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RAPID COMMUNICATION

The high resolution melting PCR protocol for rapid identification of single nucleotide substitutions in cytochrome *c* oxidase subunit II of *Globodera pallida* populations assigned to three pathotypes as an attempt of their differentiation

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Abstract

The potato cyst nematode (PCN), *Globodera pallida*, originates from South America and is considered one of the most severe agricultural pests of potato crops and other Solanaceae plants globally. Based on their virulence and ability to reproduce on various potato cultivars, the populations of *G. pallida* are divided into three pathotypes, Pa1– Pa3. In this study, comparative sequence analyses of the fragment of mitochondrial cytochrome *c* oxidase subunit II (*mtCOII*) gene for eight populations of *G. pallida*, representing three pathotypes, Pa1, Pa2 and Pa3, indicated genetic diversity between them. However, we did not identify significant mutations distinguishing Pa2 from Pa3. Interestingly, two single nucleotide substitutions, T441C and A468G, were characteristic only for populations assigned to Pa1. On this basis, we developed high resolution melting (HRM) PCR protocol. As a result, the melting curves obtained for samples of Pa1 populations varied from those obtained for populations designed as Pa2 and Pa3, allowing their differentiation. Thus, the HRM protocol developed here enables a rapid, very sensitive and low-cost screening assay for SNPs identification in *mtCOII* of *G. pallida* pathotypes. In effect, it might also be a helpful molecular tool in pathotype differentiation. However, further verification of the correlation of the occurrence of single nucleotide mutations in *mtCOII* in particular pathotypes should be carried out on a much larger number of samples of *G. pallida*, to determine if these mutations are characteristic only for this pathotype.

Keywords: *Globodera pallida*, high resolution melting (HRM), *mtCOII* genetic diversity, pathotypes

The species *Globodera pallida*, next to *G. rostochiensis*, belongs to potato cyst nematodes (PCN), which are the most economically important pests of Solanaceae plants. Due to their damaging potential, PCNs significantly decrease potato production. According to the available literature, *G. pallida* and *G. rostochiensis* are responsible for a significant decrease in worldwide potato yields (Hoolahan *et al.* 2012). PCNs occur in Europe, Asia, Africa, America, and Oceania. Due to their very high expansion and aggressiveness both species have the status of quarantine pests (EPPO 2017).

In addition to morphological identification, several molecular techniques are successfully applied for routine PCN species detection and differentiation. Molecular tools such as conventional PCR or multiplex reactions (Bulman and Marshall 1997; Zouhar and Ryšánek 2000; Vejl *et al.* 2002), a real-time PCR using SYBR Green (Bates *et al.* 2002; Madani *et al.* 2005) or TaqMan hybridization probes (Nowaczyk *et al.* 2008; Nakhla *et al.* 2010) are helpful for identifying species. Recently, Subbotin and co-workers showed high genetic diversity of *cytb* and *COI* genes, which can be used as markers to differentiate *Globodera* species (Subbotin *et al.* 2020). Extensive studies have also been conducted on the genetic diversity of various populations of potato cyst nematodes showing high

intra-species genetic variability of PCN. The populations of *G. pallida* are classified into three pathotypes (also known as virulence groups), Pa1–Pa3 (Kort *et al.* 1977). Kort and co-workers proposed a European international scheme for identifying pathotypes that utilize the ability to multiply on different potato lines, characterized by genes resistant to PCN (Kort *et al.* 1977; Hinch *et al.* 1998). However, this scheme is not useful for Andean regions of South America, where six various pathotypes were indicated (Saenz and De Scurrah 1977). The genetic diversity observed in PCN populations was studied using electrophoretic techniques: SDS-PAGE (Bakker *et al.* 1992), isoelectric focusing (Fox and Atkinson 1984) showing several IP-protein variants between populations of different pathotypes, as well as molecular methods such as restriction fragments length polymorphism (Burrows and Boffey 1986; Schnick *et al.* 1990) and random amplified polymorphic DNA (Folkertsma *et al.* 1994; Thiéry *et al.* 1997; Sedlák *et al.* 2004; Nowaczyk *et al.* 2011). The populations of *G. pallida* are characterized by genetic variations within genes constituting molecular markers for diagnostics, such as internal transcribed spacer (ITS) regions, microsatellites and mitochondrial DNA (mtDNA) (Hoolahan *et al.* 2012). The above-mentioned molecular techniques are laborious and require a significant amount of DNA. Hence, developing a rapid, sensitive method for identifying cyst nematode pathotypes in the field is crucial for choosing the most resistant potato cultivar to be cropped. In this study, nucleotide sequence analyses of partial *mtCOII* gene of eight populations of *G. pallida,* previously assigned to three pathotypes, showed some genetic differences. On this basis, we designed the protocol of the real time PCR high resolution melting (HRM) with primers flanking the point mutations. We tried to determine the usefulness of this method in the differentiation of the *G. pallida* pathotypes.

We tested eight populations of *G. pallida* cysts, collected before 2010, previously assigned to a particular pathotype. Nematode cysts (4–6) were crushed and digested overnight at 37°C in T1 lysis buffer with proteinase K (Macherey-Nagel). The genomic DNA was then isolated using the NucloSpin Tissue Kit (Macherey--Nagel). Alternatively, 1–2 cysts were crushed with a pipette tip and suspended in 10 µl of distilled water and used as a template (Nowaczyk *et al.* 2008). As the first step, the fragment of *mtCOII* (450 nt) was amplified using a combination of three primers, GpaCOIIc 5'AAGGGGTTTGGTTGGACT3' and GpaCOIIB 5'CTGGTAAGGCCCAAGAATG3' with an annealing temperature of 50.5°C, alternatively GpaCOIIA 5'GTTTGGTTGGACTGGTTTC3' and the aforementioned GpaCOIIB at 48°C. The PCR reactions were done in 20 µl, and the mixture contained 1X buffer (Novazym) with 2.5 mM $MgCl_2$, 0.2 dNTP, 0.5 µM primers, 0.2 Allegro Taq DNA polymerase (Novazym) and about 10 ng of DNA template. The thermal profile consisted of initial denaturation at 94°C for 2 min, followed by 30–35 cycles of denaturation at 94°C for 30 s, annealing at appropriate temperature for 30 s, elongation at 72°C for 40 s, and the final extension was 5 min at 72°C. The obtained products were analyzed by agarose electrophoresis and visualized in UV light after Midori Green (Nippon) staining. Specific products were excised, eluted from gel using Wizard SV Gel and PCR clean-up system (Promega) and subsequently sequenced in both orientations. The obtained sequence results were analyzed in Bio-Edit software and compared with sequences available in the GenBank database. Sequence comparisons of analyzed nematode populations showed two unique single nucleotide polimorphism (SNPs) localized at positions T441C and A468G, characteristic only for populations assigned as Pa1. On this basis, the HRM real-time PCR reaction was developed. For this purpose, the specific primers flanking the region with the above-mentioned substitutions in *mtCOII* were designed. The test was done in 10 µl and the reaction mixture contained the following reagents: 1X buffer with 2.5 mM $MgCl_2$, 0.2 dNTP, 0.5 µM primers hrmGpa1 5'ATTTTCGGCTCCTGGAGGTA3' and hrmGpa2 5'CTGGTAAGGCCCAAGAATGA3', 1X Eva Green (Biotium) and 1 µl of crushed cyst suspension as a template. The thermal profile consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles, denaturation at 95°C for 10 s, annealing at 57°C for 10 s, and elongation at 72°C for 10 s. All analyzed positive and negative samples (without a template, NTC) were amplified in triplicate in a Corbett Rotor Gene™ 6000 thermal cycler (Qiagen), and subsequently, the amplicons were melted. The obtained amplification plots and the melting curves were visualized and analyzed in the Rotor-Gene 6000 software 1.7 (Qiagen) and presented in Figure 1.

Sequence data analyses of the partial *mtCOII* gene revealed genetic diversity between analyzed populations of *G. pallida*. The populations representing Pa2 and Pa3 were characterized by a very high sequence identity ranging from 98 to 99%. In contrast, the similarity with populations assigned to Pa1 ranged 95–96% of nt sequence identity. Several point substitutions and single nucleotide deletion in the analyzed sequences of Pa1, Pa2 and Pa3 were indicated (Fig. 1A), among which two SNPs were common only for populations classified as Pa1 (Fig. 1A). HRM real-time assay resulted in a unique melting curve profile of populations classified as Pa1, allowing their differentiation from populations Pa2/Pa3. The melting point for Pa1 was 82.3°C, and for Pa2 and Pa3, it was 81.3°C (Fig. 1B).

Based on data from available literature, Pa2 and Pa3 of *G. pallida* (next to some *G. rostochiensis*) are

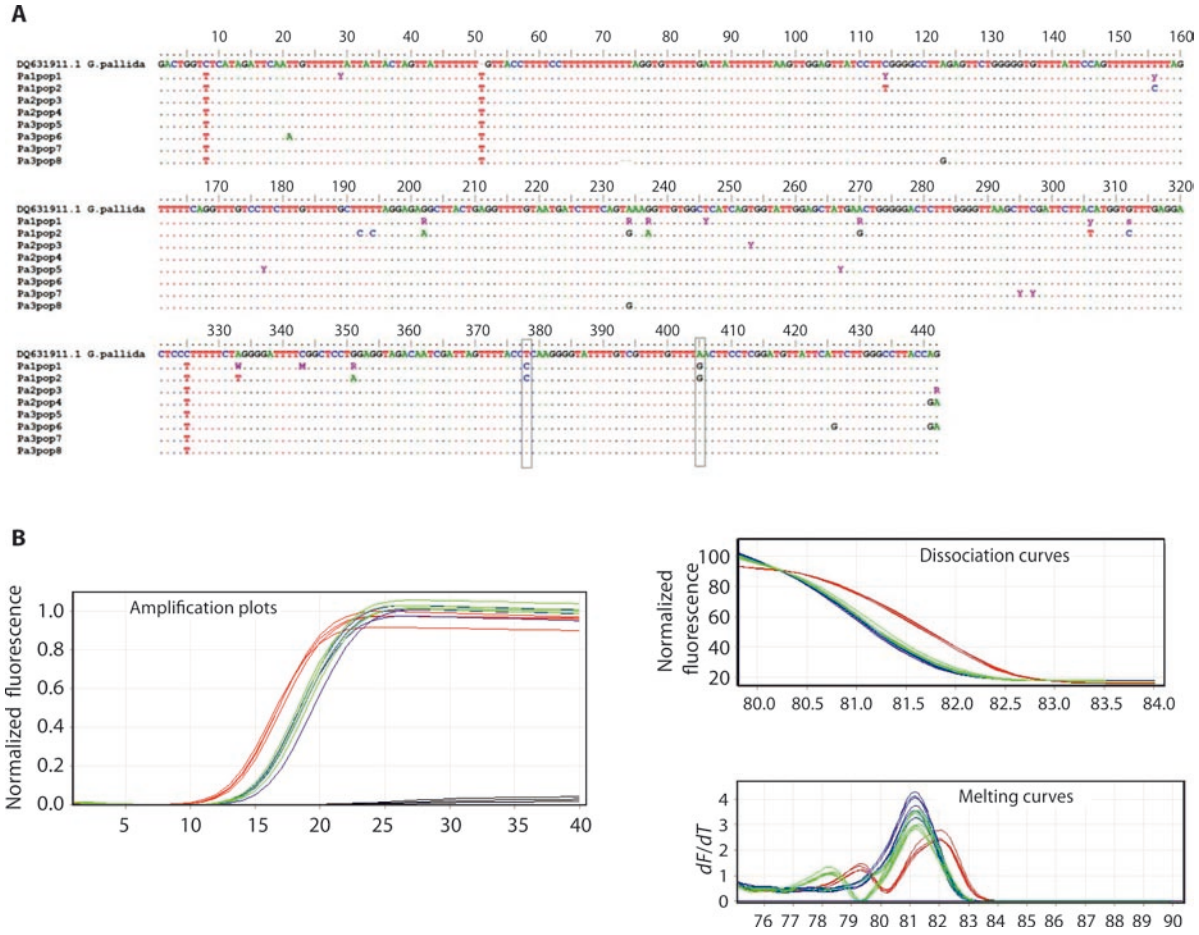

Fig. 1. Genetic differences in the fragment of the *mtCOII* gene in three pathotypes of *Globodera pallida* populations. Panel A: The alignment of the consensus of a partial region of analyzed *mtCOII* gene of three pathotypes of *G. pallida*. The nucleotide substitutions are indicated. Gray boxes mark two characteristic SNPs. Panel B: HRM real-time PCR results based on the fragment of the *mtCOII* gene; left – the amplification plots; right – the normalized dissociation curves (upper) and the melt rate profiles (bottom) of analyzed *mtCOII* amplicons for all studied populations classified as particular pathotypes. Nematode populations assigned to Pa1 – red curves, Pa2 – blue curves, and Pa3 – green curves; black lines – no template control, NTC. The images visualized by using the Rotor-Gene 6000 software 1.7 (Qiagen)

genetically closely related to each other. Previously reported differences do not warrant differentiation into Pa2 and Pa3 pathotypes (Phillips and Trudgill 1983, 1998; EPPO 2017). Our results somewhat confirm those observations. Based on our observations, the HRM real-time protocol developed in this work might be a sensitive and rapid method for the differentiation of Pa1 from Pa2/Pa3. However, to unequivocally state that the correlations of identified mutations are unique only for Pa1 populations, more samples of *G. pallida* from various localizations should be analyzed. Subsequently, the developed protocol will be usable as a method of distinguishing between pathotypes, which is crucial for potato crop protection against PCNs.

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