Original Article

Molecular Genetic Analysis of Helminths of Southern Uzbekistan Chickens (Galliformes)

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Abstract

The analysis of sources on helmintofauna of the chickens (Galliformes) of Southern Uzbekistan is presented in the article, besides information about the material and methodology of research, molecular and genetic analysis methods are given.

Keywords: Tetrameres, Oxyspirura, Chloroform, Electrophoresis, Ascariasis, Geminolepidosis, Phenol.

INTRODUCTION

In our republic, special attention is paid to the creation of new productive breeds resistant to various diseases in the field of agriculture, including poultry, comprehensive research of helminths that parasitize them, and improvement of measures to combat them. In this regard, preventive measures have been developed to prevent chicken helminthosis, which negatively affects the health and productivity of poultry and chickens. Accordingly, it is of great theoretical and practical importance to establish the species composition, ecological characteristics, distribution and life cycles of chicken-like helminths distributed in the southern regions of Uzbekistan, as well as to improve the methods of combating them. The Strategy of Actions for the further development of the Republic of Uzbekistan defines such tasks as "expanding scientific research works on the creation and introduction into production of high-productivity animal breeds that are resistant to diseases and pests, adapted to the local climate and ecological conditions". In the successful solution of these tasks, among other things, the identification of the helminth fauna of the hen-like family of South Uzbekistan, the assessment of their ecology and importance, and the development of measures to combat them are of great scientific and practical importance. Endohelminths parasitizing chicken-like birds in the southern regions of Uzbekistan were collected as materials for research. During 2010-2021, researches were carried out at the Department of Zoology of Termiz State University and the Laboratory of Molecular Zoology of the Institute of Zoology of the Academy of Sciences of the Republic of Uzbekistan.

RESULTS AND DISCUSSION

In order to carry out the research work, in 2010-2021, stationary research in mountain and sub-mountain areas of the districts of Surkhandarya and Kashkadarya region (Termiz, Jarkurgan, Boysun, Muzrobot, Dekhkanabad and Shahrisabz) in the southern regions of Uzbekistan, based on the route method from the river valleys of partial plain zones, 248 chickens, 52 partridges, 68 pheasants, 47 quails, 12 chickens, and the analysis of the collected helminthological materials was carried out according to generally accepted methods (Scryabin 1928, Dubinina, 1971, etc.).

The most effective method of diagnosing helminths is the method of K.I. Skryabin [1; 45-c.], which is a complete and incomplete helminthological examination method. In this method, all the organs and tissues of the animal were specially processed and carefully examined, because the body of the bird may contain mites that feed on ectoparasites. After removing the skin of the examined chickens, first the main attention was paid to the subcutaneous tissue, then the organs of the chest and gastrointestinal tract, starting from the esophagus to the rear exit hole, were cut into separate containers, and the food and dung masses in them were washed and left in the container, the inner mucous wall of the stomach and intestines was scraped and they were also processed in a container together with food and faecal masses by the method of serial washing, that is, the sample was washed several times (3-5 or more times) with plain water until the supernatant became clear. The sediment was examined thoroughly, using magnifiers when necessary.

In the same way, the respiratory organs: starting from the larynx (trachea), all the bronchi and bronchioles of the lungs were

cut, the lung parenchyma was divided into small pieces and washed successively in water, the precipitate was examined under a microscope, and eggs, larvae and adult helminths were collected and 427 samples from 5 species belonging to 1 family of chickens were collected by the method of complete helminthological examination (Table 1).

In the method of incomplete helminthological examination, some organs and tissues are examined by incomplete helminthological examination. It is considered a simplified helminthological examination and is carried out only when the location of helminths is clear. For example: ascariasis and geminolepidosis, which are parasitic in 57 incompletely helminthologically examined chickens, are mainly parasitic in the intestines. Therefore, the intestines of chickens are taken out and examined separately. In the process of dissecting the body of chickens, helminths, which differ in size and color from organs and tissues, are isolated and used in the preparation of a temporary or permanent drug. Nematodes were fixed in Barbagall liquid, trematodes and cestodes in 70% ethyl alcohol.

Table 1: Chickens studied in South Uzbekistan species composition

Familia	**Kind**	**Checked sample**	**Number of damaged birds**
Phasianidae	*Alectoris chukar* G.R.Gray, 1843-	52	21
	Gallus – gallus domesticus	248	178
	Coturnix coturnix Linnatus, 1758-	47	19
	Phasianus colchicus Linnaeus, 1758-	68	40
	Ammoperdix griseogularis J.E.Gray, 1830	12	1
Total:		**427**	**259**

Processing of parasitological material was carried out in laboratory conditions. Permanent and temporary preparations were studied using stereoscopic LOMA MB S-10, MBR-3, MBS, inverted SK2-TR (Olympus Japan), binocular VL-2200 (Olympus Japan) microscopes.

METHODS OF MOLECULAR AND GENETIC ANALYSIS

DNA isolation - in our research genomic DNA is isolated from the tissue of partridge and pheasant digestive system nematodes. In this case, the nematodes pre-soaked in 96% or 70% ethyl alcohol are thoroughly washed with distilled water and each one is placed in an eppendorf tube containing 100 µl of lysis buffer. For the isolation of genomic DNA from nematodes, it is preferable to use their head (from the head of the body to the end of the esophagus), which is cut under a binocular magnifying glass, using an eye scalpel or a razor blade (the tail side of male nematodes is left for morphological and molecular analysis).

In our research, genomic DNA was isolated from species belonging to the genera Tetrameres Creplin, 1846 and Oxyspirura. In this case, the bodies of Tetrameres fissispina and Oxyspirura sp., which were previously soaked in 70% or 96% ethyl alcohol, were thoroughly washed with distilled water, and 3 copies of each species were taken and placed in 1.5 ml eppendorf tubes, and 100 µl of lysis buffer was added.

3 different methods were mainly used to extract DNA from the samples.

DNA isolation using the phenol-chloroform method is a classic method for isolation of nucleic acids. This technique involves the digestion of biological materials by detergents in the presence of proteinase K and the separation of nucleic acids using phenol and chloroform. The *PhCh (*phenol-chloroform) -extraction method of nucleic acids from the tissues of Tetrameres fissispina and Oxyspirura species consists of the following steps:

A piece of tissue weighing 0.05 g is taken from *Tetrameres fissispina* and *Oxyspirura species.*

1. *Tetrameres fissispina and Oxyspirura species* are homogenized in a buffer solution containing 40 mM tris-HCl (pH = 8.0), 0.5 mM EDTA, 1xSSC (100 µg of tissue and buffer; 500 µg ratio).
2. Add up to 0.5% SDS and proteinase K to a final concentration of 1 mg/ml, mix and incubate at 37°C for 2-3 hours or leave for 18 hours.
3. Add 5 M sodium acetate to the test tube and mix until the final concentration is 0.1 M. Then 1:1 ratio of phenol, tris saturated HCl (pH=8.0) was added and stirred for 10-15 minutes.
4. Add chloroform in a ratio of 1:1 and mix for another 5-10 minutes.
5. The resulting mixture is centrifuged at a temperature of 4°C for 20 minutes (at a speed of 4000 revolutions for one minute).
6. The above fraction *with* dissolved DNA (supernatant) is withdrawn using a pipette.
7. The process of *separation* from protein with phenol-chloroform is repeated until it is completely free.

8. Chloroform was added to the separated supernatant in a ratio of 2:1, mixed for 10-15 minutes, and then centrifuged for 15 minutes (at 4000 *rpm* for one minute) to obtain the upper phase.
9. 5 M sodium acetate was added to 1 ml of dissolved DNA and mixed to a final concentration of 0.2 M.
10. Two volumes of 96% ethanol were added and mixed continuously until the DNA precipitated.
11. It is cooled at -20°C for one day, then centrifuged at 15,000 rpm for 15 minutes (until it cools down to 0°C).
12. The supernatant is removed, the DNA precipitate is washed in a 70% ethanol solution.
13. The ethanol solution is drained, the DNA is air-dried until the ethanol solution evaporates, and dissolved in deionized water.

The concentration of the obtained DNA preparations is determined in a spectrophotometer. The concentration of isolated DNA was 2.0-2.3 μg/μl. The concentration of the obtained DNA preparations is determined in a spectrophotometer. The concentration of isolated DNA was 2.0-2.3 μg/μl.

DNA isolation using the Diatom DNA Prep firm set. This method is one of the methods of extraction of nucleotides using reagents or kits. This kit allows for the isolation of DNA from various natural materials, as well as the rapid purification of DNA from clinical samples. The mechanism of action is based on the use of a lysing reagent with guanidinethiothionate, which leads to cell lysis, cell solubilization, and cell nuclease denaturation. In the presence of a lysing (decomposing) reagent, DNA is actively absorbed in the NucleosTM sorbent kit, then it is easily washed from proteins and salts in an alcohol solution. The DNA isolated from the sorbent can be used in PCR.

Contents of the set: decomposition reagent, salt buffer Nucleos sorbent suspension, "Extra Gen" ion exchange mixture suspension.

The procedure for extracting DNA from tissues of *Tetrameres fissispina* and *Oxyspirura species* using the Diatom DNA Prep 200 reagent set includes the following steps.

1. According to the manual, the working solution of the buffer is prepared.
2. A piece of tissue weighing 0.05 g was cut from the body of *Tetrameres fissispina* and *Oxyspirura* species, placed in a 1.5 ml test tube (it is better to take the head of the nematode, because the tail part is of particular importance in the morphological identification of the species), ground with 800 μl of disintegration reagent and mixed by hand 5-10 times.
3. The mixture is placed in a thermostat with a temperature of 65°C for 5-7 minutes.
4. The mixture in the test tube is centrifuged at a speed of 5000 rpm for 10 seconds.
5. The supernatant is taken into a clean test tube, and the pre-homogenized Nucleos suspension is added to it in the amount of 20-40 μl.
6. The tube should be mixed with a rotator at a speed of 10-20 rpm or by hand, then at a speed of 5000 rpm for 10 sec. centrifuged and the supernatant removed.
7. Add 400 μl of dissolution reagent to the precipitate and vigorously mix with a vortex until homogenous.
8. 1 ml working solution of saline buffer is added to the mixture, mixed 5-10 times, centrifuged at a speed of 5000 rpm for 10 seconds, and then the supernatant is removed.
9. Step 7 is repeated.
10. The precipitate is dried at a temperature of 65°C for 4-5 minutes.
11. "Extra Gen" suspension is poured into this test tube in the amount of 50-100 μl and mixed until it becomes homogeneous, then it is placed in a thermostat at a temperature of 65°C for 5 minutes and mixed again.
12. Then it is centrifuged at a speed of 10,000 rpm for 1 minute.
13. The supernatant is taken into a clean test tube and stored at -20°C.

The concentration of isolated DNA is 0.12-0.17 μg/μl.

In most cases, the DNA extracted from the "Fast" PgCh – method must be cleaned of protein and carbohydrate residues. This can be cleaned using the Nucleos suspension in the Diatom Reagent Set, starting with step 5 of the method (Figure 1).

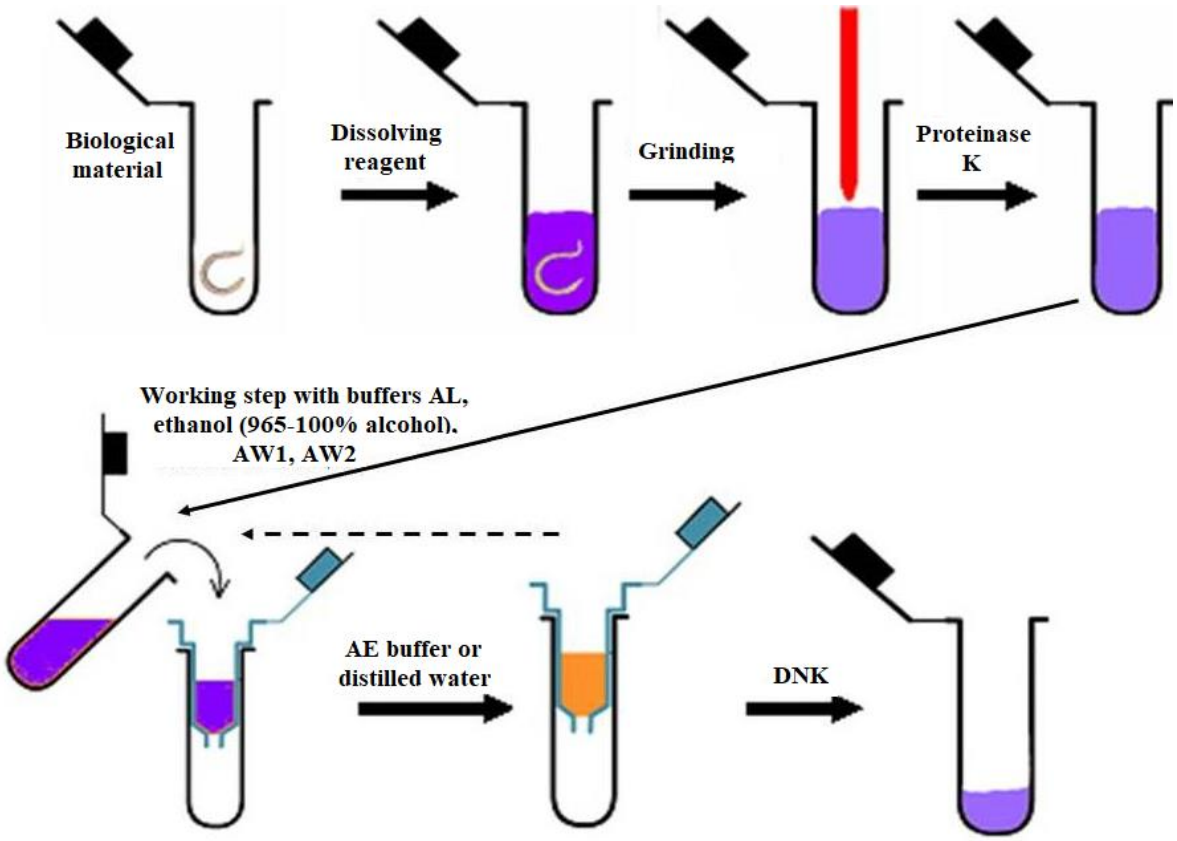

Figure 1. DNA isolation procedure (diagram)

DNA isolation using Dneasy Tissue Set (QIAGEN GmbH, Germany) *Tetrameres fissispina* and *Oxyspirura* species are isolated or 1-2 larvae or eggs are taken with a pipette and placed in a 1.5 ml Eppendorf test tube. Samples should not weigh more than 10 mg.

1. 180 µl of ATL buffer is added to the biological material.
2. Added 20 µl proteinase K and vortex for 15 sec. is mixtured. Test tubes are incubated in a thermostat at a temperature of 55°C until complete decomposition of biological material. Samples can be left for 1-3 hours or overnight for 18 hours.
3. Vortex for 15 sec, then add 200 AL buffer, vortex for 15 sec, incubate at 70°C for 10 min.
4. Then add 200 µl of ethanol (96-100% alcohol) and mix in a vortex until a homogeneous solution is formed.
5. Each homogenate is carefully placed in filter epindorff test tubes (Dneasy Mini spin column) and centrifuged for 1 minute at a speed of 8000 rpm.
6. The clear liquid transferred to Epindorf is transferred to 2 ml test tubes, 500 µl of AW1 is added to it and centrifuged at 8000 rpm for 1 minute.
7. Transfer the clean liquid to 2 ml filter tubes, add 500 µl of AW2 buffer and centrifuge at 14,000 rpm for 3 minutes until the liquid is completely separated from the filter.
8. Transfer the filter Epindorf to another 1.5 ml or 2 ml test tube and add 50-100 µl of AE buffer or distilled water, then incubate for 2 minutes at room temperature and centrifuge at 8000 rpm for 1 minute. The DNA dissolved in the buffer is stored at -20°C.
9. The eluate process can be repeated according to step 8.

Polymerase chain reaction (PCR) is an amplification method

The genome DNA of Tetrameres fissispina and Oxyspirura sp species isolated for amplification was amplified using the Silex reagent kit - 10x PCR buffer, dNTP solution, Taq polymerase, primers (ITS2 F -5` TAACAAGGTTTCCGTAGGTGAA 3`, ITS2 R -5` GCTGCGTTCTTCATCGATGC 3`), (Table-2) used in molecular taxonomy to study the nucleotide sequence of the ITS-2 region of the chromosome and using distilled water.

PCR was performed using an automatic programmable amplifier (Touchgene Gradient, UK).

PCR was carried out according to the following scheme: step 1 - denaturation of DNA at 95°C for 3 minutes, step 2 - denaturation of DNA at 93°C for 20 seconds, step 3 - DNA denaturation at 55°C for 30 seconds annealing of primers, step 4 -

elongation at 72°C for 2 minutes, step 5 - chain elongation at 72°C for 10 minutes. From the second to the fourth step, the process was repeated up to 30 times in a loop form.

The master-mix was prepared according to the manufacturer's reagent set protocol (Table 2).

Table 2: List of reactants for master mix

Water (ster.)	13.2 µl
10x PCR buffer	2 µl
dNTP	3.2 µl
From each primer	0.25 µl
Taq polymerase	0.2 µl
Total:	19.2 µl

Agarose gel electrophoresis method. Gel – electrophoresis method was used to check the separation of DNA in samples after polymerase chain reaction. This is an analytical (analytical) method that is used to separate, align, and purify DNA fragments. DNA electrophoresis is carried out in the horizontal direction [2; p. 288].

Gel composition includes: 1X TAE (pN 8.1), agarose, ethidium bromide. To prepare an agarose gel and perform electrophoresis on PCR products, the following sequence was performed.

1. Before placing the gel in the electrophoresis bath, wells were made by installing plate-mirror combs to insert the samples. The lower teeth of the combs should be placed at a distance of 2 mm from the base of the gel with a total volume of 50 ml (placed at a distance of 1 mm from the base of the gel with a total volume of 150 ml).
2. To prepare 50 ml of 2% agarose gel, add 50 ml of 1X TAE and 1 g of agarose. A starting concentration of 1X TAE is prepared from a 50X TAE solution (Tris, 0.5M EDTA pH 8.0, glacial acetic acid).
3. The mixture of TAE with agarose placed in the flask is heated until the solution reaches a homogeneous state, that is, there should be no undissolved particles of agarose.
4. After this process, it was cooled to 50°C and 0.5 µl of ethidium bromide was added.
5. The entire gel volume was poured into the electrophoresis bath. After the gel had cooled (30-45 min at room temperature), the combs were slowly removed and 1X TAE buffer was poured into the electrophoresis bath until the gel was completely covered.
6. After 10-15 minutes, 2.5 µl DNA marker DNA Ladder 100pb (Promega) was added to one of the wells.
7. For DNA separation, the voltage should not exceed 5 volts per centimeter of gel.
8. After 40-45 minutes, the DNA in the gel is viewed and photographed under UV light, and the results are recorded.

DNA purification. The desired DNA fragments resulting from electrophoresis were cut from the gel using a scalpel and placed in a 1.5 ml Epindorf test tube. DNA was extracted from the gel and purified according to the instructions produced by the company "Silex M" (Fig. 2).

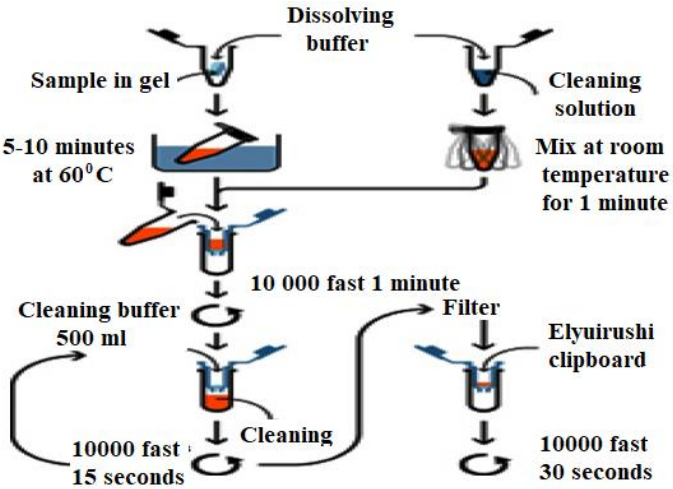

Figure 2. DNA purification workflow diagram

Sequencing - determining the nucleotide sequence of DNA. Prior to sequencing the gel-purified PCR products, gel-purified DNA concentrations were measured and sequenced using the PCR primers (Table 3).

Table 3: Nematode species submitted to sequencing

Nematode species	DNA concentration (ng/µl)	The amount of DNA sequenced
Tetrameres sp	10,1	2,1
Oxyspirura sp	15,2	0,9

DNA sequencing using ABI PRISM® BigDye™ Terminator v. 3.1 was performed using reagent set, and reaction plots were recorded on an ABI PRISM 3100-Avant automatic sequencer.

The sequence data was obtained in "ab1" format and "Chromas version 1.45" [3; 1592-1595-p.] was analyzed using the program. In order to correct the errors of the data obtained from the sequence, the results of the sequence trained using the correct and reverse primers were converted into FASTA-format. Then "Clustal X version 1.81" [4; 502-506-p.] was carried out using the program. "Gendoc version 2.5.000" [5; 1999.] unnecessary nucleotides were removed using the program. "ForCon version 1.0 for Windows" [6; 3381-3391-p.] was implemented in the program. Phylogenetic analyzes PAUP* 4.0b10 [7; 10-14 p.] was implemented in the program. It should be noted that this program works only on the Nexus file.

In our molecular studies, the nucleotide sequence in the ITS2 region of rDNA of *Tetrameres fissispina* and *Oxyspirura sp* species was amplified. The PCR products were then examined by gel electrophoresis and the samples were photographed (Figure 1). According to the results of electrophoresis fractions, it can be seen that about 250-260 pairs of nucleotides were separated in the samples.

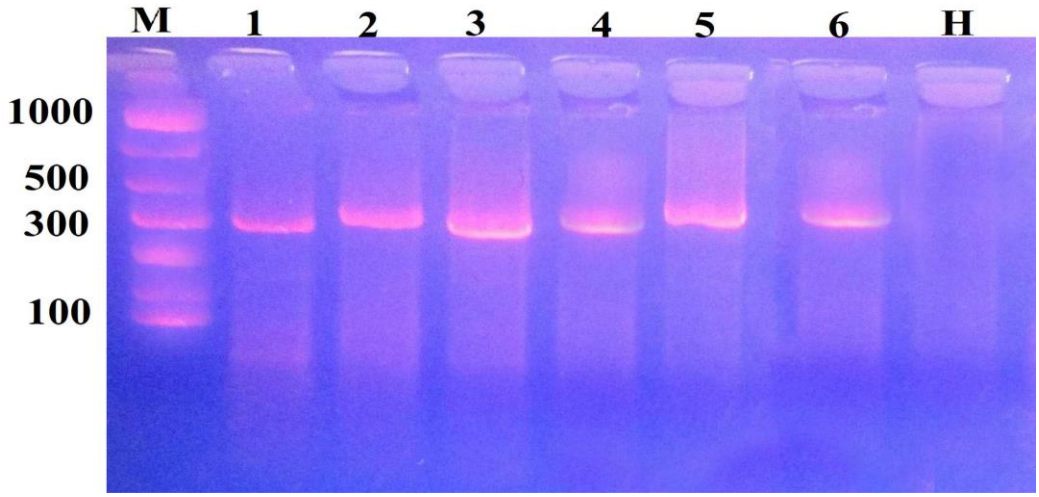

Figure 3. M-marker, 1-3- Tetrameres sp, 4-6- Oxyspirura sp, N- control.

Based on the chromatograms obtained from the sequencing, the sequence of 246 pairs of nucleotides belonging to the rDNA ITS2 region was determined from the samples. Comparing these obtained data with the International Nucleotide Sequence Database (GenBank) data, the following results were obtained (Figure 2).

When the DNA sample of Tetrameres sp. was compared with the data in the international GenBank database, the similarity between the nucleotides of the species *Tetrameres fissispina* (accession number-MN305802) was about 99-100%. It can be seen from the table that the insignificant difference is that thymine is substituted for guanine in 107 nucleotides, and adinine nucleotides are substituted for thymine in 155 nucleotides. The total nucleotide difference between them was 0.8%. Thus, we can say that this specimen of Tetrameres sp is the type of *Tetrameres fissispina.*

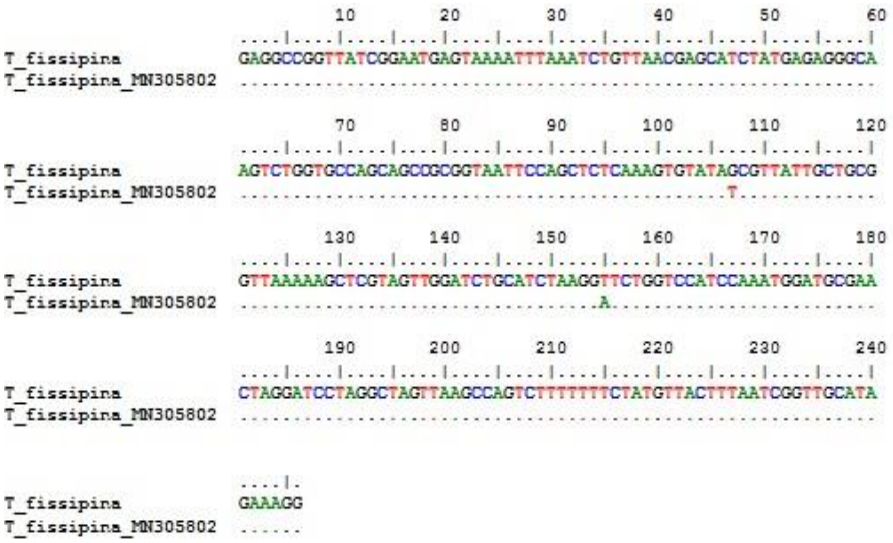

Figure 4. Fragment of the rDNA ITS region of Tetrameres sp and Tetrameres fissispina species (from 5' to 3'-terminal direction).

According to Oxyspirura (Drasche in Stossich, 1897) genera, *Oxyspirura sp*. species, 96% close to *Oxyspirura mansoni* (accession number LC538186) in the international GenBank database was found.

There were 9 differences between the nucleotides of *Oxyspirura sp*. and *Oxyspirura mansoni* species, and these differences were found to be differences in nucleotides 26, 81, 120, 157, 160, 162,186, 202 and 225 (Figure 3).

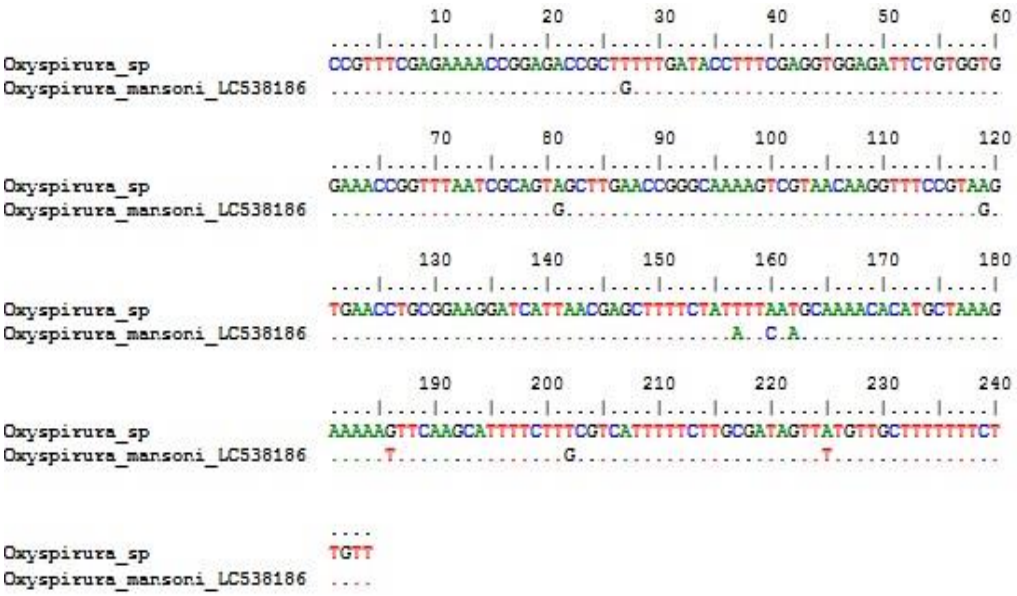

Figure 5. Fragment of the rDNA ITS region of Oxyspirura sp and Oxyspirura mansoni species (5' to 3'-terminal direction).

CONCLUSION

As a result of the experiment, it was concluded that when comparing the obtained data with the International Nucleotide Sequence Database (GenBank) data, *Tetrameres sp.* species *Tetrameres fissispina* (accession number-MN305802) is 99-100% close and *Oxyspirura sp* species is 96% close to *Oxyspirura mansoni* (accession number-LC538186).

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