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Morphological and molecular identification of potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida* in Bulgaria

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Abstract

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The potato cyst nematodes (PCNs), *Globodera rostochiensis*and *Globodera pallida* are economically important parasites of the potato and are recognized as quarantine pests. In Bulgaria *G. rostochiensis* was identified for the first time in 1978, while *G. pallida* in 1992. Identifications of PCNs populations have been based only on morphological characters and *G. rostochiensis* is prevailing species in potato fields. The present work was aimed to introduce a faster and more reliable method for identifying and differentiating the PCN species in three potato-producing regions in Bulgaria. For the morphological identification of both PCN species, 30 cysts and 30 second-stage juveniles (J_{2S}) were studied. For a more accurate and rapid identification of *G. rostochiensis* and *G. pallida*, polymerase chain reaction (PCR) based method was applied. Multiplex PCR reaction was performed with primers designed for a small region between internal transcribed spacer 1 and the 5.8 S ribosomal RNA gene regions in order to identify the *Globodera* species. The mean morphometric values of the 30 experimental cysts and J_{2S} were common within the expected range for *G. pallida* and *G. rostochiensis* with some variations observed**.** The present results demonstrate the occurrence of both species of PCNs, as *G. pallida* was identified in 24 from the soil samples and *G. rostochiensis* in 6 of them. The correct and rapid identification of PCN species in Bulgarian potato fields is essential for adopting more effective measures for its control.

Keywords: PCR identification; *Globodera pallid; Globodera rostochiensis;* potato; cyst nematodes

Introduction

Potato cyst nematodes (PCN) – *Globodera rostochiensis* (Wollenweber, 1923) Behrens and *Globodera pallida* (Stone, 1973) Behrens, 1975), are the major pests from this group that cause the highest potato losses (Van Riel & Mulder, 1998). Both species of the PCNs are regulated by the European Directive 2007/33/EC from June 11, 2007. *Globodera rostochiensis* and *G. 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).

Globally, PCNs are established in almost all potato-producing countries. EPPO reports that PCNs have been established in 71 countries for *G. rostochiensis* and 44 countries for *G. pallida* (EPPO, 2014).

In Bulgaria *G. rostochiensis* was first identified in 1978 in the region of Samokov (Stoyanov, 1980). The species spread very quickly and is one of the most important pests of potatoes in all potato-producing regions in the country (Stoyanov, 1980; Samaliev, 2011). The second type of pota-

to cyst nematode *G. pallida* has been identified in the region of Yundola (Samaliev et al., 1992; Samaliev et al., 1995) and then in the region of Smolyan, and Pazardzhik but the distribution is localized in distinct areas in mixed population with *G. rostochiensis* (Samaliev, 1998, 2011).

However, in the course of time, the proportions of both species are changing because of the use of potato varieties resistant to *G. rostochiensis*. The increase in population of *G. pallida* versus *G. rostochiensis* is also due to the lesser impact of conventionally applied nematocides that are likely to have less soil persistence than the longest period of living of *G. pallida*'s invasive J_2s (Whitehead et al., 1994). Because of high economic losses that they cause in the potato production their correct identification is of rising importance.

The main method of distinguishing the two species of PCNs in Bulgaria is morphological, which is based on the different characteristics of second-stage juveniles and perinea area of the cysts, and with usage of plants from the International host test.

To achieve more accurate and rapid identification of potato cyst nematodes in recent years morphological analyzes are combined with molecular ones. Several laboratory-based methods were developed such as enzyme-linked immunosorbent assays, protein analysis by isoelectric focusing, and techniques based on polymerase chain reaction (Ibrahim et al., 2001) and Real-time PCR (Bates et al., 2002; Madani et al., 2008; Quader et al., 2008) in order to distinguish the two species of potato cyst nematodes and can also be used to directly identify PCNs without morphological characterization (Reid et al., 2010).

The aim of the present work was to introduce faster and more reliable method for identifying and differentiating the two species of PCNs in three potato-producing regions in Bulgaria.

Materials and Methods

Soil samples were collected during 2016 and 2017 after the harvest of the potato crops in three potato-producing regions of Bulgaria in western (Koprivshtitsa and Samokov), central (Ravnogor and Dragor) and southern (Smolyan, Momchilovtsi, Davidkovo, Stoikite and Rudozem) parts of the country. The total number of soil samples was 30. Soil samples 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.

For the morphological identification, 30 cysts and 30 second-stage juveniles (J_{2S}) were studied. The morphological identification of the two species of potato cyst nematodes is based on differences in the main characteristics of cysts and J_2s– body length, tail length, hyaline region length, stylet length for the juveniles and distance fenestra to anus, fenestra diameter, Granek's ratio (the distance from the anus to the nearest edge of the fenestra), number of cubicula ridges between the vulva and anus for the cysts.

The perineal regions were obtained by cutting the cysts with an ophthalmic scalpel and mounted into fixed microscopic preparations. The J_{2S} were pre-fixed and then also included in preparation of permanent collection slides, used the rapid method of Sainhorst (Sainhorst, 1959, 1962). The studied cysts and J_2swere examined with Olympus CX 22 LEDRFS1 microscope with IDS uEye digital camera. Measurements of the main cysts and J_2s characteristics were made in µm using Quick Photo Camera 3.1 software. All measurements of the cysts and J_2s were compared to the corresponding values for both species from reference data (EPPO, 2013).

DNA extraction and PCR amplification

Total genomic DNA was prepared from cysts with eggs and juveniles as well as from individual J_2s, used for the morphological characterization described above. For the molecular analyses, 5 J_2s were individually hand-picked and placed into 20-µl 10X PCR buffer (Sci Tech S) in a 1.5 ml Eppendorf tube. The nematodes were ground with pestle and 5 µL of Proteinase K (20 µg μL^{-1}) was added to each tube, incubated for 1 h at 60°C and 10 min at 94°C, briefly cooled on ice followed by centrifugation to remove debris. Five µl of the supernatant were used directly for PCR and the remainder was stored at −20°C for further use.

A three primer system was designed for the present study composed of: universal primer UNI 5'-GCAGTTGGCTAGGGATCTTC-3', which binds to the same locus in both species, *G. pallida* specific primer GPA1 5' GGTGACTCGACGATTGCTGT-3' and *G. rostochiensis* specific primer GRO1 5'-ATGTTGTACGTGCCGTACCTT-3' (Zouhar et al. 2000). PCR was performed in 25 µL reaction volume containing 5 µL templates DNA, 0.2µM of each primer, 12.5 µl PCR ready Mix (Bioline Meridian Life Science Inc.). PCR was carried out in a QB-96 Thermal Cycler (Quanta Biotech, London, UK). The cycling program consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 62°C, and extension at 72°C for 1 min, with a final elongation step for 5 min at 72°C. After the completion of PCR, the products

were separated electrophoretically using a 1.5% agarose gel cast in TBE buffer, stained with Ethidium Bromide and photographed under UV light. DNA fragment sizes were determined by comparing with the 100bp DNA marker.

Results

Morphological identification

Measurements of cysts and J_2s of potato cysts nematodes from three main potato-producing region of Bulgaria are presented in Table 1 and Table 2. The results obtained from the morphological identification indicate the presence of two species genus *Globodera* (*G. rostochiensis* and *G. pallida*). The main features that distinguish both species of PCNs did not always match with their values from reference data.

Samples 1, 2, 3, 4, 6, 7, 9, 10, 11, 12, 15, 16, 17, 18, 19, 21, 22, 24, 25, 26, 27 and 30 were morphologically identified as *Globodera pallida* (Table 1 and Table 2).The measured mean values for second-stage juveniles such as body length 476.06 µm (395.22 – 519.85), and length of the stylet 22.87µm (19.67 – 25.29) are within the range compared to their values of reference data (Table 2) The mean values of

Table 1.Morphological and morphometric measurements of *Globoderarostochiensis* and *Globodera pallida* cysts and second-stage juveniles from samples of Bulgarian populations. All measurements are in µm

Samples number n*	Second-stage juvenile				Cyst			
	Body length	Tail length	Hyaline region	Stylet length	Fenestra to anus	Fenestra diam.	Granek's ratio	No cuticular ridges
1.	491.75	59.01	25.29	22.48	36.81	20.51	1.82	9
2.	463.65	53.39	28.10	25.29	38.74	21.82	2.12	8
3.	491.75	50.58	22.48	22.48	39.34	25.29	3.09	8
4.	519.85	50.58	19.67	25.29	50.58	36.53	3.09	9
5.	477.70	30.91	11.24	25.29	70.25	22.48	5.34	15
6.	483.32	30.91	16.86	22.48	56.20	30.91	3.93	7
7.	488.94	36.53	16.86	19.67	53.39	33.72	3.93	8
8.	477.70	36.53	16.86	22.48	87.11	28.10	8.15	18
9.	492.65	33.78	16.86	20.32	52.19	31.92	3.93	8
10.	500.18	39.34	22.48	25.29	67.44	25.25	5.90	9
11.	483.32	42.15	25.29	25.29	78.68	22.48	5.62	10
12.	488.94	44.96	28.10	22.48	64.53	19.67	5.34	8
13.	474.89	47.77	28.10	22.48	109.59	14.05	7.02	18
14.	480.51	53.39	25.29	22.48	106.78	16.86	6.74	13
15.	474.89	53.39	28.10	22.48	81.49	25.29	7.02	10
16.	476.38	53.55	25.64	21.74	50.90	20.25	3.06	8
17.	455.88	49.13	26.03	22.76	36.58	18.60	2.44	8
18.	454.18	44.39	23.62	22.62	43.78	22.43	3.03	8
19.	465.21	45.14	22.66	22.82	49.86	23.84	3.36	9
20.	427.25	39.44	21.52	21.12	103.53	19.35	7.84	18
21.	469.85	32.45	19.62	24.64	56.42	20.16	4.31	11
22.	483.77	35.30	22.28	22.82	46.73	23.63	3.38	7
23.	440.26	48.19	22.14	19.24	91.39	17.43	6.32	13
24.	462.98	34.63	19.46	19.92	58.84	24.43	4.43	10
25.	491.35	36.76	18.43	22.24	69.03	24.03	5.98	16
26.	395.22	35.26	17.29	20.44	46.73	23.63	3.37	7
27.	457.06	45.11	24.83	23.76	74.22	24.12	6.35	10
28.	427.47	39.64	21.72	21.21	109.59	14.05	7.02	18
29.	440.25	48.20	22.15	19.24	91.30	17.43	6.32	13
30.	479.08	40.03	26.32	23.82	75.84	20.24	5.66	10
GRO**	392-468	44-51	20-27	20-22	51-70	***	3.0-4.5	17-20
GPA**	452-486	50-53	26-27	23-24	48-54	***	2.1-2.5	12

*number of studied cysts/J_2; **according to OEPP/EPPO Bulletin (2013); ***no data

Table 2. Morphological and morphometric characteristics of second-stage juveniles and cysts from Bulgarian populations compared to characteristics of OEPP/EPPO Buletin (2013). Measurements of second-stage juveniles and cysts from Bulgarian populations are in µm and represented as mean ± s.d. (range)

	*G. pallida** (n = 24)	*G. rostochiensis** (n = 6)	*G. pallida***	*G. rostochiensis***
Second-stage juveniles				
Body length	476.06 ± 23.10 (395.22 – 519.85)	448.43 ± 23.45 (427.25 – 480.51)	452- 486	392- 468
Tail length	42.24 ± 8.23 (30.91 – 59.01)	46.10 ± 5.49 (39.44 – 53.39)	50-53	44 – 51
Hyaline region	21.84 ± 4.50 (11.24 – 28.10)	23.48 ± 2.65 (21.52 – 28.10)	26-27	20-27
Stylet length	22.87 ± 1.72 (19.67 – 25.29)	20.96 ± 1.45 (19.24 – 22.48)	23-24	20-22
Cysts				
Fenestra to anus	57.73 ± 14.92 (36.58 – 87.11)	102.03 ± 8.57 (91.30 – 109.59)	48-54	51-70
Fenestra diam.	24.55 ± 4.62 (18.60 – 36.53)	16.52 ± 2.09 (14.05 – 19.35)	***	***
Graneks's ratio	4.36 ± 1.62 (1.82 – 8.15)	6.87 ± 0.56 (6.32 – 7.84)	2.1-2.5	3.0-4.5
No. cuticular ridges	9.62 ± 2.84 (7.00 – 18.00)	15.50 ± 2.73 (13.00 – 18.00)	12	17-20

* values from Bulgarian populations of *G. pallida* and *G. rostochiensis,* **according to OEPP/EPPO Bulletin (2013), ***no data

the other characteristics of second-stage juvenile, such as tail length and hyaline region of the tail have a differences compared with their values indicated in the literature data (EPPO, 2013). The measured mean values for cysts such as fenestra to anus 57.73 µm (36.58 – 87.11), and number of cuticular ridges 9.62 µm (7.00 – 18.00) are within the range compared to their values of reference data (Table 2). The mean values of the other characteristics of cysts – fenestra diameter and Graneks's ratio have a differences compared with their values indicated in the literature data (EPPO, 2013).

The remaining samples – 5, 8, 13, 14, 20, 23, 28 and 29 – were morphologically identified as *Globodera rostochiensis* (Table 1 and Table 2). The mean values of the characters of second-stage juveniles – body length 448.43 µm (427.25 – 480.51); tail length 46.10 µm (39.44 – 53.39); hyaline region of the tail 23.48 µm (21.52 – 28.10) and stylet length 20.96 µm (19.24 – 22.48), completely match with the mean values of reference data (Table 2). The measured mean values for cysts such as number of cuticular ridges 15.50 µm (13.00 – 18.00) are within the range compared to their values of reference data (Table 2). The mean values of the other characteristics of cysts – fenestra to anus, fenestra diameter and Graneks's ratio vary most compared with their values indicated in the literature data (EPPO, 2013).

Therefore, from the morphological analyses it is also clear that the basic characteristics distinguishing the two species of PCNs may vary. This makes it very difficult to determine *G. pallida* and *G. rostochiensis* in an individual study of cysts and second-stage juveniles.

PCR Indentification

The multiplex PCR with the primers UNI, GPA1 and GRO1 allows distinguishing *G. rostochiensis* and *G. pallida* producing fragments of 391 bp for *G. pallida* and 239 bp for *G. rostochiensis* respectively. Figure 1 presents electrophoretic profiles from multiplex PCRs of the analyzed PCN samples. The three primers amplify products from a small region between internal transcribed spacer 1 (ITS1) and the 5.8 S ribosomal RNA gene region that was shown to be highly valuable.

This region was identified as suitable for classifying, molecular diagnostics and phylogeny reconstructions of diverse organisms at various taxonomic levels, including plant-parasitic nematodes (Marek et al., 2010; Douda et al., 2013). In our study we found this variation to be useful for fast determination of PCNs population structure. The method proved highly sensitive as presence of only one cyst of different species in the collection of cysts from particular locality appeared detectable (data not shown). The performed multiplex PCR also allows correctly identifying each *Globodera* species from mixed populations in one reaction tube and single assay (Figure 2).

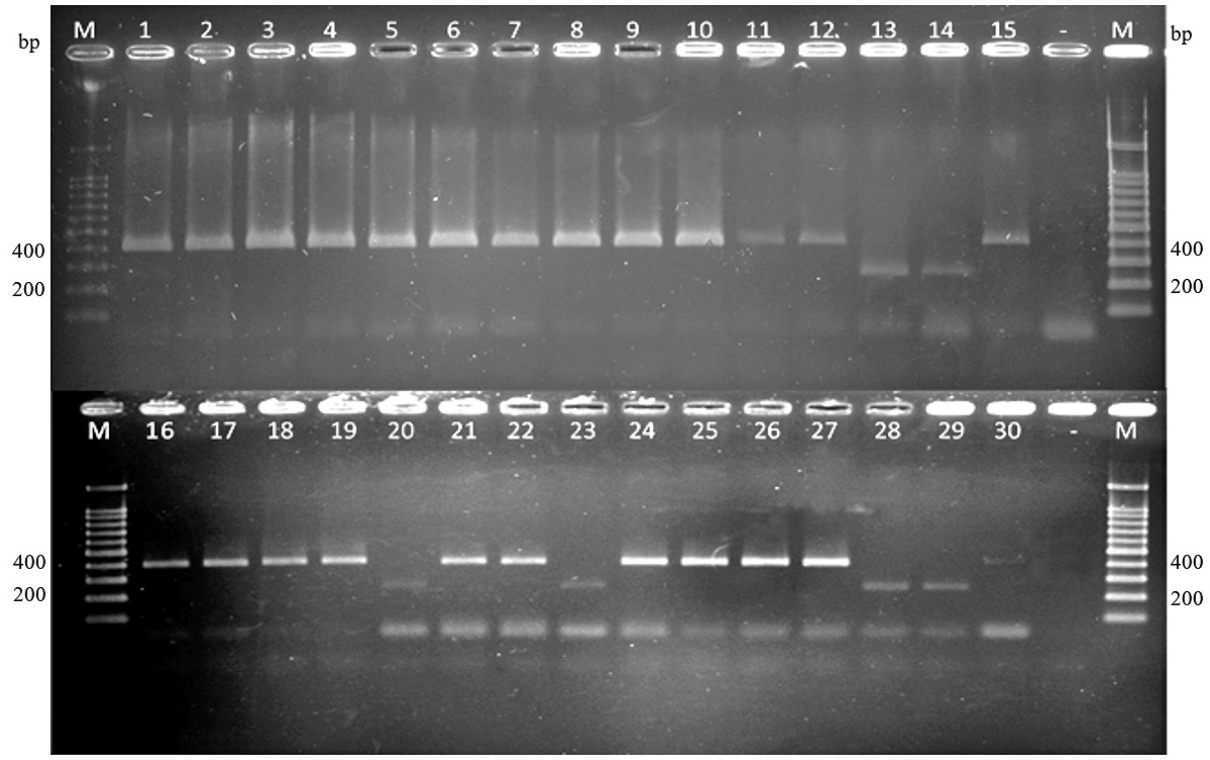

Fig. 1. Molecular differentiation of PCN populations. Products from multiplex PCR using primers UNI, GPA1 and GRO1. Line M –100bp Molecular marker. Lines 1-15, 16-30 – PCN samples, Line – negative control

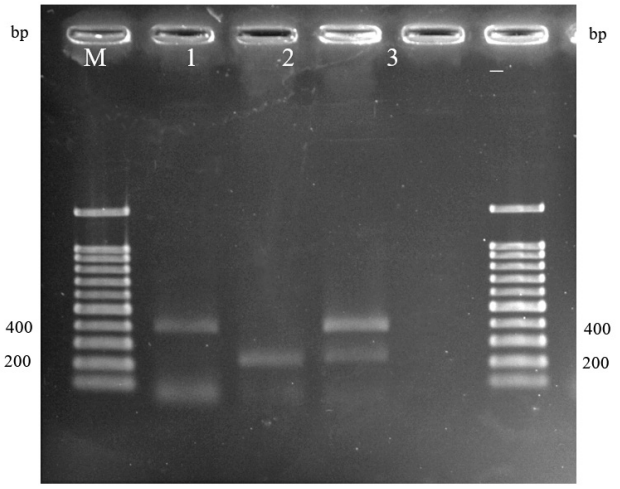

Fig. 2. Multiplex PCR using primers UNI, GPA1 and GRO1. Line M – 100bp Molecular marker, Line 1 – *G. pallida*, Line 2 – *G. rostochiensis*, Line 3 mixed populations (*G. pallida*/*G. rostochiensis*) – negative control

Comparing the data obtained from morphological analysis with the data obtained from PCR analyses shows that they did not always match. In the present study samples 5 and 8 are morphologically defined as the *G. rostochiesis* (morphometric data are typical for this species of PCNs) but the amplified PCR products correspond and identified the samples as *G. pallida*.

Discussion

The results of this investigation indicate the presence of *G. rostochiensis* and *G. pallida*, in the main potato-growing regions of Bulgaria. These data are in conformity with Samaliev's results, reported during the period 2006-2008 about the distribution of the both species of PCNs on potato plants (Samaliev, 2011).

The study of the basic morphological characteristics distinguishing the two species of PCNs from potato fields vary and make it difficult to identify and distinguish potato cyst nematode species. Therefore, for the more successful and accurate determination of the PCNs it is therefore

imperative to apply molecular identification methods. The method applied here for PCR species-specific discrimination of PCN is reliable and easily applicable for rapid identification in laboratory conditions. Multiplex PCR with species-specific primers (UNI, GPA1 and GRO1) allows both species to be distinguished. Samples 13, 14, 20, 23, 28, 29 were identified as *G. rostochiensis* as they contained 239 bp PCR product expected for that species (Figure 1). The other 24 samples were identified as *G. pallida* with amplified product of 391 bp. However, despite the development of molecular identification methods, the morphological approach remains useful as a complementary diagnostic technique. The method of DNA extraction allows detection and identification of just 5 nematodes per sample which makes it appropriate for more sensitive and accurate discrimination of PCNs.

The present study establishes a change in the composition of both species of PCNs with predominance of *G. pallid*a as compared to the data from 2006 – 2008 periods when the prevalent species of PCN was *G. rostochiensis* (Samaliev, 2011). We suppose that the change in composition of both PCNs species with predominance of *G. pallida* is most likely due to the use of potato varieties resistant to *G. rostochiensis* and with only a partial resistance to *G. pallida*. This apparently allows for better development and reproduction of the latter pest, thus increasing its proportion in the PCNs population.

Conclusions

The method used in the present study is appropriate for distinguishing of PCN species by multiplex PCR. Furthermore, it brings a benefit of amplification of different products from DNA mix thus allowing for detection of mixed populations of PCN.

Based on the combined data from morphological characteristics and molecular methods applied, the potato cyst nematodes found in Bulgaria were found to contain *G. pallida* as the most common PCN nematode species in the surveyed regions.

The correct and rapid identification of PCNs in Bulgarian potato fields is essential for adopting more effective measures for its control.

References

Bates, J. A., Taylor, E. J. A., Gans, P. T. & Thomas, J. E. (2002). Determination of relative proportions of *Globodera* species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. *Molecular Plant Pathology, 3,* 153–161.

Buhr, H. (1954). Studies on potato nematodes – I. The "paper strip method". Simplified procedure for examining soil samples for their stock with nematode cysts. *Nachrichtenblatt für den Deutschen Pflanzenschutzdienst* , *8,* 45–48.

Council Directive 2007/33/EC of 11 June 2007 on the control of potato cyst nematodes and repealing Directive 69/465/EEC. *Official Journal of the European Union.*

Douda, O., Marek, M., Zouhar, M. & Ryšánek, P. (2013). Insights into the structure and phylogeny of the 28S rRNA expansion segments D2 and D3 of the plant infecting nematodes from the genus *Ditylenchus (Nematoda: Anguininae). Phytopathol. Mediterr., 52,* 84–97.

EPPO (2013).Diagnostic Protocol PM 7/40(3) for *Globodera rostochiensis*and *G. pallida*. *Bulletin OEPP/EPPO Bulletin, 43*, 119–138.

EPPO (2014). PRQ-EPPO Plant Quarantine Data Retrieval System 5.3.1.

EPPO (2017). EPPO A1 and A2 lists of pests recommended for regulation as quarantine pests as approved by EPPO Council in September 2017, PM 1/2 (26).Paris, France, European and Mediterranean Plant Protection Organization. *EPPO Standards*.

Ibrahim, S. K., Minnis, S. T., Barker, A. D., **Russell, M. D., Haydock, P. P., Evans, K., Grove, I. G., Woods, S. R., & Wilcox, A.** (2001). Evaluation of PCR, IEF and ELISA techniques for the detection and identification of potato cyst nematodes from field soil samples in England and Wales. *Pest Manag Sci*., *57(11),* 1068-74.

Madani, M., Ward, L. J. & De Boer, S. H. (2008). Multiplex real-time polymerase chain reaction for identifying potato cyst nematodes, *Globodera pallida* and *Globodera rostochiensis*, and the tobacco cyst nematode, *Globodera tabacum*. *Canadian Journal of Plant Pathology*, *30,* 554–564.

Marek, M., Zouhar, M., Douda, O., Mazáková, J. & Ryšánek, P. (2010). Bioinformatics-assisted characterization of the ITS1-5.8S-ITS2 segments of nuclear rRNA gene clusters and its exploitation in molecular diagnostics of European crop-parasitic nematodes of the genus *Ditylenchus*. *Plant Pathol., 59,* 931–943.

Quader, M., Nambiar, L. & Cunnington, J. (2008). Conventional and real-time PCR-based species identification and diversity of potato cyst nematodes (*Globodera* spp.) from Victoria, Australia*. Nematology, 10,* 471–478.

Reid, A., Kenyon, D. M., Evans, F. F., Mulholland, V., Pickup, J., Blok, V. C., Paterson, A. & Phillips, **M. S.** (2010). Development of a high through put method for the detection and species determination of PCN. *Aspects of Applied Biology*, *103, 13*–16.

Samaliev, H. (1998). Distribution and virulence of *Globodera pallida* (Nematoda: Heteroderidae) in Bulgaria. *Plant Science*, *35,* 66-69 (Bg).

Samaliev, H. (2011). Plant-parasitic nematodes associated with potatoes (*Solanum tuberosum* L.) in Bulgaria. *Plant Science*, *48 (5*), 470-474.

Samaliev, H., Baicheva, O. & Alecsiev, A. (1992). Identification of *Globodera pallida* in Bulgaria.*VII Congress of Infec-*

tion and Parasitic Diseases, Velingrad, Bulgaria (Abstract).

Samaliev, H., Baycheva, O. & Alexiev, A. (1995). Identification of potato cyst nematode (*Globodera pallida*) in Bulgaria. *Higher School of Agriculture-Plovdiv, Scientific Works*, *XL (3)*, 251-255.

Seinhorst, J. W. (1959). A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica*, *4,* 67–69.

Seinhorst, J. W. (1962). On the killing, fixing and transferring to glycerin of nematodes. *Nematologica*, *8,* 29–32.

Stoyanov, D. (1980). Parasitic nematodes of crop plants and their control. Agricultural Academic Press, Plovdiv, 196.

Van Riel, H. R. & Mulder, A. (1998). Potato cyst nematodes (*Globodera* species) in Western Europe. In: Marks R. J. & Brodie B. B. (eds.) *Potato cyst nematodes: Biology, distribution and control*, 271–298. CAB International, Wallingford.

Whitehead, A. G., Nichols, A. J. F. & Senior, J. C. (1994). The control of potato pale cyst nematode (*Globodera pallida*) by chemical and cultural methods in different soils. *J. Agric. Sci., 123, 207-218.*

Zouhar, M., Ryšánek, P. & Kočová, M. (2000). Detection and differentiation of the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida* by PCR. *Plant Prot. Sci., 36,* 81–84.

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