ALI SMO PRIPRAVLJENI NA NOVE VRSTE OGORČIC IZ RODU *Globodera*?

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IZVLEČEK

V tem delu razpravljamo o naši pripravljenosti na nove vrste ogorčic iz rodu *Globodera*, ki ogrožajo pridelavo krompirja v Sloveniji. Ogorčice vrst *Globodera pallida* in *G. rostochiensis*, znane pod skupnim imenom krompirjeve ogorčice (PCN), resno ogrožajo pridelavo krompirja po svetu. Prva najdba krompirjevih ogorčic v Sloveniji datira v l. 1971, ko so našli eno cisto *G. rostochiensis*. Od leta 1999 smo vrsto *G. rostochiensis* zasledili še večkrat, predvsem v osrednji in severni Sloveniji. Med uradnim nadzorom PCN smo jeseni 2011 prvič v vzorcih iz Slovenskih njiv našli vrsto *G. pallida*. Dve cisti smo izločili iz vzorca zemlje z njive za jedilni krompir v bližini Ivančne Gorice v osrednji Sloveniji. Ogorčico smo identificirali z morfometrijskimi analizami ter potrdili s tremi neodvisnimi molekularnimi metodami. Z metodama PCR-RFLP in sekvenciranje smo ogorčico identificirali kot evropski tip. Krompir in krompirjeve ogorčice izvirajo iz Južne Amerike. Razpon virulence pri PCN na tem območju je veliko večji kot pri evropskih populacijah. Vnos nove populacije PCN iz Južne Amerike v Evropo bi predvidoma vodil v zlom odpornosti proti evropskim genotipom PCN v evropskih sortah krompirja. Orodja za karakterizacijo populacij PCN so v razvoju in večinoma temeljijo na razlikah v mtDNA. V letu 2012 je bila opisana nova vrsta *G. ellingtonae*. Vrsta je sorodna in podobna PCN, našli pa so jo v Oregonu in Idahu (ZDA) na njivah, kjer so gojili krompir in druge poljščine. PCR-RFPL metodo, ki smo jo razvili na KIS, uporabljamo za identifikacijo PCN kot tudi vrst *G. tabacum* in *G. achilea*. S to metodo bi predvidoma ločili tudi vrsto *G. ellingtonae*, saj je bilo to možno z *in silico* verzijo te metode.

Ključne besede: *Globodera ellingtonae*, *Globodera pallida*, *Globodera rostochiensis*, identifikacija, virulence

ABSTRACT

ARE WE READY FOR THE NEW NEMATODE SPECIES OF THE *Globodera* GENUS?

In this work we discuss our readiness to identify the new *Globodera* nematodes potentially endangering potato production in Slovenia. *Globodera pallida* and *G. rostochiensis*, commonly known as potato cyst nematodes (PCN), are considered to be the most important nematode threat to potato production worldwide. The first report of the PCN in Slovenia dates back to 1971, when a single cyst of *G. rostochiensis* was detected. Since 1999 *G. rostochiensis* was detected several times, mainly in the central and northern parts of the country. During the official PCN systematic survey in autumn 2011, *G. pallida*, was found for the first time in a soil sample in Slovenia. Two viable cysts were extracted from the infested soil sample originated from a ware potato field near Ivančna Gorica, central Slovenia. The nematode was identified by morphometrical analyses and confirmed by three independent molecular methods. PCR-RFLP method and sequencing identified this population as the European type. South America is the origin of potatoes as well as PCN. The range of

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virulence of PCN present in that area is far greater than that present in European populations. Introduction of a new PCN population from South America to Europe would very likely lead to a break of resistance to European PCN genotypes. Tools for characterization of populations of PCN are now starting to become available, mainly based on differences in mtDNA. A new species *G. ellingtonae* was described in 2012. This species, similar and closely related to PCN was found in Oregon and Idaho (USA) from agricultural fields, where potato and other crops have been grown. With the PCR-RFLP method developed and used at KIS for identification of PCN as well as *G. tabacum* and *G. achilea,* we should be able to differentiate *G. ellingtonae* as we have shown with the *in silico* version of this method.

Key words: *Globodera ellingtonae, Globodera pallida, Globodera rostochiensis,* identification, virulence

1 INTRODUCTION

Potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* are considered the most economically important plant parasitic nematodes of potato plants and can reduce yields by 70% (Brown and Sykes 1983; Greco *et al*., 1982). Both species are present world-wide in over 50 potato-growing countries including Slovenia (Baldwin and Mundo-Ocampo, 1991) and are listed as quarantine organisms in Europe. The first report of the PCN in Slovenia dates back to 1971, when a single cyst of *G. rostochiensis* was detected (Hržič, 1971). Since 1999, *G. rostochiensis* was detected several times, mainly in the central and northern parts of the country (Širca *et al*., 2010). Interceptions of *G. pallida* in imported consignments of ware potato occurred several times, but had not been detected in soil in Slovenia until recently. During the official PCN systematic survey in Slovenia in the autumn 2011, the pale potato cyst nematode, *G. pallida*, was found for the first time in a soil sample (Širca *et al*., 2012).

In addition to *G. rostochiensis* and *G. pallida*, the genus *Globodera* comprises more than 10 species, including *G. artemisiae*, *G. achilleae*, *G. hypolysi*, *G. leptonepia*, *G. 'mexicana'*, *G. millefolii*, *G. mirabilis*, *G. pseudorostochiensis*, *G. tabacum* species complex and *G. zelandica*, but none of these species is damaging the potato production. However, a new species *G. ellingtonae* was described in 2012 (Handoo *et al*., 2012). The species is similar and closely related to PCN. It was found in Oregon and Idaho (USA) from agricultural fields, where potato and other crops have been grown. Morphologically, *G. ellingtonae* is a round-cyst species that differs from the related species *G. pallida, G. rostochiensis, G. tabacum complex* and *G. mexicana* by its distinctive J2 tail, and by one or another of the following: shorter mean stylet length in J2, females and males; number of refractive bodies in the hyaline tail terminus of J2; cyst morphology including Granek's ratio; number of cuticular ridges between the anus and vulva; and in the shape and length of spicules in males. Since morphological character can overlap between species molecular identification of the PCN and closely related species is usually necessary.

In this work we present the tools and methods at the Nematology laboratory of the Agricultural Institute of Slovenia to identify the new *Globodera* nematodes potentially endangering potato production in Slovenia.

2 MATERIAL AND METHODS

2.1 *G. pallida* identification and characterisation

Two viable cysts were extracted from the infested soil sample originated from a ware potato field near Ivančna Gorica, central Slovenia. The posterior part of the cysts containing eggs and juveniles were used for morphometrical analysis, while the anterior parts were used for DNA extraction and molecular analyses. The ribosomal internal transcribed spacer (ITS)

region was amplified using ITS5 and PITSp4 primers and detected in real-time PCR as described by Bačić *et al*. (2008). The ribosomal DNA region extending from the 3′ end of the 18S ribosomal subunit, including all of ITS1, 5.8S, and ITS2, to the 5′ end of the 28S ribosomal subunit was amplified using primers described by Ferris *et al*. (1993). The amplicon was analysed by PCR-RFLP method (Širca *et al*., 2010) and sequenced. The similarity of the obtained sequence to other sequences in the public domain was performed by BLASTn. To further characterize this population, we have determined sequences of the cytochrome b (*cyt*B) gene as described by Picard et al. (2007) and the S222 non-coding region on the mitochondrial DNA (mt DNA) as described by Grujić (2010). To determine the spread of *G. pallida* infestation in Slovenia an additional 69 samples were taken from the surroundings of the field where *G. pallida* was first detected.

2.2 *G. ellingtonae* identification

The first molecular method of choice for PCN identification in our laboratory is ITS region amplification with real-time PCR (Bačić *et al*., 2008). According to data published by Handoo *et al*. (2012) the PCN species specific primers from this method do not anneal and allow amplification in *G. ellingtonae*. We can therefore conclude that the method would not allow *G. ellingtonae* rDNA amplification and species identification. In our laboratory the second molecular method of choice for *Globodera* species identification (also in in cases where morphological analysis indicates PCN, but these were not confirmed by real-time PCR) is the PCR-RFLP method (Širca *et al*., 2010). We have performed an *in silico* version of this PCR-RFLP method using computer software BioEdit in order to check if this method would identify *G. ellingtonae* correctly.

2.3 mtDNA sequences for characterization of PCN populations

Sequence of *cyt*B gene was determined in 10 populations of *G. rostochiensis* from Slovenia, Croatia, Bosnia and Herzegovina, and Serbia using the primers developed by V. Blok *et al*. (unpublished).

3 RESULTS AND DISCUSSION

3.1 First finding of *Globodera pallida* in Slovenia

Two viable cysts extracted from the infested soil sample originated from a ware potato field near Ivančna Gorica, central Slovenia. The species identification by morphometrical analyses and confirmed by three independent molecular methods proved the presence of *G. pallida*. The determined sequence of rDNA containing ITS1, 5.8S rRNA gene and ITS2 (acc. no. HF583248) revealed unequivocal similarity to *G. pallida*. Restriction of the rDNA sequence extending from the 3′ end of the 18S ribosomal subunit, including all of ITS1, 5.8S, and ITS2, to the 5′ end of the 28S ribosomal subunit with the PCR-RFLP revealed that the Slovenian population of *G. pallida* is of the European type. To further characterize this population, we have determined sequences of the *cyt*B gene (acc. nos. HF583256 - HF583265) and the S222 non-coding region on the mtDNA (acc. nos. HF583249 - HF583255). Both sets of determined sequences (*cyt*B gene and S222 non-coding region) exhibited 98.7 or higher identity. Viable *G. pallida* cysts were found in the five of additional 69 samples taken from two neighboring fields (one of grassland and the other of clover).
Three fields, totaling 1.9 ha, were declared as *G. pallida*-infested. The eradication of the pest will take place by enforcing strict phytosanitary measures. Ware potatoes originating from areas where the pests occur is considered to be the most probable pathway for the introduction of *G. pallida* in Slovenia. A ware potato processing facility is situated in very close proximity

to the infested fields. The waste waters from potato tuber washing were discharging onto the grassland, never used for potato or other field crop production in which the *G. pallida* infestation was found. The facility processes imported ware potato from several European and non-European countries. This case demonstrates that ware potato may pose a serious risk for the introduction of such pests, and should be therefore subjected to more intensive phytosanitary inspection.

3.2 New *Globodera* species from USA - *G. ellingtonae*

Sixty-four *G. ellingtonae* rDNA sequences were downloaded from the public DNA database and used in the *in silico* RFLP analysis. The results showed four different restriction patterns in *G. ellingtonae*, which differed from the patterns of *G. pallida*, *G. rostochiensis*, *G. tabacum* and *G. achillea* (Table 1).

Table 1: *In silico* cleavage and fragment sizes of *Globodera rostochiensis, G. pallida, G. tabacum, G. achilleae* and *G. ellingtonae* rDNA-ITS sequences with *Alu*I, *Hinf*I, *Mbo*I, *Mse*I and *Rsa*I restriction enzymes.

Species with sequence acc. no. and source	Fragment size	*Alu*I	*Hinf*I	*Mbo*I	*Mse*I	*Rsa*I
G. rostochiensis AY700060 Slovenia: Libeliče EF153839 USA: New York FJ212163 Canada: Newfoundland DQ847119 Russia: Moscow region FJ212166 Canada: Quebec DQ847118 UK: Scarcliffe	 874 874 874 874 874 873	381, 310, 148, 35	856, 18	522, 170, 165, 16	449, 425	538, 221, 106, 9
G. pallida EF153838 UK: York EF153836 USA: Idaho FJ212165 Canada: Newfoundland EU855119 Poland	 876 876 877 876	459, 382, 35	706, 152, 19	431, 171, 165, 93, 16	426, 392, 58	539, 328, 9
G. pallida DQ097514 Argentina EU006706 Peru	 874 876	459, 382, 35	706, 152, 19	524, 170, 165, 16	450,426	539, 222, 106, 9
G. tabacum FJ667946	877	458, 383, 36	860, 17	524, 172, 165, 16	452, 425	541, 222, 105, 9
G. achilleae AY599498 Slovenia: Zadraga	888	888	888	530, 177, 165, 16	460, 428	549, 218, 112, 5, 4
G. ellingtonae GQ896542, GQ896546, GQ896547	873	457, 380, 36	559, 152, 145, 17	521, 171, 165, 16	449, 424	538, 124, 105, 97, 9
G. ellingtonae GQ896543	873	811, 62	559, 152, 145, 17	495, 197, 165, 16	449, 424	538, 124, 105, 97, 9
G. ellingtonae GQ896544	873	457, 380, 36	704, 152, 17	521, 171, 165, 16	425, 400, 48	537, 222, 105, 9
G. ellingtonae GQ896545 *	873	457, 380, 36	856, 17	521, 171, 165, 16	449, 424	547, 221, 105

It should be noted, that one pattern (marked by asterisk in Table 1) was very similar to restriction pattern in *G. tabacum*. The subtle difference of maximum 6 bp in one fragment would not be recognized using standard agarose gel electrophoresis for visualization of restriction patterns, but might be possible with the more precise method of DNA size analysis using capillary electrophoresis or sequencer, the latter in combination with Terminal Restriction Fragment Length Polymorphism (TRFLP). A third alternative method that could

be used would be sequencing of the rDNA amplicon. The sequence obtained could be used for *in silico* restriction and BLAST comparison to all other known sequences.

With the PCR-RFLP method developed and used at KIS for identification of PCN as well as *G. tabacum* and *G. achilea*, we would most probably be able to differentiate *G. ellingtonae* as we have shown with the *in silico* version of the this method. However such a test should be performed on biological material as soon as it becomes available as well.

3.3 Risk of non-European PCN populations

The Panel on Plant Health of the European Food and Safety Authority (EFSA) has recently delivered a meaningful scientific opinion where the risks to plant health posed by European versus non-European populations of the PCN *G. pallida* and *G. rostochiensis* to solanaceous plants in the EU are discussed (EFSA Panel on Plant Health, 2012). Main facts and predictions described there are recapitulated as follows: it was generally accepted that PCN originated from the Andean Highlands of South America, where they co-evolved with their plant hosts (potatoes and other members of the family Solanaceae). Their initial introduction into Europe consisted of a small number of cysts that represent a restricted proportion of the gene pool and virulence present in South America. The PCN introduced into Europe are thought to have originated from a highly restricted region in the south of Peru. All PCN currently present in Europe represent a minor subset of the full biological diversity present in South America. The range of virulence of PCN present in South America is far greater than that present in European populations. There is only a limited range of control options currently available for PCN. The use of resistant cultivars resulted in a decline in PCN populations and is recognized as the most important and effective control option in Europe. It was assessed that further introductions of PCN from South America are almost certain to have different virulence characteristics from the PCN currently present in Europe. This will lead, in time, to the currently available resistance to PCN (which has been bred against populations currently present in Europe) being overcome. The impact on the potato is therefore expected to be major. It has been demonstrated unequivocally that South American populations of both species of PCN are virulent to the resistance sources used in European cultivars and that selection for virulence can occur. The PCN populations originating from South America are considered a potential threat to potato cultivation control in Europe if introduced here. As new South American genotypes are very likely to have a similar potential for establishment and spread as existing European genotypes, the potato varieties currently grown in Europe will not be resistant to new virulent genotypes. As resistant varieties take a very long time to develop, the consequences of a new introduction of South American PCN would be major. The Panel therefore concluded that it is very important to maintain the current phytosanitary measures to prevent the entry of South American PCN.

Several studies have investigated differences between European and South American populations of PCN including our studies of variability of cell wall degrading proteins (Gerič Stare *et al*., 2011, 2012). No diagnostic test that distinguishes these populations or that identifies populations with specific virulence characteristics has been developed. However, various parts of the mtDNA have been used in phylogenetic studies of *G. pallida* and *G. rostochiensis* both from their centre of origin and from regions into which they have been introduced (Armstrong *et al*., 2007, Plantard *et al*., 2008). Although these tests have the capacity to differentiate European and South American populations (with the exception of the South American populations from which the European introduction was derived), they do not differentiate on the basis of virulence characteristics.

As the new tools for characterization of PCN populations will most likely be based on differences in mtDNA sequences, our group has studied parts of mtDNA in PCN from Balkan region. We have determined variability within two regions of mtDNA in Slovene populations of *G. pallida* (*cyt*B gene: acc. nos. HF583256 - HF583265, S222 non-coding region: HF583249 - HF583255). Further we have shown that *cyt*B gene in *G. rostochiensis* in ten populations from Slovenia, Croatia, Bosnia and Herzegovina, and Serbia is identical (acc. nos. HF913246 - HF913255).

4 CONSLUSIONS

In 2011 *G. pallida* was detected in Slovenian soils for the first time. The KIS Nematology laboratory identified this new PCN species in Slovenia quickly and correctly; the diagnostic report was sent out six days after receiving the soil sample.
Additionally, our laboratory participates in yearly proficiency tests for extraction and identification by morphometrical and molecular methods for PCN. In comparison to other European nematology laboratories participating in the test, our laboratory has always ranked in the top quarter among more than 20 laboratories.
In 2012 a new *Globodera* species, *G. ellingtonae* originating from USA was described. With the methods currently used at KIS for *Globodera* species identification we would presumably be able to differentiate the new species from the PCN.
Introduction of a new PCN population from South American to Europe would very likely lead to break of resistance in potato to European PCN genotypes. We recognize the need to develop new molecular methods for characterization of PCN populations and identification of *Globodera* species.

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