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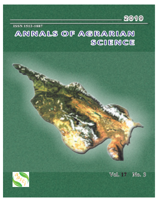

Molecular characterization and phylogenetic relationships of *Globodera rostochiensis* isolates in two geographically distinct regions (Samckhet-Javakheti and Svaneti) of Georgia

D. Gaganidze[a,b*], E. Abashidze[a], N. Nazarashvili[a], M. Aznarashvili[a,b]

[a]State Laboratory of Agriculture; 49, Godziashvili Str., Tbilisi, 0159, Georgia

[b]Agricultural University of Georgia, Sergi Durmishidze Institute of Biochemistry and Biotechnology; 240, David Aghmashenebeli Alley, Tbilisi, 0159, Georgia

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ABSTRACT

Molecular characterization of *Globodera rostochiensi* was performed by the amplification and sequencing D3 expansion region of the large subunit ribosomal gene (28S rDNA).Molecular analysis of *G. rostochiensis* had not been previously performed in Georgia. Sequence analyses of D3 expansion region of the rRNA using computer program BLASTN shown (94-99%) identity with D3 expansion region to all known isolates of *G. rostochiensis*. Based on analysis of *G. rostochiensis* isolates D3 expansion regions are grouped in three major clades (A, B and C) on the phylogenetic tree.Clade A are divided in three subclades, clade C are divided in two subclades. Isolates from Samtckhet-javakheti population are in subclade 1 of clade A and in subclade 1 of clade C. Isolates from Svaneti populations are in subclade 2 of clade A and in clad B. In Clade C subclade 2 is presented by three isolates from Svaneti and by one isolates from Samckhet-Javakheti. Our investigation showed of high genetic variation of D3 region of rDNA of the isolates of *G. rostochiensis* in two geographically distinct origins (Samckhet-Javakheti and Svaneti) of Georgia.

Keywords: *Globodera rostochiensis*, Molecular characterization, Phylogenetic tree, rDNA, D3 expansion region, Polymerase Chain reaction (PCR).

*Corresponding author: Dali Gaganidze; E-mail address: d.ghaghanidze@agruni.edu.ge

Introduction

Potato is one of the main agricultural crops in Georgia. Georgia produces early and late potato varieties in almost all regions. Potato production area equals to 25,000 ha and its average yield is 20-25 t/ha. In traditional potato growing areas (Svaneti, Samckhet javaheti and Kvemo Kartli) the yeld is higher than 30-35 t/ha.

Among the plant-pests that limit potato production and quality, the potato cyst nematodes (PCN) are the harmful around the world. Yield losses caused by PCN are estimated up to 30% [1,2] .Two species of PCN – *Globodera pallida* [3,4] and *Globodera rostochiensis* [4,5] are recognized as plant quarantine pests and are added to the EPPO A2 list [6]. *G. rostochiensis* is present in all EU countries.It is globally recognized as one of the most important factors of yield loss [5]. Cyst nematodes live on the roots of host plants and can damage them to the extent of causing growth retardation, water st ress, nutrient deficiency, early wither and ultimately yield loss [7].

PCN are among the most difficult plant pests to control. Cysts protected by the durable wall can survive for over 30 years [8]. Control measures for cyst nematodes are: use of healthy planting material, crop rotation, chemicals, solarisation, bio-fumigation, weed removal. Currently, the most reliable control method against the cyst nematodes is breeding of resistant potato cultivars [9]. Council Directive 2007/33 /EEC, which establishes the measures against populations of PCN in order to determine their distribution and prevent their spread (Council Directive 2007/33 /EC), regulates control of PCN

(*G. pallida* and *G. rostochiensis*). There was no legislative regulation of these pests before 2016 in Georgia. The Government Resolution #302 from July 1, 2016, developed within the action plan of DCFTA (Deep and Comprehensive Free Trade Area) Agreement for legal acts approximation to EU .

Our rout surveys conducted in two geographically distinct regions of Georgia producing potatoes - Samtskhe - Javakheti and Svaneti revealed potato cyst nematodes (PCN). Morphological, morphometric and molecular analyses of PCN confirmed that belongs to *Globodera rostochiensis* [10,11].

Two genomic regions have been routinely characterized among nematode taxa: the ribosomal RNA array and the mitochondrial genome. The ribosomal RNA genes and their intervening sequences are the best-characterized gene regions in Nematoda [12].

In Georgia, is not information on the molecular characteristics of cyst nematodes. Therefore, the aims of this study were to molecular characterization and phylogenetic analysis of isolates of *Globodera rostochiens*is within the Georgian population (Svaneti and Samckhet Javakheti) using sequence of D3 expansion regions of 28S rRNA ribosomal nuclear RNA gene.

Materials and Methods

DNA Extraction and detection *Globodera rostochiensis* by multiplex PCR

DNA isolation from individual cysts was conducted using the Nematode DNA extraction& purification kits (Clear Detection) according to manufacturer's protocol. A multiplex PCR test were used for molecular identification. PCR was performed by the universal ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3) and cyst nematodes' (*G. pallida, G. rostochiensis*) spe[13] cific primers (PITSp4: 5′-ACAACAGCAATCGTCGAG-3'; PITSr3: 5′-AGCGCAGACATGCCGCAA-3) [13].

PCR was conducted in thermocycler (SimpliAmp™ Thermal Cycler _Applied Biosystems").

PCR mixture in a total volume of 25µl contained 25-50 ng DNA, and 0.25 µM ITS5, PITSp4R and PITSp4 primers. Positive controls contained G. pallida and G. rostochiensis from the Cheech collection of bank of Plant Pathogens DNA as matrices. Negative control contained PCR grade water instead of DNA Cycling conditions were: one cycle 95^{0}C for 5 min, 35 reaction cycles of 95^{0}C for 1 min, 55^{0}C for 30 s min, 72^{0}C for 1 min, followed by a final extension of 10 min at 72˚C and 4˚C hold.

Analyses of obtained PCR fragments were conducted by horizontal electrophoresis on 1,5% agarose gel containing ethidium bromide (0.2µg/ml) in TAE buffer (40mM Tris acetate, pH 8.0, 2mM EDTA-Na2) [14]. Visualization of fragments was done on trans-illuminator Benchtop UV. The sizes of the PCR products were determined by comparing the bands with a 100bp DNA ladder (Biolabs).

Amplification DNA fragments for phylogeny

D3 expansion region was amplified using primer pairs: D3A (5' GACCCCTCTTGAAACACGGA-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3' [15].

PCR condition were: 94°C for 5 min s; 30 cycles (94°C for 1 min 62°C for 1 min

and 72°C for 1 min); final extension 5 min at 72 °C.

PCR products of the were cleaned up and sequenced using an ABI 3500xL Genetic Analyze

Sequence and Phylogenetic Analysis

Obtained sequencing results were analyzed by computer program BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cg).

Phylogenetic analyses to resolve the relationships between the isolates were conducted in MEGA7 [16] using both distance- and character-based methods.

The evolutionary history was inferred using the UPGMA method [17]. The bootstrap consensus tree inferred from 400 replicates is taken to represent the evolutionary history of the taxa analyzed [18]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Tamura-Nei method [19] and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 248 positions in the final dataset.

Results and discussion

Nematode identification

Identification of all the samples from the two Globodera populations (Samtskhe - Javakheti and Svaneti) i.e. *G. rostochiensis* (20 isolates) were confirmed by conventional multiplex PCR with ITS 5 universal and PITSp4 , PITSr3 specific primers of the cyst nematodes' (G. pallida, *G. rostochiensis*). Size of PCR fragment 434 bp confirms that PCN samples from two populations Samtskhe- Javakheti and Svaneti belongs to *G. rostochiensi* (Fig. 1).

Molecular characterization and sequence analysis

Molecular characterization of *Globodera rostochiensis* was performed by the amplification and sequencing D3 expansion region of the 28S rRNA.

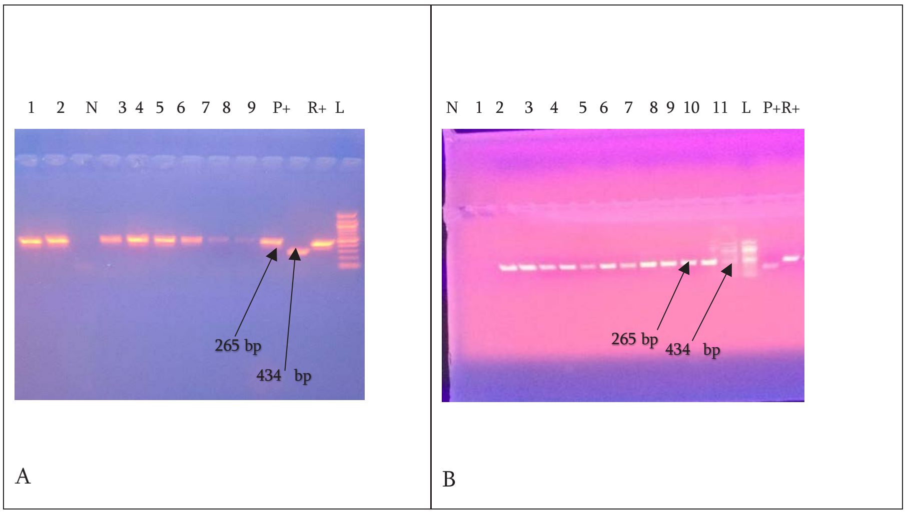


Fig. 1. *Multiplex polymerase chain reaction (PCR) of G. rostochiensis isolates using primers PITSt4, PITSr3, and ITS5. A. 1-9 (GL10, GL11, GL12, GL13, GL14, GL15, GL16, GL17, GL18,) G.rostochiensis isolates from Samckhet Javakheti; P+ G.pallida DNA (positive control)); R+G.rostochiensis DNA (positive control); L--100bp DNA ladder; N-negative control (nuclease free water); B.1-11 (GL1, GL4, GL5, GL8, GL9, GL19, GL20, GL21. GL22, GL23, GL24) G.rostochiensis isolates from Svaneti); R+G.rostochiensis DNA (positive control); N-negative control (nuclease free water); L--100bp DNA ladder; P+ G.pallida DNA (positive control).*

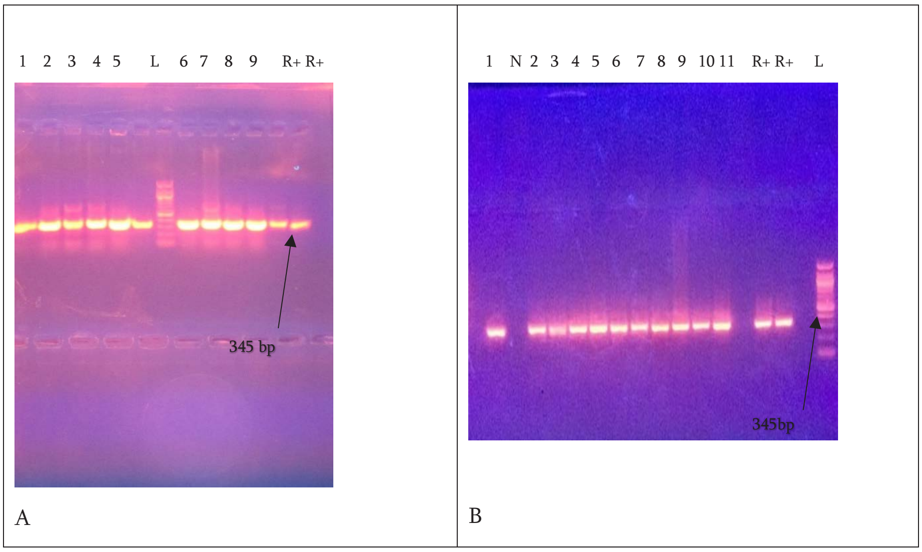


Fig. 2. *Amplified D3 expansion region of 28sRNA of Globodera rostochiensis izolates . A. 1-9 (GL10, GL11, GL12, GL13, GL14, GL15, GL16, GL17, GL18) G.rostochiensis isolates from Samckhet Javakheti; R+G.rostochiensis DNA (positive control); L--100bp DNA ladder. B.1-11 (Gl1, GL4, GL5, GL8, GL9, GL19, GL20, GL21GL22, GL23, GL24)) G.rostochiensis isolates from Svaneti); R+ G.rostochiensis DNA (positive control); N-negative control (nuclease free water); L--100bp DNA ladder.*

Amplicon size of the D3 region is 345 bp (Fig. 2).

Sequence date of D3 expansion region of the rRNA of *Globodera rostochiensis* were compared to those referenced in the NCBI database of other populations using computer program BLASTN. . High identities (94-99%) of the D3 rRNA sequences indicates that Georgian isolates of *G. rostochiensis* are similar to all known isolates of *G. rostochiensis* .

The sequences of D3 expansion regions were aligned using CLUSTAL V [20] The sequence lengths and frequencies of nucleotide distribution for the different isolate of *Globodera rostochiensis* are shown in Table 1.

Pairwise distance of D3 expansion region of *Globodera rostochiensis* isolates are presented in Fig. 3. The number of base substitutions per site

Table 1. *Sequence lengths and nucleotide composition of ITS1-5,8S region of G. rostochiensis isolates from Samckhet Javakheti and Svaneti*

Isolates of Globodera Rostochiensis	D3 expention region bp	T(U)%	C%	A%	G%
GL1	302,0	20,5	23,2	24,2	32,1
GL4	304,0	20,7	23,0	24,0	32,2
GL5	304,0	20,7	23,0	24,3	31,9
GL8	304,0	20,7	23,0	24,0	32,2
GL9	305,0	21,0	23,0	23,9	32,1
GL10	299,0	21,1	23,4	23,4	32,1
GL11	299,0	20,1	23,4	23,7	32,8
GL12	302,0	20,9	23,2	23,8	32,1
GL13	303,0	21,5	23,1	23,4	32,0
GL14	304,0	21,4	23,0	23,4	32,2
GL15	307,0	20,2	22,8	24,8	32,2
GL16	304,0	20,7	23,4	24,7	31,3
GL17	310,0	14,8	30,0	20,3	34,8
GL18	301,0	20,6	23,3	25,2	30,9
GL19	272,0	22,8	23,5	24,3	29,4
GL20	305,0	20,3	23,0	24,3	32,5
GL21	306,0	20,9	22,9	24,2	32,0
GL22	290,0	21,4	23,4	24,5	30,7
GL23	305,0	21,3	23,0	24,6	31,1
GL24	306,0	20,9	22,9	24,2	32,0
avg	301,6	20,6	23,5	24,0	32,0

The average nucleotide composition is as follows: 20,6% T(U), 23.5% C, 24,0% A and 32% G% (Table1).

from between sequences according to the Tamura-Neil model is shown below the diagonal. The number of base differences per sequence from between sequences is shown above.

Pierwase distance of D3 expansion region between Georgian *G.rostochiensis* isolates show that GL16 (Samckhet-javakheti) differs from GL17 (Samckhet-javakheti) and GL19 (Svaneti); base differences per sequence =0,5, 0,1 respectively; number of nucleotide substitutions per site =0.0 (Fig. 3). GL17 (Samckhet-javakheti) differs from GL18 (Samckhet-Javakheti) and GL19, GL20 , Gl21, Gl22, GL23, GL24 (Svaneti); base differences per sequence =0.5, 0. 3, 0.5, 0.5, 0.5, 0.5, 0.5 respectively; number of nucleotide substitutions per site =1.8. GL19 (Svaneti)) differs from GL20, GL21, GL22, GL23, GL24 (Svaneti) - base differences per sequence =0.1. GL18 (Samckhet-javakheti) differs from GL19 (Svaneti) base differences per sequence =0.1 (Fig. 3).

Phylogenetic analysis

Based on analysis of *G.rostochiensis* isolate`s D3 expansion regions are grouped in three major clades (A, B and C) on the phylogenetic tree (Fig. 4).. Clade A are divided in three subclades, clade C are divided in two subclades. Isolates (GL13, GL14, GL16, GL18) from Samtckhet-javakheti population are in subclade 1 of clade A and isolates (GL10, GL15) in subclade 1 of clade C. Isolates (Gl 11, GL 12, GL24, GL4) from Svaneti populations are in subclade 2 of clade A and isolates (GL1, GL20, GL5, GL8) in clad B. In Clade C subclade two is presented by three isolates (GL22, GL23, GL19) from Svaneti and by one isolates (GL17) from Samckhet-Javakheti.

Our investigation showed of high genetic variation of D3 region of rDNA of the isolates of G. rostochiensis from different geographic origins (Svameti, Samckhet-Javakheti) of Georgia.

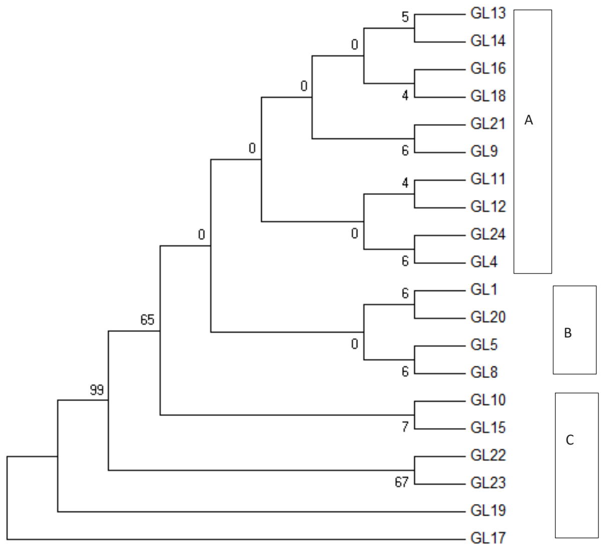
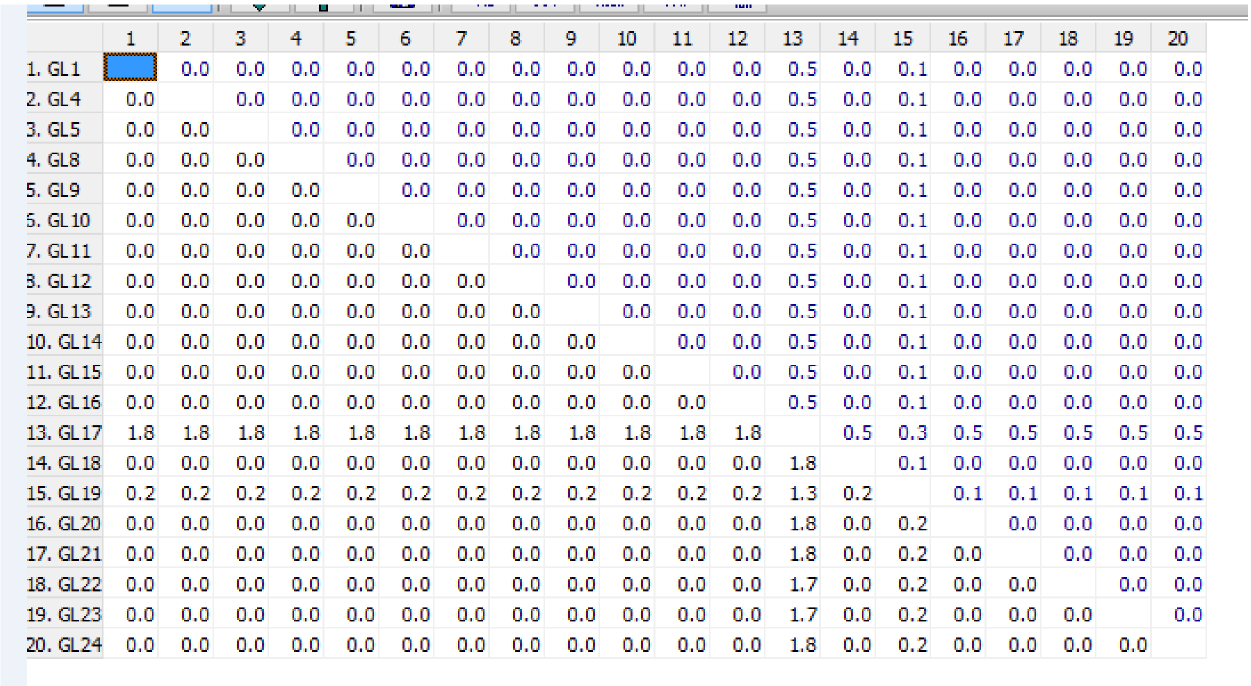


Fig. 4. *Phylogenetic relationships between isolates of G. rostochiensis from different geographic origins (Svameti, Samckhet-Javakheti) of Georgia.*



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. GL1		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
2. GL4	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
3. GL5	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
4. GL8	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
5. GL9	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
6. GL10	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
7. GL11	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
8. GL12	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
9. GL13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
10. GL14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
11. GL15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
12. GL16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.0
13. GL17	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8		0.5	0.3	0.5	0.5	0.5	0.5	0.5
14. GL18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8		0.1	0.0	0.0	0.0	0.0	0.0
15. GL19	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	1.3	0.2		0.1	0.1	0.1	0.1	0.1
16. GL20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.2		0.0	0.0	0.0	0.0
17. GL21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.2	0.0		0.0	0.0	0.0
18. GL22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.2	0.0	0.0		0.0	0.0
19. GL23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.2	0.0	0.0	0.0		0.0
20. GL24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.2	0.0	0.0	0.0	0.0	

Fig. 3. *Pairwise distances between D3 regions of of Globodera rostochiensis isolates.*

The evolutionary relationships between the potato cyst nematodes (G. rostochiensis and G.pallida) found in Georgia and studed in other countries will be shown by the phylogenetic analyses, which enhances the knowledge in the evolutionary genetics.

Molecular characterization and phylogenetic analysis of PCN by rDNA fragment sequencing methods is important as far as similar studies have not been held in Georgia yet.

Ribosomal RNA genes encoding 5.8S, small subunit 1 8S and large subunit 28S have been widely used to inter phylogenetic relationships among closely and distantly related taxonomic lineages [21].

D3 expansion segments of the 28S ribosomal RNA molecule have been used as meaningful genetic markers for resolving phylogenetic relationship at lower and higher taxonomic levels and developing species- specific primers [15, 22-27].

Nucleotide sequences of the D3 expansion region of 28S rDNA corresponding to positions 3304-3648 are available for some nematode species [28, 29]. This region was successfully amplified from formalin-fixed nematodes and could be useful for nematode identification [29]. Although this region is specific at the species level in the genus Pratylenchus, for example, and may be used for species identification [15, 21]. it is unlikely to be useful for the identification of Globodera species parasitizing in solanaceous plants as it is highly conserved [22].

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