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Research Article

FIRST REPORT ON LOCAL ENTOMOPATHOGENIC NEMATODE STEINERNEMA FELTIAE IN UZBEKISTAN

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ABSTRACT

In 2020-2023, a study was conducted to study the distribution and biological diversity of steinernematid and heterorabtid entomopathogenic nematodes (EPN) in three regions of Uzbekistan.

Steinernematids are more common than heterorhabditids, and S. feltiae is the most common species. Sampling sites were grouped into 4 habitats: barren land, gardens, fields, residential homesteads; the soil composition of each plot was determined and the influence of the habitat and soil composition of each species was evaluated.

KEYWORDS

4 habitats: barren land, gardens, fields, residential homesteads; the soil composition.

INTRODUCTION

Pest control of agricultural crops is one of the global problems of today's food production. In this regard, it is important to develop countermeasures by protecting the ecological environment and soil health. The use of entomopathogenic - beneficial nematodes as biological control measures is used in world experience and is highly effective [15].

Beneficial nematodes occur naturally in the soil and are used to control insect pests and their larvae. Beneficial nematodes enter and feed on their hosts through the mouth, anus, or

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respiratory openings. This leads to the emergence of specific bacteria from the intestinal tract of the nematode [13]. These bacteria leave the body cavity of nematodes, spread inside insects and multiply very quickly. The bacteria convert the insect tissue into food that is easily absorbed by the nematodes, and the insects die within a few days.

Beneficial nematodes can be used for biological control of more than 200 insect pests belonging to 100 genera that live in the soil and pass the larval stage in the soil [3].

They are distributed globally, but the biodiversity and distribution of their species in some countries and regions are still unexplored.

In particular, until now, no specific scientific information has been provided about scientific research related to entomopathogenic nematodes in Uzbekistan. This shows that the study of these beneficial organisms and the development of technologies for their use as objects of biological control are relevant.

The purpose of this research is to identify local entomopathogenic nematodes distributed in the territory of Uzbekistan, to analyze them from a molecular and genetic point of view, and to publish preliminary information about them.

Entomopathogenic nematodes (EPN) range in size from 0.3 to 10 mm. In Greek dictionaries, the term entomopathogenic nematodes is derived from the words "entomos", "pathe", "guenos" - meaning a group of nematodes capable of causing disease in insects.

They belong to two families, Steinernematidae, i.e. Steinernema (100 valid species) and Heterorhabditidae, i.e. Heterorhabditis, comprising 16 well-characterized species globally (Kaya & Stock, 1997).

MATERIALS AND METHODS

Soil samples were collected from the southern part of Uzbekistan in 2020 and 2023. Sampling was carried out in spring and autumn from different fields. A total of 1200 soil samples were taken. Soil was collected from the upper 10 - 30 cm layer and a total of 1 kg of soil was sampled from five parts of each plot.

The samples were stored in polyethylene bags in the refrigerator until extraction. Samples were processed within two days of collection [2]. Each sample was thoroughly mixed and small samples were taken from each soil sample for soil type, pH analyses.

Using wax moth larvae (Galleria mellonela) using the "bait" method developed by Kaya and Stock (1997), beneficial nematodes in the soil were collected in special plastic containers with 5-10 larvae per soil sample and stored in the dark at room temperature (20-22 ° C) was stored for 14 days [12].

At two-day intervals, dead insects from the samples were collected and placed in a “modified White trap” (Kaya & Stock, 1997) to collect nematodes from the insect body. Collected infective stage nematode larvae were stored in special culture flasks in an isotonic solution in a refrigerator at 4°C.

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All larvae and 50 adult nematodes obtained in the experiment were fixed and transferred to anhydrous glycerol. Nematodes were examined under a special biological MBC 10 microscope, morphological and morphometric analyzes were performed.

Molecular genetic methods

Species were selected for molecular-genetic research from the samples of nematodes belonging to the genera Heterorhabditis Poinar, 1976 and Steinernema Travassos, 1927 collected from the study area. Diatom DNA Prep kit was used to extract genomic DNA from selected samples [25].

DNA isolation using the Diatom DNA Prep kit

This method is one of the methods of extraction of nucleotides using reagents or kits [1].

This kit allows for the isolation of DNA from various natural materials, as well as the rapid purification of DNA from clinical samples. This method differs from the FX method by its speed (30 min. - 1.5 time is spent on 1 sample), and the absence of use of toxic (poisonous) reagents. The mechanism of action is based on the use of a lysing reagent with guanidinethiocyanate, 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 [27]. DNA separated from the sorbent can be used in PCR [4].

The composition of the kit: disintegration reagent, saline buffer Nucleos sorbent suspension, "Extra Gen" ion exchange mixture suspension.

The method of DNA extraction from nematode tissues using the Diatom DNA Prep 200 reagent kit includes the following steps [14].

1. A buffer working solution is prepared according to the instructions.

2. A piece of tissue weighing 0.05 g is cut from the body of the nematode in a 1.5 ml test tube (it is better to take the head part of the nematode, because the tail part is important for morphological identification of the species) and grind it with 800 µl of the disintegration reagent and use the hand for 5-10 mixed once.

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 for 10 seconds at a speed of 5000 rpm.

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. Mix the tube with a rotator at 10–20 rpm or by hand, then spin at 5000 rpm for 10 sec. centrifuged and the supernatant removed.

7. 400 µl of dissolution reagent is added to the precipitate and vigorously mixed with a vortex until homogenous.

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8. Add 1 ml working solution of saline buffer to the mixture and mix 5-10 times, 5000 rpm. centrifuged at 10 s for 10 sec, 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 reaches a homogeneous state, then it is placed in a thermostat at a temperature of 65°C for 5 minutes and mixed again.

12. Then 10000 rpm for 1 minute. centrifuged at high speed.

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.

DNA extracted from the "fast" FX method must be cleaned of protein and carbohydrate residues in most cases. This can be cleaned using the Nucleos suspension in the Diatom reagent kit, starting from step 5 of the method (Figure 1).

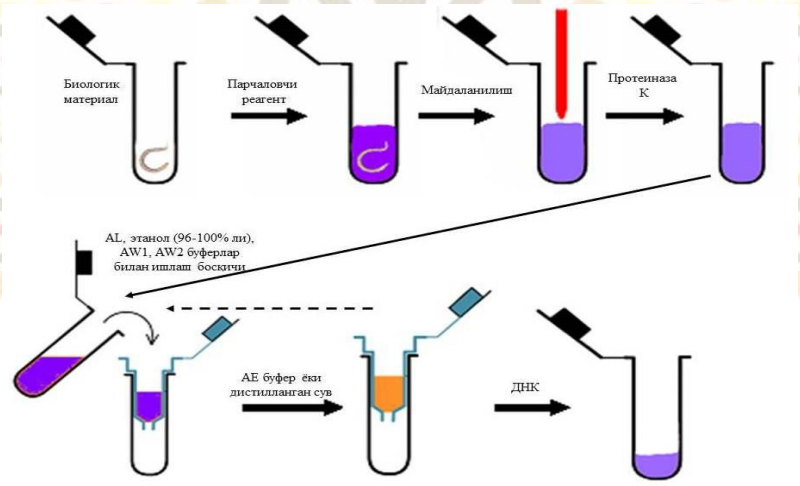

Figure 1. DNA isolation procedure (diagram)

PCR-amplification method. In order to study the nucleotide sequence of the COI region of the chromosome, the genomic DNA of the insects isolated for amplification was amplified using the reagents of the "Silex" company - sterilized water, 10x PCR buffer, dNTP solution, Taq-polymerase and the following primers used in molecular taxonomy (Table 1) .

Table 1

Primers used in Taq polymerase and molecular taxonomy

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No	Primer Name (Correct Primer)	Primer Name (Correct Primer)
1.	FWD_533: 5′- CAAGTCTTATCGGTGGATCAC 3′	REV_534 5′- GCAATTCACGCCAAATAACGG-3′

Polymerase chain reaction (PCR) was performed using a programmable autoamplifier (Touchgene Gradient, UK). Master-mix was prepared from the following reagents based on the company's instructions (Table 2).

Table 2

List of reactants for master mix

Water (ster.)	13.8 µl
10x PCR buffer	2 µl
dNTP	0.6 µl
From each primer	1.5+1.5 µl
Taq polymerase	0.6 µl
Total:	20 µl

PCR was carried out according to the following scheme: 1 - step - denaturation of DNA at 95°C for 3 minutes, 2 - step - denaturation of DNA at 93°C for 20 seconds, 3 - step - binding of primers to DNA at 55°C for 30 seconds , 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 35 times in a loop form (Table 3).

Table 3

PCR temperature and time

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Reaction (Program)	*Stage*	*Temperature (°C), stage cycle*		*Time*
I	*Initial denaturation*	95		*3 minutes*
	Denaturation	93	*45 cycles*	*20 seconds*
	Finish	48		*30 seconds*
	Elongation	72		*2 minutes*
	Finish the chain	72		*10 minutes*

Agarose gel electrophoresis method. After completion of the polymerase chain reaction, gel electrophoresis was used. This method is an analytical method used to isolate, align, and purify DNA fragments. DNA electrophoresis is carried out in the horizontal direction [8].

The gel contains: 1X TAE (pN 8.1), agarose, ethidium bromide. Preparation of agarose gel and electrophoresis of PCR products were carried out in the following sequence.

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 were 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).

50 ml of 1X TAE and 1 g of agarose were added to prepare 50 ml of 2% agarose gel [9]. A starting concentration of 1X TAE is prepared from a 50X TAE solution (Tris, 0.5M EDTA pH8.0, glacial acetic acid). The mixture of TAE with agarose placed in the flask is heated until it reaches a homogeneous state (there should be no undissolved particles of agarose in the solution). After this process, it was cooled to 50°C and 0.5 µl of ethidium bromide was added. 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. After 10-15 minutes, 2.5 µl DNA marker DNA Ladder 100pb (Promega) was added to one of the wells [22].

For DNA separation, the voltage should not exceed 5 volts per centimeter of gel. After 40-45

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minutes, the gel was viewed and photographed under ultraviolet and transilluminator light, and the results were recorded [6] .

DNA purification. The desired fragments resulting from electrophoresis were cut from the gel using a scalpel and placed in a 1.5 ml eppindorf tube. A kit of reagents produced by Sileks M (Moscow, Russia) was used to extract DNA from the gel, following the manufacturer's instructions [5,8].

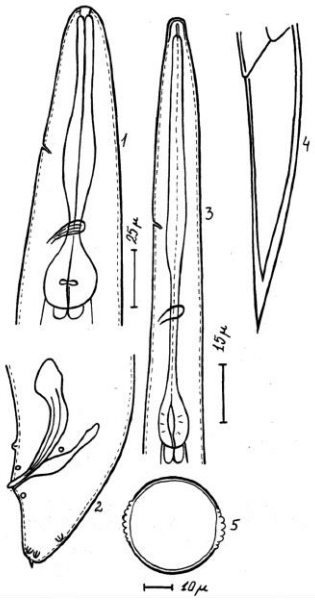
Sequencing - Determining the Nucleotide Sequence of DNA - When sequencing gel-purified PCR products, gel-purified DNA concentrations were measured and sequenced using PCR primers.

In order to correct the errors of the data obtained from the sequence, the results of the sequence, which were trained with the help of forward and reverse primers, were converted into FASTA-format. Then, to combine the results of two chromatography, it was done using the program "Clustal X version 1.81" [Swofford D.L., 1998]. "Gendoc version 2.5.000" [McCarthy; URL: www. Chris. com.] program, unnecessary nucleotides are removed. "ForCon version 1.0 for Windows" [Raes J., 1999] program was used to convert to Nexus-format [7].

RESULTS AND DISCUSSION

Steinernemu feltiae (Filipjev, 1934)

Diagnostics. Males: Males of this species also have six labial and four circular cephalic papillae on the head. The exit opening is located in front of the nerve ring. D% - 60 (51 - 64). It has a long beaked tail. The head part of the spicule is elongated, the ratio of the length and width of the spicule is about 1.5 - 2.0 nm. A spicule shaft is present, without a beak [4,5].



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2- rasm. Steinemema feltiae

1,2 - the front and rear ends of the male body of the first generation; 3,4 - the front and back ends of the body of the infectious larva; 5 - the lateral part of the body of the infectious larva

The length of the spicule is 70 (65 - 77) nm, the ratio of the length of the spicule to the width is 6.0 (5.8-6.2). SW = l,l (1.0 - 1.3); GS = 0.59 (0.52 – 0.61). The gubernaculum is laterally boat-shaped, with a narrow neck area. The stem is short, Y-shaped [23, 24]. Infectious larvae: Average body length 849 (736 - 950) nm. EP = 62 (53 -67) m, E% = 78 (69 - 86). T = 81 (70 - 92) nm.

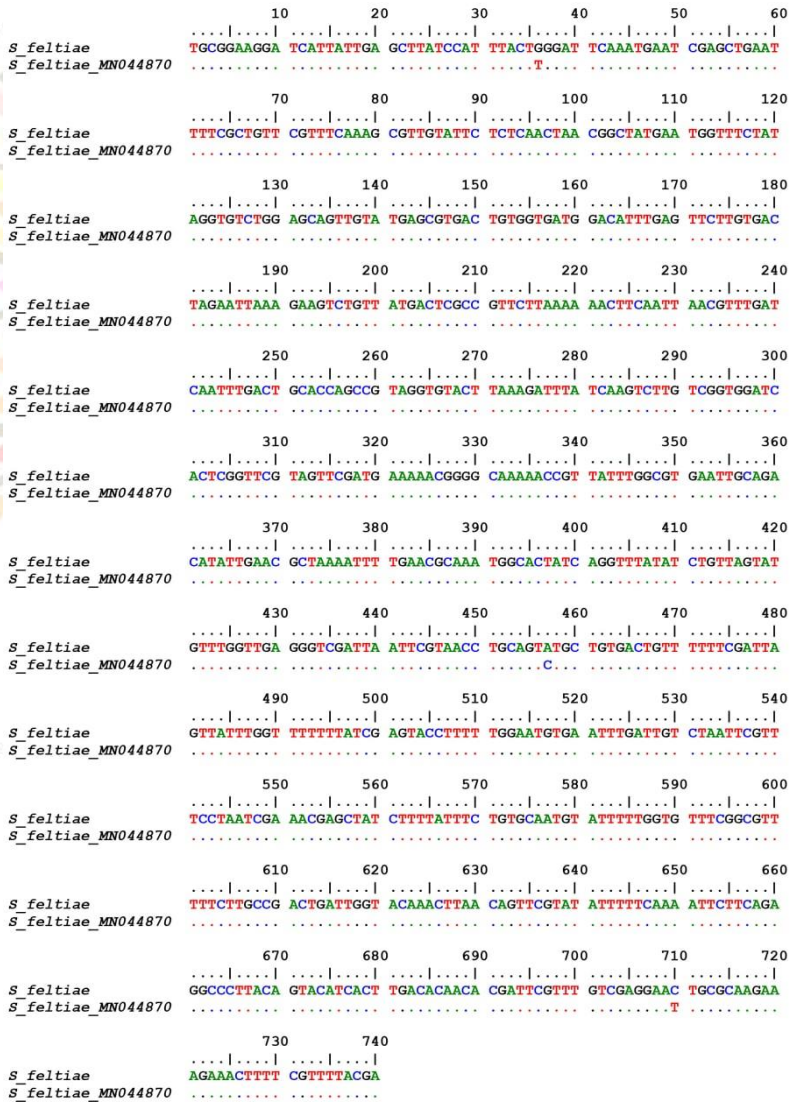

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Figure 2. Comparison of the nucleotide sequence of the pDNA ITS region of species belonging to the genus Steinernema Travassos, 1927, based on the sequence material.

Izox: Steinernema feltiae and Steinernema feltiae (accession number: MN044870) species rDNA ITS region in the 5' to 3'-end direction, identical nucleotide bases marked with dots.

CONCLUSION

As can be seen from the table above, 99.6% nucleotide similarity with Steinernema feltiae (accession number: MN044870) obtained from the international bioinformatics gene database was found, and the studied species from the molecular genetic point of view is Steinernema feltiae. found its proof.

There are 3 nucleotide differences between these species, which are T-thymine instead of G-guanine at 36 nucleotides, C-cytosine instead of A-adenine at 457 nucleotides, and T-thymine instead of C-cytosine at 710 nucleotides. It was explained by the exchange of nucleotides.

These obtained data were placed in the Genbank database of the National Center for Biotechnology Information (Genebank, NCBI) and the accession number (OP380866) was obtained.

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