DOI: 10.22620/agrisci.2020.27.004

ISSR MOLECULAR MARKERS FOR THE STUDY OF THE GENETIC DIVERSITY IN BULGARIAN POPULATIONS OF PCN FROM GENUS *GLOBODERA*

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Abstract

Genetic diversity of potato cyst nematodes from genus *Globodera* was studied using ISSR molecular markers. Potato cyst nematodes are characterized by different virulence with regard to the host resistance genes. One of the primary tasks related to best pest control is associated with the determination of the parasite populations genetic diversity. Our findings indicated that by applying ISSR markers, it was possible to distinguish between closely related and morphologically similar species of potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida*. At present, genetic research on local PCN populations has not been done and therefore valuable information is missing.

Keywords: PCN (Potato Cyst Nematode), ISSR, genetic diversity, *Globodera rostochiensis*; *Globodera pallida*

INTRODUCTION

The potato cyst nematodes (PCN) *Globodera rostochiensis* and *Globodera pallida* are the most important pests feeding on potato roots (Evans and Trudgill 1992). The symptoms of infection are not specific and are similar to those caused by other biotic or abiotic stress agents. Attacked potato plants are smaller, tubers are much smaller and their yield is much lower than that obtained from healthy plants. Both species of the PCNs are regulated by the European Directive 2007/33/EC from June 11, 2007. *Globodera rostochiensis* and *Globodera pallida* are also part of A2 List – quarantine pests present in that area, but not widely distributed there and being officially controlled. The A2 List is a part of EPPO Standard PM 1/2(26) and includes the pests which EPPO recommends to be regulated as quarantine pests, in the national phytosanitary regulations of EPPO Member Governments (EPPO 2017).

PCN can be controlled by different defence methods (chemically, biologically or by crop rotation and trap cropping). However, growing resistant cultivars is believed to be economically the most effective and environmentally safe method of protecting potato crops against PCN (EPPO/OEPP 2004).

Resistance to the potato cyst nematodes, *Globodera rostochiensis* and *Globodera pallida*, is a major objective in potato breeding programmes in Europe. Kort et al. (1977) recognized five *G. rostochiensis* and three *G. pallida* pathotypes and their pathotype scheme are now widely used. PCN pathotype identification has been done based on the differential responses on potato clones. The pathotypes are characterized by their ability to multiply on certain tuberous Solanum clones and hybrids used in breeding. Five pathotypes are recognized within *G. rostochiensis* (Ro1Ro5 international notation) and three in *G. pallida* (Pa1-Pa3). Some of these pathotypes are recognized by their almost total inability to multiply on specific cultivars of potato (single-gene resistance).

A number of biochemical and molecular techniques have been used to investigate genetic variability in potato cyst nematodes with a view to find markers related to pathorypes or to find groupings that relate to biological characteristics (A.C. Boucher et al.,2013, Vivian et.al., 1995). The Inter-simple Sequence Repeats (ISSR) PCR technique has been shown to have a wide application for the analysis of genetic variation at subspecies level, particularly in investigations of population structure and differentiation.

Although this species has a huge economic importance, information on population genetics is still very limited. Identifying current diversity and relation among populations is very important to prevent selection for more virulent pathotypes.

In the present study, we tested ISSR DNA markers in order to study genetic diversity into *Globodera* populations from three major potato producing region in Bulgaria.

MATERIALS AND METHODS

Soil samples were collected after the harvest of the potato crops in three potato-producing regions of Bulgaria: Sofia (Koprivshtitsa and Samokov), Smolyan (Smolyan, Rudozem, Davidkovo and Momchilovtsi), Pazardzhik (Ravnogor and Dragor). The soil samples were collected at depths of up to 15 cm by means of a probe (with a capacity of maximum 20 g) and then were processed in the laboratory of Nematology at Agricultural University – Plovdiv. Extraction of the cysts from the soil was performed by the Baunacke method, modified by Buhr (1954). The second-stage juveniles were obtained after cutting the cysts with an ophthalmic scalpel for their observation and determination. The cysts and J_2s thus obtained were stored at room temperature until used in the morphological and PCR analyses.

Total genomic DNA was prepared from cysts filled with eggs and juveniles as well as from individual juveniles, used for the previous morphological characterization. DNA from cyst was isolated using innuPREP Plant DNA Kit (Analytik Jena) according to manual. DNA was isolated from five to ten previously characterized as *G. pallida* larvae second-stage, individually selected and transferred to 20 µl of a standard 10xPCR buffer. Two µL of Proteinase K (20 µg μL^{-1}) was added to each tube and incubated for 1 h at 60 °C and 10 min at 94°C and briefly cooled on ice followed by centrifugation to remove debris. From 1–3 µL of the supernatant were used directly for PCR and the remainder were stored at −20 °C until use.

Table 1. ISSR primers used in a genetic diversity study.

Primer name	DNA sequence 5' – 3'	Lenght (bp)	Melting temperature (^{0}C)
ISSR N7	AG(8)CTG	19	48.2
ISSR N11	AG(8)YT	18	51.4
ISSR N11	GA(8)YC	18	53.9
ISSR N15	CT(8)RG	18	53.9

PCR reactions were performed in 25 µl reaction volumes, where for each reaction: PCR master mix (Bioline) 12.5 µl; ISSR primer – 1.5 µl; sterile distilled water – 10 µl; 2 and 1 µl genomic DNA was used.

ISSR PCR reactions were performed under the following regime: denaturing – 94^{0}C for 3 min; 40 cycles of: 940C – 1 min, primer annealing temperature – 30 sec, elongation at 72^{0}C – 45 sec, followed by a final extension at 720C – 4 min.

ISSR analyses were performed by PCR reactions in QB-96 Thermal Cycler (Quanta Biotech, London, UK). Sequences of ISSR primers used to perform PCR reactions are listed in Table1.

Amplified bands were scored as 1/0 (presence/absence) of homologous bands for all samples.

STATISTICAL ANALYSIS

Statistical analysis was carried out by hierarchical clusters with the "SPSS for Windows" statistical package.

RESULTS AND DISCUSSION

Being highly polymorphic, microsatellites are very informative markers and have been extensively used in diversity studies. Total levels of polymorphism were evaluated from produced multilocus anonymous dominant markers. Molecular data gathered throughout the current study was used for calculating relative genetic distances and producing hierarchical clusters with the "SPSS for Windows" statistical package.

In order to obtain additional information related to the pathotype population composition, the ISSR analysis was performed on samples pre-established as G. pallida (PA2 and Pa3). (data not shown).

The use of the selected ISSR primers resulted in producing in total 45 polymorphic bands. Clearly detectable amplified ISSR ranged from 300 to 2200 bp in size. The average numbers of clear bands generated per polymorphic primer were 6, with a maximum of 12 for primer N7. (Fig.1)

ISSR dataset from 4 tested primers grouped all populations in two clusters, according to their geographical origin. These results suggested potentially two different pathotypes or biological entities exsisting in *Globodera* species. The highest genetic similarity coefficient was observed between the populations from Momchilovtsi and Davidkovo. The lowest genetic similarity coefficient was observed between the populations from Davidkovo and Koprivshtica from the first cluster and Smolyan and Ravnogor for the second. These results correspond to the samples analyzed from the certain region.

The ISSR primers provided the clearest banding pattern including a large number of

scorable bands that were among those selected to generate the dendrogram Fig.2

To our knowledge, this is the first study for the genetic structure of *Globodera* populations revealed by ISSR markers at a geographical level.

It was proved that ISSR markers were efficient for detecting genetic variation among the different geographic populations in Bulgaria.

In addition, when the genetic structure of the species or genus to be analyzed is not well-known, this technique is particularly useful because it is simple cost, effective and reliable.

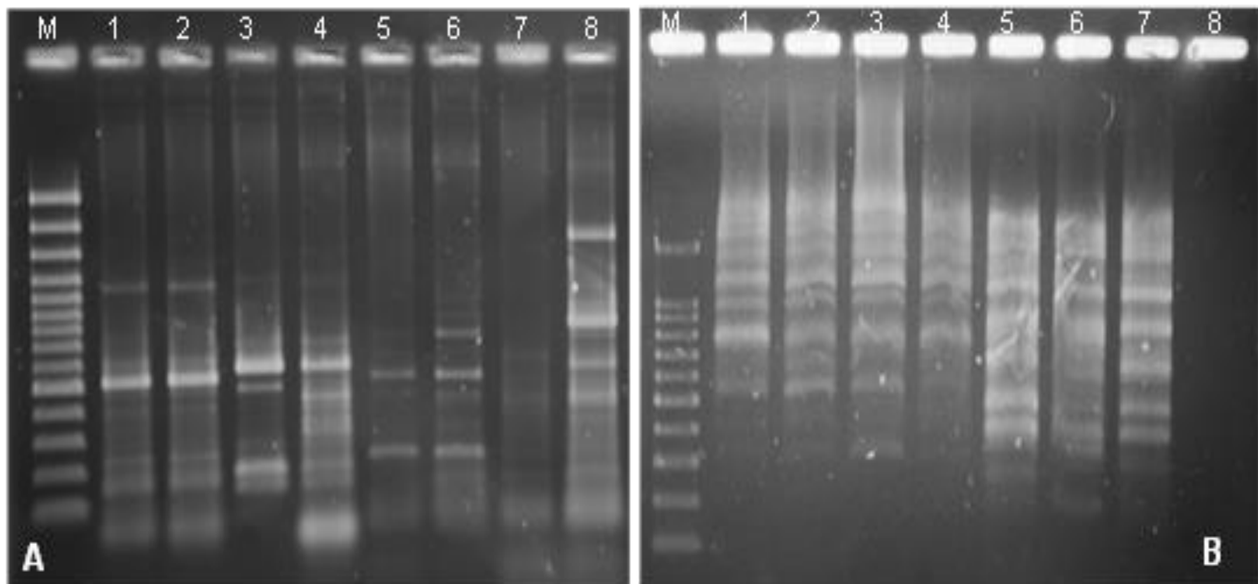

Fig. 1. Polymorphism within the *Globodera* samples with ISSR primers
A – ISSR N11; B- ISSR N7. Lane M–100 bp DNA ladder; 1- Koprivshtitsa; 2- Samokov; 3- Momchilovtsi 4- Smolyan; 5 – Dragor; 6 – Ravnogor; 7- Davidkovo; 8 – Rudozem

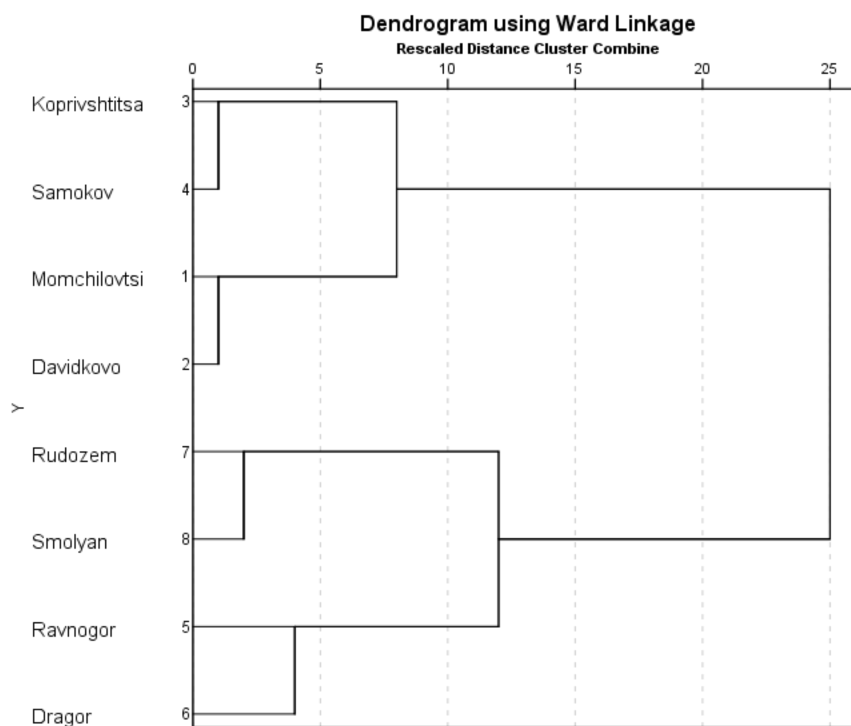

Fig. 2. Phylogenetic relationships among *Globodera* populations based on their Jacard's genetic similarity values using ISSR markers.

CONCLUSIONS

Conducted molecular analyzes demonstrated that the ISSR marker system is capable of identifying genetic diversity within the *Globodera* species.

Developed ISSR method will be used to establish genetic fingerprint of the Bulgarian nematodophauna and will be used for future investigation on PCN.

The study could have an important role for the control of the Bulgarian PCN populations and the development of quarantine measures.

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