

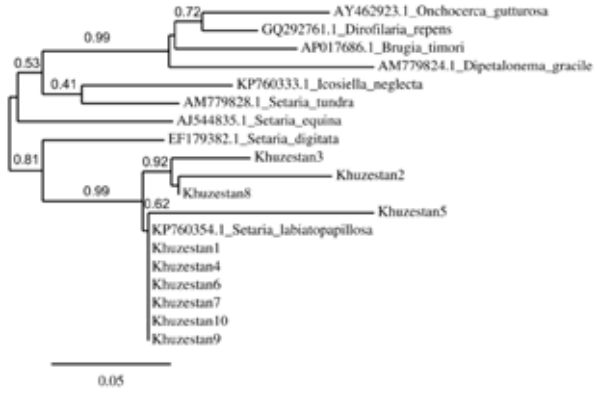


Fig. 5. Neighbor-Joining phylogenetic relationship of 10 isolates of *S. labiatopapillosa* from cattle of Ahvaz, Iran. The analysis was based on 12s rDNA gene sequences (450 bp). Percentage bootstrap support from 1000 replicate samples is indicated at the right of the supported node. Accession numbers for sequences obtained from GenBank are shown, followed by name of Filarioidea species. The scale bar indicates distance.

DISCUSSION

Setariosis is one of the most important parasitic disease in cattle which caused by different species of *Setaria* in different countries of the world. The implementation of molecular techniques for the identification of the parasite species is an important requirement to plan effective control strategies for the emerging parasite infections. This study was undertaken to evaluate the morphological and phylogenetic characteristics of *Setaria* spp. in cattle and buffaloes in Southwest of Iran (Khuzestan region) The climatic conditions of Southwest of Iran favour the presence of mosquitoes at almost 5 months of the year. Sixteen species of mosquitoes belonging to five genera, including *Aedes* (2 Species), *Anopheles* (5 Species), *Culex* (6 Species), *Culiseta* (2 Species) and *Ochlerotatus* (1 Species) were reported from this region which can transmit *Setaria* spp. (Navidpour et al., 2012; Nasirian et al., 2014; Maghsoodi et al., 2015; Farhadinejad et al., 2015).

In this study, the overall prevalence of Setarial infection in cattle was 12.3%, which reflects the considerable prevalence rate of infection in cattle. There were no significant association between seasons and infection which might be due to high longevity of adult worms, absence of periodicity of microfilaria in the blood and seasonal distribution of mosquitoes as well. Our findings showed that the prevalence rate of infection in cattle below 1 year of age was higher than other age groups. This might be attributed to the weakness of the immune system in younger animals and providing more opportunities for the development of the parasite. It seems that the onset of the parasite infection and its complete development occur in calves; thus allowing them to continue in older animals.

Despite the fact that female animals are slaughtered at an older age, no significant differences were observed between the gender and the infection. Several surveys have been carried out to determine the prevalence of *Setaria* spp. around the world. The prevalence ranges from 11.11% to 47% in different regions (North, Northwest and East) of Iran have previously been reported (Bazargani et al., 2008; Khedri et al., 2014; Dawoodi, 2014). In previous studies, *S. digitata, S. marshalli,* and *S. labiatopapillosa* have been isolated from cattle and buffaloes from different regions of Iran, while in our study, *S. labiatopapillosa* was the only isolated *Setaria* species from cattle of southwest of Iran. The difference between the findings may be due to differences in climate, the presence of a suitable intermediate hosts and livestock management systems in various regions.

Setariosis in buffaloes has been reported from

different countries such as India and Iran. The prevalence rate of infection varies from 0.9% to 54% (Dawoodi, 2014; Siddiqui et al., 1996; Patnaik, 1989; Chauhan and Pande, 1980) but in this study, no infection with *Setaria* parasite was observed in buffaloes. The reasons for the lack of setarial infection in buffaloes in this area have not been clarified yet. It may be related to presence of genetic resistance, differences in the structure of the skin and the absence of specific intermediate host for development and transmitting the parasite to the buffaloes. To strengthen one of the previous assumptions it has been found that buffaloes have genetic resistance to some internal and external parasitic infection such as tick infestation, fascioliasis and theileriosis (FAO, 2007).

The present study provides detailed information about the ultrastructures of *S. labiatopapillosa*. Conventionally, the light microscope is used to identify the *Setaria* spp. but, sometimes due to the low-resolution images, an accurate identification of some species (i.e., *S. labiatopapillosa* and *S. marshalli*) becomes difficult and leads to a misdiagnosis; on the contrary, ultrastructural images prepared by the SEM are of high quality and facilitates the identification of the *Setaria* spp. (Almeida et al., 1991; Ronghang and Roy, 2013; Kumar and Kumar, 2016). Some morphological features such as amphid, ventral bands, patterns of cloacal papillae in male worms and the genital pore in female worms were clearly visible with SEM. These structures were not detectable with light microscope. It should be noted that SEM (Scanning Electron Micrographs) of samples which were prepared with the osmium tetroxide lacked adequate quality sometimes were collapsed and some structures such as amphids were not detectable while these problems did not exist in samples that were only fixed with 5% glutaraldehyde. According to our results, it seems that the use of osmium tetroxide is not suitable for the preparation of *Setaria* spp.

Different studies have shown that larval stages of filarial species usually cannot be differentiating by classical morphology. Analysis of genetic markers can also be reliable for the accurate differentiation of *Setaria* spp. and the determination of genetic diversity in parasites originated from different geographical areas. In the present study, molecular characteristics and genetic variations of *Setaria* spp. isolated from cattle and originated from southwestern Iran were determined by PCR-sequencing of the 12S rDNA and *cox1* genes. In fact, previous studies have shown that these sequences provide reliable genetic markers for the accurate differentiation and identification of *Setaria* spp. The analyses confirmed that all the sequences from the cattle and localities are identical to those of previously submitted to GenBnak for *S. labiatopapillosa*. These results indicated that this species is the main *Setaria* species involved in the spread of Setariosis in southwestern Iran.

The 12S rDNA sequences of *S. labiatopapillosa* obtained in this study showed nucleotide variations in 53 positions. The comparisons of all obsereved *cox1* sequences with related sequences of *cox1* of *S. labiatopapillosa* from other geographical areas showed lower nucleotide differences in compared with 12S rDNA. Yatawara et al. (2007) noted that mitochondrial *cox1* and 12S rDNA genes are conserved in *S. digitate*. They also mentioned that *S. digitata* and *S. labiatopapillosa* appear to be sister species.

The phylogenetic tree constructed using *cox1* and 12S rDNA genes of several other filarial nematodes showed that the Khuzestan isolates share a common branch with *S. labiatopapillosa.* Low intra-specific variation was observed in 12S rDNA but not in *cox1*. In fact, groups of multiple closely related genotypes of *S. labiatopapillosa* obtained in the present study are broadly sympatric. Such pattern is expected for species with high gene flow, whose populations have not been sundered by long-term biogeographic barriers.

The genetic characterization of *Setaria* Spp. present in southwestern Iran is useful to achieve the basic information necessary for the field control of this parasite and may have implications for the diagnosis and control of the disease. To better understand the genetic variability and population genetic structure of *Setaria* spp. in Iran and in oth-

er neighboring areas a wide range of isolates from different hosts and geographical localities and the use of more variable genetic markers are needed

In conclusion, our results showed that infection of cattle with *S. labiatopapillosa* is common in southwestern Iran while buffalo may be free from filarioid nematodes. From the above findings, it can be proved that DNA sequences of 12S rDNA and *cox1* genes are useful molecular tools for accurate identification of *Setaria* species. Further studies are necessary in order to recognizing the molecular and morphological characteristics of other filarial species infecting cattle in Iran, its vectors and possible prevention.

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CONFLICT OF INTEREST STATEMENT

The authors confirm that there is no conflict of interest.

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