Eur J Plant Pathol (2014) 139:245–252
DOI 10.1007/s10658-014-0385-x

SHORT COMMUNICATIONS

A new chorismate mutase gene identified from *Globodera ellingtonae* and its utility as a molecular diagnostic marker

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Accepted: 2 January 2014 / Published online: 21 January 2014

Abstract *Globodera ellingtonae*, a new cyst nematode species recently detected in Oregon and confirmed to reproduce on potato, shares key morphological features with the two species of potato cyst nematode (PCN; *G. rostochiensis* and *G. pallida*) which are of quarantine concern. Currently no methods are available for the molecular diagnosis of this new *Globodera* species. In this study, we cloned a chorismate mutase gene (*Ge-cm-1*) from *G. ellingtonae*. Our detailed sequence analysis identified two different *Ge-cm-1* mRNA transcripts, named as *Ge-cm-1* and *Ge-cm-1-IRII*, of which *Ge-cm-1-IRII* differs from *Ge-cm-1* by a 93-base pair (bp) insertion. Comparison of the *Ge-cm-1-IRII* transcript with the genomic sequence of *Ge-cm-1* revealed that *Ge-cm-1-IRII* was an alternatively spliced transcript generated by intron retention, further confirming a previous discovery that alternative splicing of *CM* genes is conserved among *Globodera* species. The genomic sequence of *Ge-cm-1* contains three introns with intron 1 showing significant divergence compared to those of *CM* genes from the two PCN species as well as a related species *G. tabacum*. Based on the sequence variations, we designed PCR primers and a TaqMan probe specific for *Ge-cm-1* and developed a TaqMan qPCR assay that provides reliable and sensitive identification of *G. ellingtonae*. Due to the fact that multiple *Globodera* species that infect potato currently occur in the U.S., this new molecular diagnostic method is valuable and should be included with other standard diagnostic methods to achieve a rapid and accurate differentiation of *Globodera* species.

Keywords Alternative splicing · Chorismate mutase · *Globodera ellingtonae* · Molecular diagnosis · Potato cyst nematode · TaqMan qPCR

Abbreviations

AS	Alternative splicing
bp	Base pair
CM	Chorismate mutase
Ge-cm-1	*Globodera ellingtonae* chorismate mutase gene
Gr-cm-1	*Globodera rostochiensis* chorismate mutase gene

Electronic supplementary material The online version of this article (doi:10.1007/s10658-014-0385-x) contains supplementary material, which is available to authorized users.

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Gp-cm-1	*Globodera pallida* chorismate mutase gene
Gt-cm-1	*Globodera tabacum* chorismate mutase gene
ITS	Internal transcribed spacer
PCN	Potato cyst nematode
qPCR	Quantitative real-time polymerase chain reaction
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal RNA

Potato cyst nematodes (PCNs; *Globodera rostochiensis* and *G. pallida*) are internationally recognized quarantine pests that infect roots of potato. It is generally believed that PCNs are indigenous to the South American Andes and were introduced to Europe and then to other parts of the world where potatoes are grown (Brodie 1999; Evans and Stone 1977; Grenier et al. 2010). Prior to 2006, only *G. rostochiensis* was present in the U.S. with a very restricted distribution (Brodie 1999; Brodie and Mai 1989). Similarly in Canada, one or both PCN species were reported only in restricted areas of Vancouver Island and Newfoundland (Rott et al. 2010; Stone et al. 1977). In 2006, *G. pallida* was detected in Idaho, which signified the first discovery of a second PCN species in the U.S. (Hafez et al. 2007; Skantar et al. 2007). In the same year, soil samples collected from a potato field in St-Amable, Quebec were identified to contain *G. rostochiensis* cysts, revealing for the first time the introduction of PCN to the inland area of Canada (Sun et al. 2007). Furthermore, in 2008, a *Globodera* species sharing similar morphological features with the two PCN species was isolated from a field in Oregon and two fields in Idaho (Skantar et al. 2011). Further detailed morphological examinations and analysis of the ribosomal RNA (rRNA) internal transcribed spacer (ITS) region revealed characteristic and molecular differences between the Oregon *Globodera* species and the related species of *G. rostochiensis*, *G. pallida*, *G. tabacum*, and *G. mexicana*, which resulted in the description of the Oregon species as *G. ellingtonae*, a new round-cyst species that reproduces well on potato (Handoo et al. 2012).

Cyst nematodes are biotrophic pathogens that secrete effector proteins into host root cells to promote successful infection (Mitchum et al. 2013). Among the many effector protein genes that have been identified, the chorismate mutase (*CM*) gene was found to be distributed in diverse cyst nematode species including *G. rostochiensis*, *G. pallida*, and *G. tabacum* (Jones et al. 2003; Lu et al. 2008; Yu et al. 2011) as well as in root-knot nematode species and a migratory endoparasitic nematode *Pratylenchus coffeae* (Mitchum et al. 2013). Our previous studies revealed that *CM* genes from *G. rostochiensis*, *G. pallida*, and *G. tabacum* are subject to alternative splicing (AS) through intron retention, which represented the first example of AS identified in plant-parasitic nematodes (Lu et al. 2008; Yu et al. 2011). Analysis of genomic sequences of these *Globodera CM* genes further identified regions unique to each of these three *Globodera* species, which subsequently led to the development of TaqMan qPCR assays that provide rapid and reliable identification of these three *Globodera* species (Yu et al. 2011).

The rRNA ITS region is commonly used as a taxonomic marker for nematode identification (Powers et al. 1997). Analysis of ITS sequences of *G. ellingtonae* revealed sequence variations different from those of *G. rostochiensis*, *G. pallida*, *G. tabacum*, and *G. mexicana* (Handoo et al. 2012; Skantar et al. 2011). However, several standard methods including conventional and multiplex PCR-ITS and PCR-ITS-RFLP (restriction fragment length polymorphism) failed to identify PCR products or banding patterns unique for *G. ellingtonae*, which might be due to the high level of intraspecific variability observed in ITS sequences of *G. ellingtonae* (Handoo et al. 2012; Skantar et al. 2011). Morphological diagnosis of *G. ellingtonae* requires a trained nematologist and can be challenging and time-consuming. Therefore, a rapid molecular diagnostic method for *G. ellingtonae* is much needed to help provide timely and accurate differentiation of *G. ellingtonae* from the two PCN species that are of quarantine concern. In this study, we cloned and characterized the *CM* gene (named *Ge-cm-1*) from *G. ellingtonae*. By comparing with the available *CM* gene sequences from *G. rostochiensis*, *G. pallida*, and *G. tabacum* (Lu et al. 2008; Yu et al. 2011), we identified unique regions in the genomic sequence of *Ge-cm-1* that were subsequently utilized for the development of a reliable and sensitive molecular method for the identification of *G. ellingtonae*.

Two different batches of *G. ellingtonae* cysts collected from the Oregon field were used for genomic DNA extraction as previously described (Yu et al. 2011). Preparasitic second-stage juveniles hatched from cysts in potato root diffusate were used for inoculation of potato

Table 1 Primers and probe used in this study

Name	Sequence (5′ to 3′)	Application
Primers		
GrCM1-atgF	ATGAATTTGTTGGTCG TTCCGT	Gene cloning
GrCM1-tgaR	TCATTCATTCAGCAGT TTCTTG	Gene cloning
GorgCM1-188F	CGTCTTTTTTACCATATT TCCAGAA	TaqMan qPCR
GorgCM1-526R	TTTGTGCGATTTTGCC TAT	TaqMan qPCR
Probe		
GorgCM1-409P	FAM-TCGTCCACTTCG GCAAAATCA-TAMRA	TaqMan qPCR

roots grown on petri-dishes. Infected potato root segments containing different life stages of *G. ellingtonae* were collected and used for mRNA extraction followed by first-strand cDNA synthesis according to Lu et al. (2008). Obtained genomic DNA and cDNA were used respectively as template for amplifying the corresponding genomic DNA and cDNA sequences of the *Ge-cm-1* gene by PCR using primers GrCM1-atgF and GrCM1-tgaR (Table 1) that were designed according to the *Gr-cm-1* gene (Genbank accession number EF437154) (Lu et al. 2008). Amplified PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced at the Cornell University DNA Sequencing Facility (Ithaca, NY).

Sequencing of more than 15 random clones from PCR reactions with first-strand cDNA generated from *G. ellingtonae* mRNA identified two cDNA clones (named *Ge-cm-1* and *Ge-cm-1-IRII*) that showed significant similarity to *CM* genes from *G. rostochiensis*, *G. pallida*, and *G. tabacum*. The *Ge-cm-1* cDNA (GenBank accession number KF360243) is 95.4 %, 94.0 %, and 96.6 % identical to *Gr-cm-1* (EF437154), *Gp-cm-1* (AJ457834), and *Gt-cm-1* (HM148920), respectively, in the open reading frame region and encodes a predicted protein (Ge-CM-1) of 286 amino acids that contains an N-terminal signal peptide (Nielsen et al. 1997) and a conserved CM domain (Fig. 1a and Supplementary Fig. 1). At the amino acid level, the predicted Ge-CM-1 protein is 88.8 %, 87.1 %, and 90.1 % identical to the *G. rostochiensis* CM protein (Gr-CM-1), the *G. pallida* CM protein (Gp-CM-1), and the *G. tabacum* CM protein (Gt-CM-1), respectively. Multiple sequence alignment performed using Clustal W (Larkin et al. 2007) and BOXSHADE (Subramaniam 1998) showed that, in addition to several polymorphic amino acid residues randomly present in the mature protein sequence, Ge-CM-1 differed from the other three *Globodera* CM proteins mainly in the signal peptide region (Supplementary Fig. 1). It would be interesting to determine if *Ge-cm-1* shows similar spatial expression in the nematode as the other *Globodera CM* genes. The *Ge-cm-1-IRII* cDNA (GenBank accession

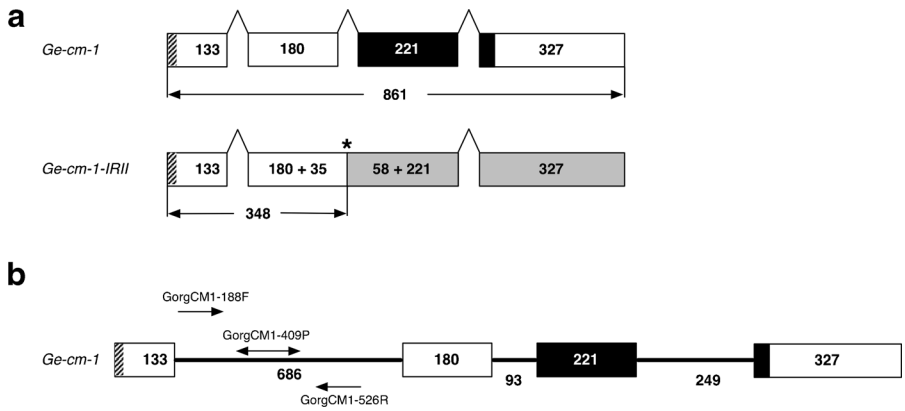

Fig. 1 Schematic diagrams of the two mRNA transcripts of the chorismate mutase gene (*Ge-cm-1*) from *Globodera ellingtonae* (**a**) and their associated genomic sequence structure (**b**). Exons and introns are indicated by *boxes* and *solid lines*, respectively. *Stippled boxes* and *black boxes* indicate the N-terminal signal peptide and the chorismate mutase domain, respectively. *Caret lines* indicate exon-exon junctions in mRNA transcripts. *Numbers* in the *boxes* and below the *lines* indicate the sizes (in base pairs) of exons and introns, respectively. *Numbers* between *arrow lines* indicated the sizes of open reading frames of *Ge-cm-1* and *Ge-cm-1-IRII*, respectively. In *Ge-cm-1-IRII*, the alternative exon 2 also includes partial sequence from intron 2 (35 bp) and its corresponding exons 3 and 4 becomes noncoding exons (*gray shaded*) because of the presence of a stop codon (indicated by an *asterisk*) introduced by retention of intron 2. PCR primers and the TaqMan probe targeting the specific regions in intron 1 that are used for the TaqMan qPCR assay are also indicated by *single* and *double arrows*, respectively

Table 2 Splice site sequences of *Caenorhabditis elegans* and intron 2 of *Globodera CM* genes

Species	5′ splice site	3′ splice site	Reference
C. elegans	AG/GURAGUUU	UUUUCAG/R	Blumenthal and Steward 1997
G. rostochiensis	AG/GUA**C**G**G**U**C**	U**GAC**CAG/A	Lu et al. 2008
G. pallida	AG/GUA**C**G**G**U**C**	U**CAC**CAG/A	Yu et al. 2011
G. tabacum	AG/GUA**CUCAC**	U**GAC**CAG/A	Yu et al. 2011
G. ellingtonae	AG/GUA**C**G**G**U**C**	U**CAC**CAG/A	This study

Nucleotides different from those in the consensus sequences of *C. elegans* are indicated in bold

R represents A or G

number KF360244) is identical to *Ge-cm-1* except for a 93-bp insertion and encodes a deduced truncated protein (Ge-CM-1t) of 115 amino acids that lacks the conserved CM domain because of a stop codon that was introduced by the 93-bp insertion (Fig. 1a).

Sequencing of more than 20 random clones from independent PCR reactions that used *G. ellingtonae* genomic DNA as template identified only one genomic sequence that matched the *Ge-cm-1* cDNA except that three introns were found as indicated in Fig. 1b. These three introns followed the 'GU-AG' rule for *cis*-splicing, interestingly however, as discovered in the other three *Globodera CM* genes (Lu et al. 2008; Yu et al. 2011), the 5′ and 3′ splice sites of intron 2 were unusual, differing in three nucleotides compared to the respective consensus sequences identified in *Caenorhabditis elegans* (Blumenthal and Steward 1997) (Table 2). Further sequence analysis discovered that the 93-bp insertion found in the *Ge-cm-1-IRII* cDNA was derived from the entire second intron of the genomic sequence, revealing that *Ge-cm-1-IRII* was a splice variant generated from intron retention, an AS event that was also identified in the *CM* genes from the other three *Globodera* species (Lu et al. 2008; Yu et al. 2011). This finding further revealed the conservation of AS of *CM* genes among *Globodera* species.

Multiple genomic sequences of the *CM* gene were identified in *G. rostochiensis* (Lu et al. 2008), *G. pallida*, and *G. tabacum* (Yu et al. 2011). Interestingly, only a single *Ge-cm-1* genomic sequence was obtained from the *G. ellingtonae* population from Oregon. The *Ge-cm-1* genomic sequence (GenBank accession number KF360245) is 89.21–93.37 %, 85.71–90.1 %, and 93.65–94.23 % identical respectively to the genomic sequences of *Gr-cm-1* (EF437152 and EF437153), *Gp-cm-1* (HM148923, HM148924, and HM148925), and *Gt-cm-1* (HM148926 and HM148927). A close comparison of these *CM* gene genomic sequences showed that the most significant sequence variation occurred in the intron 1 region (Fig. 2). Intron 2 and intron 3 were much conserved among *Globodera CM* genes of which both *Ge-cm-1* and *Gp-cm-1* shared a same nucleotide length (93 bp) in intron 2 (Fig. 1b and Yu et al. 2011).

The use of the rRNA ITS region and the D2-D3 expansion segment of 28S rRNA failed to identify molecular markers unique for *G. ellingtonae* (Handoo et al. 2012; Skantar et al. 2011). In a previous study, we have successfully used the *CM* gene as a diagnostic marker and developed highly reliable and sensitive TaqMan qPCR assays for identifying *G. rostochiensis*, *G. pallida*, and *G. tabacum* (Yu et al. 2011). Genomic sequence comparison showed significant sequence variation in intron 1 among *Globodera CM* genes. We further identified short regions in intron 1 that are unique for *Ge-cm-1* (Fig. 2) and used these unique regions to

Table 3 Nematode populations used in this study

Species	Population abbreviation	Origin
Globodera ellingtonae	Ge-1	Oregon, USA
	Ge-2	Oregon, USA
G. rostochiensis	Gr-Ro1	New York, USA
	Gr-Ro2	New York, USA
	Gr-Ro1-NL	The Netherlands
	Gr-Ro5	The Netherlands
G. pallida	Gp-ID	Idaho, USA
	Gp-Pa1	Duddingston, UK
	Gp-297	South Peru
	Gp-286	South Peru
	Gp-NF	Newfoundland, Canada
	Gp-Pa2	The Netherlands
	GPS6	South Peru
	GPS9	South Peru
Globodera sp.	GRS5	Antofagasta, Chile
G. tabacum	Gt-MO	unknown
Heterodera glycines	Hg	North Carolina, USA
H. schachtii	Hs	New York, USA

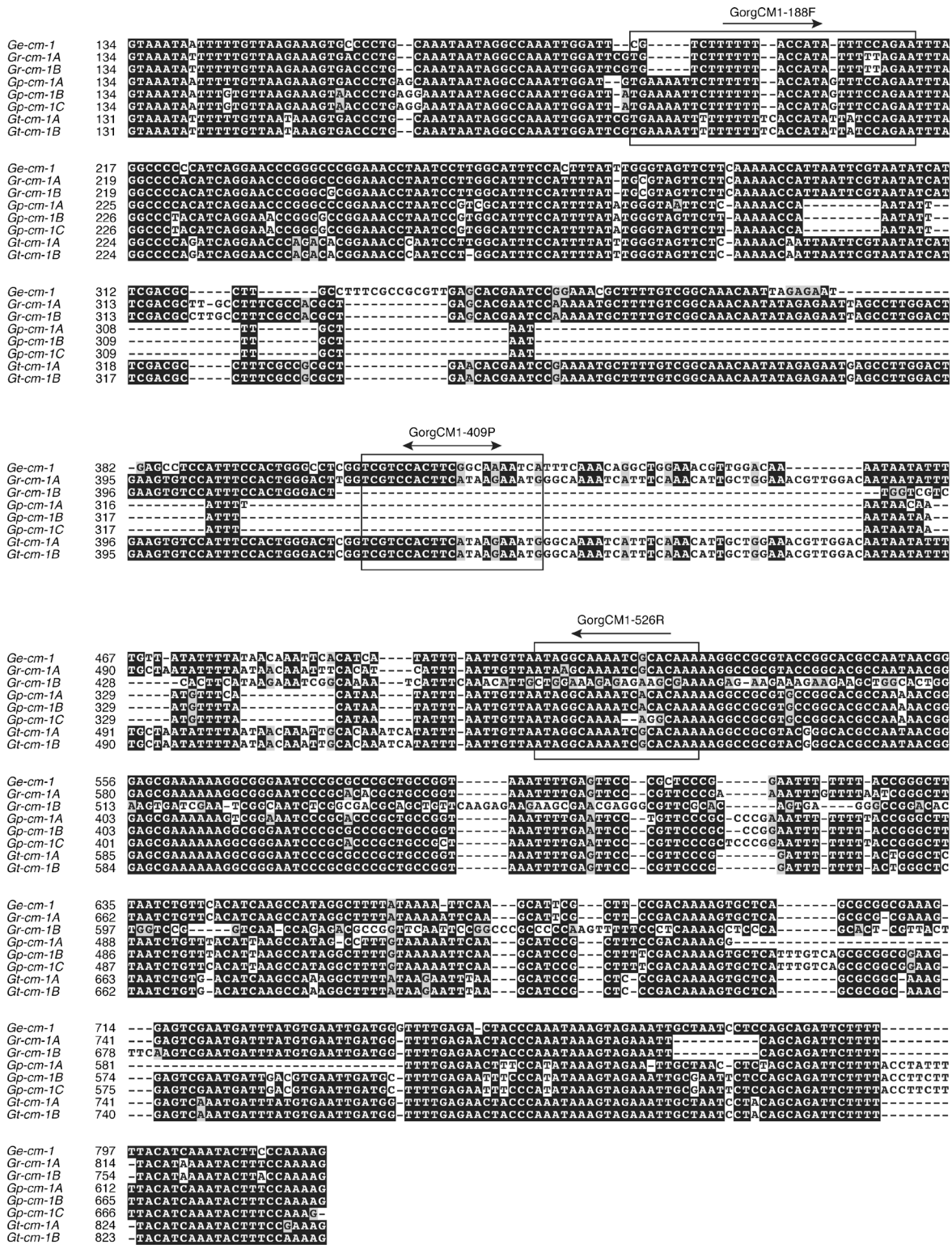

Fig. 2 Sequence alignment of intron 1 regions of chorismate mutase genes from different *Globodera* species. The alignment was made using Clustal W (Larkin et al. 2007) and BOXSHADE (Subramaniam 1998). The regions targeted for designing PCR primers and the specific TaqMan probe are indicated as shown

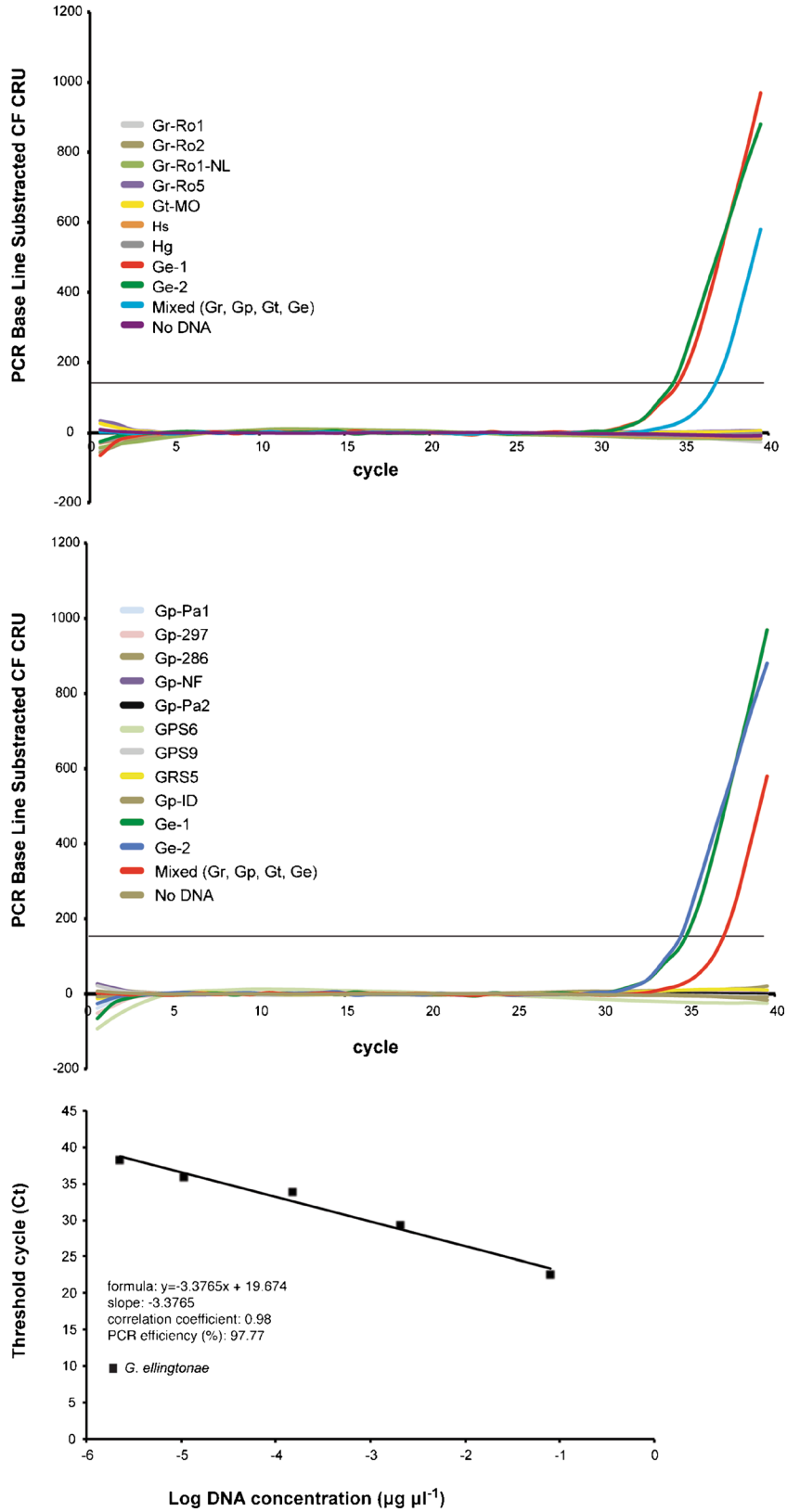

Fig. 3 Specificity and sensitivity of the developed TaqMan qPCR assay. **a** and **b** Amplification plot of the qPCR assay demonstrating specificity for the identification of *Globodera ellingtonae*. Fluorescence signals were detected only for samples containing *G. ellingtonae* genomic DNA, but not for samples containing no DNA or DNA from other *Globodera* or *Heterodera* species. Amplification curves shown in different *colours* represent each tested sample as indicated on the *left* of the graph. **c** Standard curve of threshold cycle (Ct) values plotted against the $\log_{10}$ of serially diluted *G. ellingtonae* genomic DNA. The ten-fold serial dilutions are linear over five logs. Data represent three replicates for each dilution. Similar results were obtained from two independent experiments. The detection limit of this qPCR assay for *G. ellingtonae* was calculated according to this generated standard curve

design a PCR primer and a TaqMan probe specific for *G. ellingtonae* (Table 1). The TaqMan qPCR assay was carried out in an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Yu et al. 2011). Briefly, the assay was conducted in a 96-well plate in a 25-μl reaction volume containing iQ Supermix (Bio-Rad), 500 nM of primers GorgCM1-188 F and GorgCM1-526R and 500 nM of probe GorgCM1-409P (Table 1), and 1 μl of genomic DNA. Two independent DNA samples of *G. ellingtonae* as well as DNA samples from four different populations of *G. rostochiensis*, eight different populations of *G. pallida*, one *Globodera* population from Antofagasta, Chile, and one population of *G. tabacum* that originated from diverse geographical regions (Table 3) were tested. DNA samples from *Heterodera glycines* and *H. schachtii* and PCR reactions with no DNA template were also included as negative controls. All qPCR assays consisted of three technical replicates. As expected, fluorescence signals were detected for DNA samples from *G. ellingtonae*, but no signal was detected for DNA samples from other nematode populations (Fig. 3a and b), including an Antofagasta population from Chile, which was suggested to be closely similar to *G. ellingtonae* based on ITS sequences (Handoo et al. 2012). Additionally, this TaqMan qPCR assay gave a positive detection for DNA sample from an artificially-mixed population of the four *Globodera* nematode species (Fig. 3a and b). Post-qPCR gel analysis confirmed the amplification of the expected PCR product when a fluorescence signal was detected (data not shown). To determine the sensitivity of the TaqMan qPCR assay, 10-fold serial dilutions of *G. ellingtonae* genomic DNA were prepared and used in the qPCR assay to generate a standard curve according to a published protocol (User Bulletin 2, ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Foster City, CA, USA). A linear relationship was observed between Ct values and the log concentrations of *G. ellingtonae* genomic DNA, which indicated a correlation coefficient of 0.98 and an efficiency of 97.77 % of the TaqMan qPCR assay (Fig. 3c). The lowest concentration of DNA sample analyzed that gave a florescence signal was 226 fg μl^{-1}. Therefore, the detection limit of this TaqMan qPCR assay for *G. ellingtonae* was 226 fg of DNA.

Similarly to the TaqMan qPCR assays that utilized sequence variations in the *CM* genes for differentiation of *G. rostochiensis*, *G. pallida*, and *G. tabacum* (Yu et al. 2011), this newly developed TaqMan qPCR method was confirmed to be highly specific and sensitive for detecting *G. ellingtonae* in DNA samples from individual and mixed nematode populations. The high degree of specificity and sensitivity of this TaqMan qPCR assay may allow *G. ellingtonae* detection in soil extracts that contain DNA from nematode cysts or infective second-stage juveniles. Considering that multiple *Globodera* species that infect potato are present in the U.S., the application of this method for routine detection in soil samples will help ensure accurate differentiation of *G. ellingtonae* from the two PCN species, thereby facilitating the integrity of PCN quarantine in the U.S.

Acknowledgments We would like to thank Eric Grenier for providing DNA from *Globodera* populations from South Peru, Chile and UK, Geert Smant for providing DNA from PCN populations from the Netherlands, Solke De Boer for providing DNA from a *G. pallida* population from Newfoundland, Canada, Melissa Mitchum for providing *G. tabacum*, and Eric Davis for providing *Heterodera glycines*. This study was supported by funding from USDA-ARS and USDA-APHIS.

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