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Multiplex real-time polymerase chain reaction for identifying potato cyst nematodes, *Globodera pallida* and *Globodera rostochiensis*, and the tobacco cyst nematode, *Globodera tabacum*

M. Madani, L.J. Ward, and S.H. De Boer

Abstract: Because the potato cyst nematodes (PCNs), *Globodera rostochiensis* and *Globodera pallida*, and tobacco cyst nematode (TCN), *Globodera tabacum*, may occur in the same geographic regions, their accurate identification is essential for implementing appropriate regulatory and crop loss mitigating strategies. Molecular methods for differentiating PCNs based on the internal genomic transcribed spacer (ITS) sequences have been developed but the potential of the many DNA amplification-based test formats have not yet been fully explored and most published work has not included TCN. In our study, the two PCN species and TCN could be distinguished by melting curve analysis of ITS amplicons generated in an EvaGreen-based real-time polymerase chain reaction (PCR) test. However, a multiplex real-time Taqman PCR test, which is also based on ITS sequences, was developed in this study with primers and probes modified with locked nucleic acids and proved to be superior for identification of one or more *Globodera* spp. in samples containing DNA from cysts and (or) second-stage juveniles. The test was specific for the PCN and TCN species with efficiencies of 0.91, 1.02, and 0.89 for *G. rostochiensis*, *G. pallida*, and *G. tabacum tabacum*, respectively. The multiplex, real-time PCR test was useful for distinguishing PCNs and TCN from other nematodes as well as their identification to species level in a single assay, correctly identifying each species in a DNA template from mixed populations.

Key words: potato cyst nematode, golden cyst nematode, pale cyst nematode, tobacco cyst nematode, real-time PCR, melting curve analysis, locked nucleic acid, molecular identification.

Résumé : Étant donné que les nématodes à kystes de la pomme de terre (NKP) *Globodera pallida* et *G. rostochiensis* ainsi que le nématode à kystes du tabac (NKT) *G. tabacum* peuvent être présents dans une même zone géographique, il est essentiel de pouvoir les identifier avec précision afin d'appliquer les mesures adéquates relatives aux stratégies d'atténuation en cas de pertes de cultures. Des méthodes moléculaires permettant de différencier les NKP, basées sur les séquences de l'espaceur interne transcrit (ITS), ont été développées, mais le potentiel des nombreux tests d'amplification de l'ADN n'a pas encore été complètement exploré, et la plupart des travaux qui ont été publiés sur le sujet n'ont pas encore été faits sur les NKT. Dans notre étude, les deux espèces de NKP et les NKT pouvaient être caractérisés par l'analyse de la courbe de fusion des amplicons des ITS générée par PCR en temps réel faisant appel au marqueur EvaGreen. Toutefois, une PCR multiplex en temps réel Taqman basée également sur les ITS, conçue au cours de l'étude et faisant appel à l'utilisation d'amorces et de sondes modifiées à l'aide d'acides nucléiques bloqués (LNA), s'est avérée plus efficace quant à l'identification d'une ou de plusieurs espèces de *Globodera* dans les échantillons contenant de l'ADN extrait de kystes ou de plantules ayant atteint le deuxième stade de croissance. Le test était spécifique des espèces de NKP et NKT à des taux d'efficacité de 0,91, 1,02 et 0,89 pour *G. rostochiensis*, *Globodera pallida* et *G. tabacum*, respectivement. La PCR multiplex en temps réel s'est avérée utile pour distinguer les NKP et les NKT d'autres nématodes ainsi que pour les caractériser au niveau de l'espèce en effectuant qu'un seul biotest, tout en permettant d'identifier chaque espèce à partir d'une matrice d'ADN constituée d'un mélange de populations.

Mots-clés : nématode à kystes de la pomme de terre, nématode doré, nématode blanc, nématode à kystes du tabac, PCR en temps réel, analyse de la courbe de fusion, acide nucléique bloqué, identification moléculaire.

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Introduction

There are three major groups within the nematode genus *Globodera*. They are (i) *Globodera rostochiensis* (Wollenweber) Behrens and *Globodera pallida* (Stone) Behrens, which are commonly referred to as potato cyst nematodes (PCNs), and are pathogenic to Solanaceae; (ii) *Globodera tabacum* (Lownsbery & Lownsbery) Behrens (tobacco cyst nematodes, TCN), which are a complex of three subspecies (*Globodera tabacum tabacum* (Lownsbery & Lownsbery) Behrens, *Globodera tabacum virginiae* (Miller & Gray) Behrens, and *Globodera tabacum solanacearum* (Miller & Gray) Behrens); and (iii) the composite cyst nematodes, which are economically less important and are named after the host plant on which they are found. The latter group also contains undescribed species and species that are limited to a restricted area. The composite group of cyst nematodes includes such species as *Globodera achilleae* (Golden & Klindic) Behrens, *Globodera artemisiae* (Eroshenko & Kazachenko) Behrens, *Globodera millefolii* (Kirjanova & Krall) Behrens, *Globodera hypolysi* Ogawa, Ohshima & Ichinohe, *Globodera chaubattia* (Gupta & Edward) Wouts, *Globodera mirabilis* (Kirjanova) Mulvery & Stone, *Globodera zelandica* Wouts, and *Globodera leptonepia* Cobb & Taylor) Behrens.

Economically, the most important members of *Globodera* are the PCN species *G. rostochiensis* and *G. pallida*, which are found in many potato growing regions. Potato (*Solanum tuberusom* L.) is the major host plant for these two species, and serious damage has been reported in both cool and temperate geographic regions (Marks and Brodie 1998). Other Solanaceous plants, such as tomato (*Lycopersicum esculentum* Mill.) and eggplant (*Solanum melongena* L.), as well as some weeds, have also been identified as host plants for PCNs (Sullivan et al. 2007). Occurrence of PCNs and serious damage on potato has been reported in almost all European countries (Bates et al. 2002; Fleming et al. 1998; Manduric and Andersson 2003; Manduric et al. 2004; Minnis et al. 2002). PCN has also been reported in Australia, New Zealand, Africa, the Middle East, Japan, and in North and Central America (Bulman and Marshall 1997; da Cunha et al. 2004; Ibrahim et al. 2001; Marks and Brodie 1998; Uehara et al. 2005). In Central America, infested areas have been reported in Mexico, Panama, and Costa Rica and in the United States in Long Island, western New York, and, most recently, in Idaho (Handoo et al. 2007; Marks and Brodie 1998). In Canada, one or both PCN species have been reported in restricted areas. Both *G. rostochiensis* and *G. pallida* occur in Newfoundland (Olsen and Mulvey 1962; Stone et al. 1977), but only *G. rostochiensis* occurs on Vancouver Island (Rott et al. 2008) and has recently been reported in the Saint-Amable area of Quebec (Sun et al. 2007) and in Alberta (A. Boucher, personal communication).

TCN is also of economic concern because of the damage it inflicts on commercial tobacco (*Nicotiana tabacum* L.) production, including shade-grown or broadleaf and flue-cured tobacco cultivars (Syracuse et al. 2004). Moreover, TCN exerts a synergistic effect on wilt disease of broadleaf tobacco caused by *Fusarium oxysporum* Schltdl. (LaMondia 1992). The presence of TCN has been reported from France, Italy, Spain, China, Pakistan, Morocco, Argentina, the United States (LaMondia 1992; Syracuse et al. 2004; Wang et al. 1999), and from the province of Quebec, Canada (Belair and Miller 2006).

Identification of the genus *Globodera* is traditionally based on seven important characteristics that include morphology of the vulval cone top in the perineal region of female nematodes (number of ridges and Granek's ratio), and morphometric characteristics (length of body, tail, and stylet and shape of stylet and knobs) of second-stage juveniles (J2; Manduric et al. 2004; Skantar et al. 2007). However, in both cyst and J2 populations, there is a high percentage of variation in morphometric characters and large overlap in morphological features among species that makes unequivocal identification difficult (Manduric et al. 2004; Subbotin et al. 2000). It is also impossible to distinguish the three subspecies of *G. tabacum* based on morphology alone (Mota and Eisenback 1993). Laboratory-based methods such as enzyme-linked immunosorbent assays, protein analysis by isoelectric focusing, and techniques based on the polymerase chain reaction (PCR) have been developed for routine identification, but results have occasionally been equivocal (Ibrahim et al. 2001).

However, the full potential of PCR-based strategies for discriminating *Globodera* spp. identities has yet to be explored. Thiery and Mugniery (1996) initially described a method using restriction fragment length polymorphism (RFLP) in ribosomal DNA (rDNA) of *Globodera* spp., and subsequently, a conventional PCR assay was developed to distinguish between the PCN species (Bulman and Marshall 1997). Recently, Skantar et al. (2007) also used conventional PCR to confirm the identity of *G. pallida* in Idaho. Conventional PCR methods require postamplification gel electrophoresis of DNA amplicons to visualize results, making them more time-consuming and laborious than newer techniques such as real-time PCR. Real-time PCR methods have the particular advantage of providing results immediately with neither the need for further postamplification steps nor the need for opening tubes containing amplified product and, thereby, decreasing the risk of cross-contamination. In the presence of double-strand binding dyes such as SYBRGreen or EvaGreen, it is also possible to characterize amplicons by analysis of melting curves. This strategy was successfully applied to differentiating *G. rostochiensis* and *G. pallida* in an Australian study published while this manuscript was in preparation (Quader et al. 2008). Real-time TaqMan PCR assays, with added specificity provided by a hybridization probe, have been described for a wide range of plant pathogens, including plant parasitic nematodes such as *Meloidogyne* spp. (Zijlstra 1997) and *Bursaphylenchus* spp. (Leal et al. 2007). Recently, real-time PCR methods were developed for quantification of *G. pallida*, *Heterodera schachtii* Schmidt, and *Ditylenchus dipsaci* (Kühn) Filipjev (Madani et al. 2005; Subbotin et al. 2005), as well as for *G. rostochiensis* and *Meloidogyne incognita* (Kofoid & White) Chitwood, but these studies did not determine whether the tests differentiated among related species (Toyota et al. 2008). Because three species of *Globodera* occur in some geographic regions including Canada and the United States, a multiplex real-time PCR method to identify *Globodera* to the species level in a single assay would be useful.

Table 1. Nematode populations examined in this study and the tests used to identify the nematodes in samples.

Source[a]	Location	No. of subsamples[b]	Species[c]	Genbank accession No.	Tests[d]
British Columbia	Saanich	1	*G. rostochiensis*	FJ212167	PCR, ITS sequence, TaqMan probe
Quebec	Field 1	9	*G. rostochiensis*	FJ212162	PCR, ITS sequence, TaqMan probe
Quebec	Field 2	1	*G. rostochiensis*	FJ212166	PCR, ITS sequence, TaqMan probe
Newfoundland	Avondale	3	*G. rostochiensis*	FJ212164	PCR, ITS sequence, TaqMan probe
			G. pallida	FJ212165	PCR, ITS sequence, TaqMan probe
Newfoundland	Long Pond	4	*G. rostochiensis*	FJ212163	PCR, ITS sequence, TaqMan probe
United States	New York	1*	*G. rostochiensis*		TaqMan probe
United States	Idaho	1*	*G. pallida*		TaqMan probe
United States	Connecticut	1	*G. tabacum tabacum*		ITS sequence, TaqMan probe
France	Ecosse	1	*G. rostochiensis*		PCR, ITS sequence, TaqMan probe
France	Chavornay	1	*G. pallida*		PCR, ITS sequence, TaqMan probe
France	Agen	1	*G. tabacum tabacuum*		ITS sequence, TaqMan probe
CL collection	United States	1	*D. dipsaci*		ITS RFLP
CL collection	United Kingdom	1	*H. schachtii*		ITS RFLP

[a]CL collection, Canadian Food Inspection Agency Charlottetown Laboratory nematode collection.
[b]Samples with asterisks consisted of only DNA; other samples consisted of cysts except vermiform *D. dipsaci*.
[c]Genera for species are *Globodera*, *Ditylenchus*, and *Heterodera*.
[d]PCR, polymerase chain reaction using the method of Bulman and Marshall (1997); ITS, internal transcribed spacer sequence giving 100% similarity using the nucleotide–nucleotide basic local alignment search tool (BLASTN) from the NCBI database; TaqMan probe, hybridization with the species-specific probe developed in this study; and ITS RFLP, ITS restriction fragment length polymorphism.

However, traditional PCR targets, such as the internal transcribed spacer (ITS) regions, of closely related species often show a high degree of homology that presents potential challenges for the design of specific probes that are practical for multiplex applications. Amplified mismatch discrimination of one to three bases is not always attainable through changes in reaction conditions alone and may require the use of modified primers and (or) probes. One such modification that is particularly suitable for improved mismatch discrimination incorporates an altered nucleotide containing a 2′–4′ *O*-methyl covalent link in the ribose ring, which locally both modifies and "locks" the conformation of the DNA, increasing hybridization affinity and efficiency. Locked nucleic acids (LNA) containing modifications at unique and adjacent positions have been shown to discriminate single- nucleotide polymorphisms and are suitable for the design of probes that target highly homologous regions of closely related species (You et al. 2006).

The objective of the present study was to evaluate PCR strategies for differentiating the three *Globodera* species that occur in Canada (*G. pallida*, *G. rostochiensis*, and *G. tabacum*) in a single assay. We explored PCR-based tests with amplicon melting temperature analysis and TaqMan technology utilizing LNA-modified primers and probes. To our knowledge, no assays have previously been published for simultaneous discrimination among the three *Globodera* spp., viz., the two PCN species and TCN.

Materials and methods

Cyst nematodes

Samples of PCN populations were acquired from two locations in Newfoundland, from one location in the Saanich Peninsula of Vancouver Island in British Columbia, and from two potato fields in Quebec recently found to be infested with *G. rostochiensis* (Sun et al. 2007) (Table 1). DNA from PCN populations in New York and Idaho were kindly provided by Xiaohong Wang (Cornell University, Ithaca, N.Y.). Populations of TCNs were not available from Canadian sources, but samples of *G. tabacum tabacum* were provided by J.A. LaMondia (Connecticut Agricultural Experiment Station, Windsor, Conn.). Samples of TCN and both PCN species were also received from D. Mugniery, Institut national de la recherche agronomique Rennes, France. For controls, samples of *D. dipsaci*, a vermiform nematode morphologically similar to second-stage *Globodera* juveniles, and *H. schachtii*, another cyst-forming nematode species, were obtained from the nematode collection held at the Canadian Food Inspection Agency Charlottetown Laboratory. Identity of previously identified PCN cyst populations were confirmed by PCR using primers PITSp3, PITSp4, and ITS 5 as described by Bulman and Marshall (1997) (Table 2). Additionally, ITS regions of Canadian PCN populations, the PCN and TCN samples from France, and TCN from the United States were sequenced and compared with Genbank sequences of the same species using the nucleotide–nucleotide basic local alignment search tool (BLASTN; Skantar et al. 2007; Subbotin et al. 2000; Szalanski et al. 1997; Uehara et al. 2005). *Heterodera schachtii* and *D. dipsaci* identity were confirmed by RFLP analysis of the ITS genes (Szalanski et al. 1997; Wendt et al. 1993).

DNA template preparation

Genomic DNA was obtained from nematode cysts and J2 populations by a method involving lyophilization and microlysis. Single cysts or J2 nematodes were first lyophilized in Eppendorf tubes containing 20–60 μL of distilled water with the following thermal profile: 30 min at –40 °C followed by 10 min intervals at each of –30, –20, –10, and 5 °C, ramping at 0.1 °C/min between each step. Lyophilized samples were ground directly in the tubes with hand-drawn glass pestles and then treated with Microlysis Plus buffer (The Gel Co., San Francisco, Calif.). A volume of 5 μL of

Table 2. Sequences of primers and probes used in this study.

Code[a]	Sequence (5′–3′)[b]	Reference
PITSp3*	AGC GCA GAC ATG CCG CAA	Bulman and Marshall 1997
PITSp4*	ACA ACA GCA ATC GTC GAG	Bulman and Marshall 1997
ITS5	GGA AGT AAA AGT CGT AAC AAG G	White et al. 1990
rDNA1	TTG ATT ACT TCC CTG CCC TTT	Vrain et al. 1992
rDNA2	TTT CAC TCG GCC GTT ACT AAG G	Vrain et al. 1992
Gfor	GTG TAA CCG ATG TTG GTG GCC	This study
Grev*	GGA CGT AGC ACA CAA GCG CA	This study
PITS-pall*	AGC GCA GAC ATG CCG **CTG**	Modified PITSp3, this study
Grost*	Cy5-GCT TCC TCC **GTT** **G**GC G-IBRQ	This study
Gpall*	TEX613-ATC GT**C** **GAG** **T**CA CCC ATT-IBRQ	This study
Gtab*	FAM-AT**A** **T**GC CGC **GG**G GTA CG-IBFQ	This study
EvaF	TCG TTG AGC GGT TGT TGC GCC TT	This study
EvaR*	ACG GCC ACG GAC GTA GCA CAC A	This study

[a]Sequences with asterisks are in antisense orientation.
[b]Boldface and underlined bases are locked nucleotides. Cy5, TEX613, and FAM are fluorescent molecules, and IBRQ and IBFQ are quencher signal molecules.

microlysis 5× buffer and 20 μL of water was added to each tube and incubated for 35 min with the following temperature treatment: 67 °C for 8 min, 96 °C for 3 min, 65 °C for 4 min, 96 °C for 2 min, 67 °C for 1 min, and 96 °C for 1 min. After a brief centrifugation to collect all the liquid, preparations were suitable for PCR amplification.

Amplification, cloning, and sequencing of ribosomal DNA

An approximately 1.2 kb fragment of ribosomal DNA including the ITS1, 5.8S gene, and ITS2 regions (Fig. 1) was amplified from each *Globodera* spp. with ITS universal primers rDNA1 and rDNA2 (Table 2), as described by Vrain et al. (1992), using Sprint Advantage Single Shot PCR Premix (Clontech, Mountain View, Calif.). Primer concentrations were 0.5 μmol/L and 2 μL of template DNA was used in final 25 μL reaction volumes. The PCR thermal cycling conditions were one cycle at 95 °C for 3 min; followed by 35 cycles at 94 °C, and 55 °C each for 1 min, and 68 °C for 2 min; and a final cycle at 68 °C for 5 min. Amplified product was separated by gel electrophoresis, excised, purified from the gel with a gel purification kit (MoBio Laboratories Inc., Carlsbad, Calif.), and cloned into the pUCm-T vector (UBI, Calgary, Alta.). Plasmids were purified with an UltraClean Standard Mini Plasmid Prep Kit (Mo-Bio), and inserts were sequenced using universal vector sequencing primers (M13F and M13R) by the Core Molecular Biology Facility of York University (Toronto, Ont.). Both forward and reverse sequences were obtained from several clones representing each *Globodera* spp.

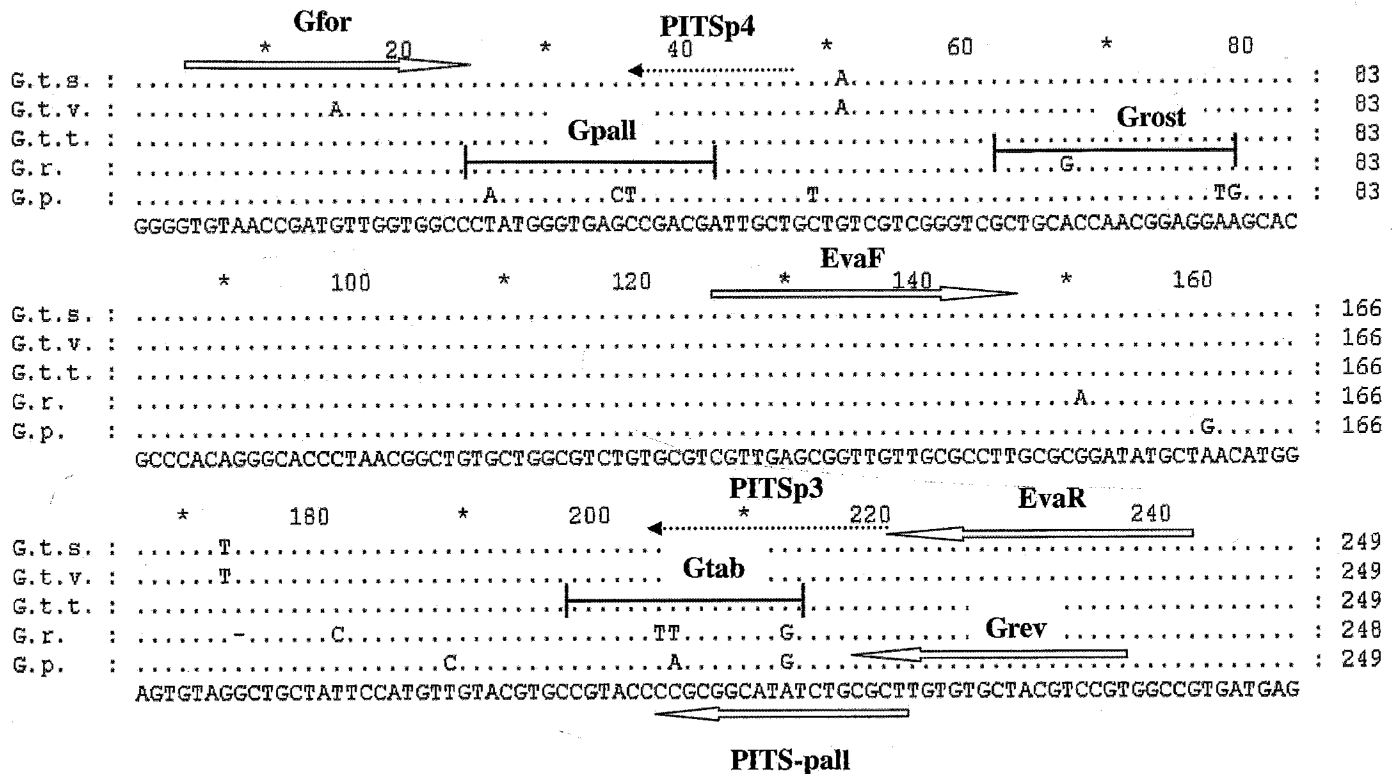
Real-time PCR

The computer software programs Chromas Lite version 2.01 (Technelysium Pty Ltd, Tewantin, Australia; http://www.technelysium.com.au/chromas_lite.html) and GeneDoc version 2.7 (Nicholas and Nicholas 1997) were used to visualize sequence chromatograms and edit DNA sequences, respectively. AllelelD (Premier Biosoft International, Palo Alto, Calif.) software was then used for alignment of new sequence data generated in this study with sequences of *Globodera* spp. in NCBI GenBank and for designing primers and probes. Primer and probe sequences were evaluated using Clone Manager (Scientific & Educational Software, Cary, N.C.) and AlleleID software on the basis of the following criteria: predicted hybridization to closely related species, potential for primer-dimer formation, length of amplicons, guanine–cytosine content, and melting temperature (T_m).

The primer set EvaF and EvaR (Table 2) was designed for use in real-time PCR using EvaGreen chemistry to amplify a 131 bp fragment from the ITS region of *G. pallida*, *G. rostochiensis*, and *G. tabacum*. Real-time PCR with these primers was carried out in a Rotor-Gene 3000 real-time thermocycler (Corbett Robotics Inc. San Francisco, Calif.). PCR reactions (final volume 25 μL) contained 0.5 μmol/L of each primer, 2 μL of template DNA, and 12.5 μL of EvaGreen (20× stock) (Biotium, Hayward, Calif.) in Sprint Advantage Single Shot PCR Premix (Clontech, Mountain View, Calif.). The reaction profile consisted of an initial denaturation–activation step for 3 min at 95 °C followed by 35 cycles of amplification using a two-step thermal profile of 96 °C for 20 s and 68 °C for 30 s and a final extension at 68 °C for 3 min. Fluorescence in the 6-carboxyfluorescein aminohexyl amidite (FAM) channel was monitored during the extension–annealing step of the amplification phase. A postamplification disassociation curve was generated for amplicons in the EvaGreen-based real-time PCR assay. DNA melting curve analysis was performed by cooling the samples at 2.5 °C/s to 60 °C and then increasing the temperature 0.2 °C/5 s to 98 °C while monitoring fluorescence in the FAM channel. The rate of change in fluorescence for each temperature was used to determine the melting point of each amplicon.

A second set of primers, Gfor and Grev (Table 2), located approximately 200 bp downstream of the 5′end of the 28S rDNA gene, was designed for use in a TaqMan PCR assay for the three *Globodera* spp. Subsequently, a third primer, PITS-pall, was designed as a modification of primer PITSp3 to ensure adequate amplification of *G. pallida* sequences in multiplex PCR. Separate TaqMan probes were designed to hybridize specifically and differentially to amplified sequences of *G. pallida*, *G. rostochiensis*, and *G. tabacum* and are

Fig. 1. Nucleotide sequence alignment of a segment of the ITS region between 18S and 5.8S genes from *Globodera pallida* (G.p.), *G. rostochiensis* (G.r.), *G. tabacum solanacearum* (G.t.s.), *G. tabacum virginiae* (G.t.v.), and *G. tabacum tabacum* (G.t.t.). Open and dotted arrows show the locations and orientations of new and published primers, respectively; solid lines depict the position of TaqMan probes. Gfor and Grev are forward and reverse primers used in the TaqMan assay (232 bp); EvaF and EvaR are forward and reverse primers used in the real-time assay with EvaGreen dye (126 bp). PITSp4 and PITSp3 are specific primers used by Bulman and Marshall (1997). PITS-pall is a modification of primer PITSp3 that was designed in this study. Gpall, Grost, and Gtab are probes for *G. pallida*, *G. rostochiensis*, and *G. tabacum*, respectively. The 5′ end of the Gfor sequence is 253 bp from 18S gene, and the 3′ end of the Grev sequence is 258 bp from 5.8S gene.



identified as Gpall, Grost, and Gtab, respectively (Table 2). To achieve a high level of specificity and probe fidelity in a genomic region with few base polymorphisms, locked nucleotides were incorporated into each of the probes (Table 2). All probes were 3′end-labeled with FQ or RQ Iowa Black quenchers and labelled with different fluorescent tags at the 5′ ends. Texas Red 613, Cy5, and FAM were used as labels for the Gpall, Grost, and Gtab probes, respectively, to distinguish amplification of DNA from the three *Globodera* spp. on separate channels of the real-time PCR thermocycler. All primers and probes used in this study were ordered from Integrated DNA Technologies (Coralville, Iowa).

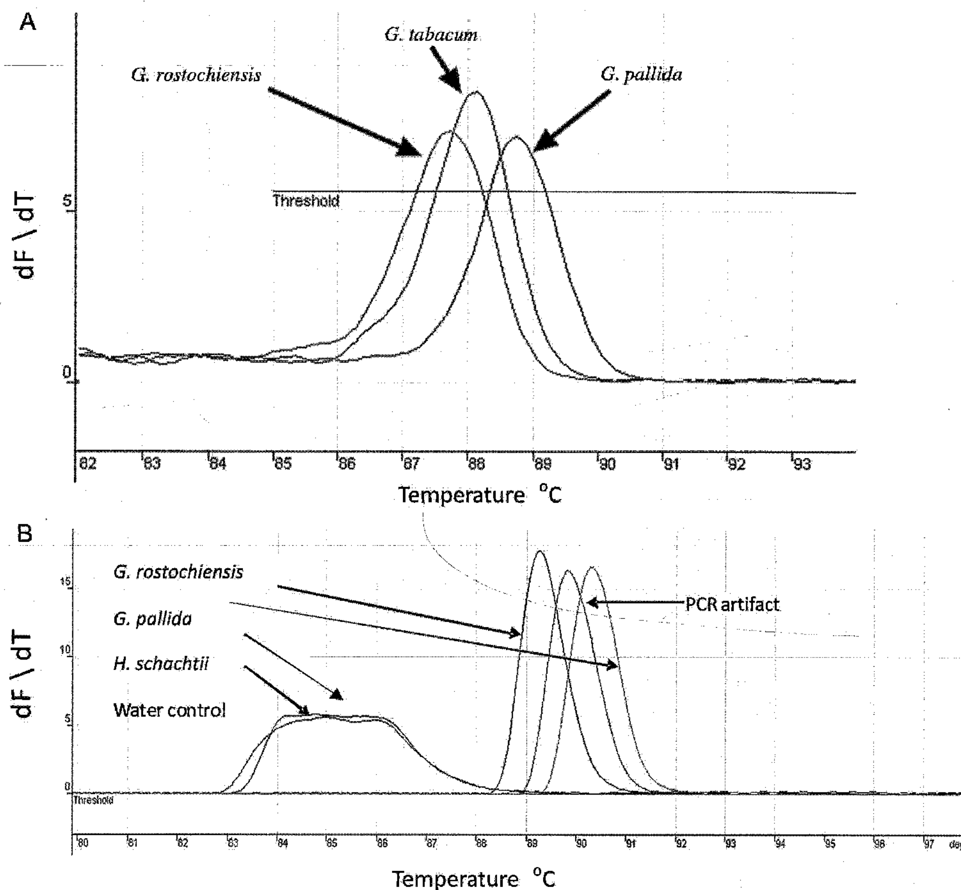
The real-time TaqMan PCR assay was performed using the Gfor, Grev, and LNA-modified PITS-pall primers in combination with the three LNA-modified probes: Gpall, Gros, and Gtab. Amplification reactions were performed in 25 μL volumes by using the Quantitect multiplex PCR kit (Qiagen Inc. Mississauga, Ont.) containing dNTPs and hotstart *Taq* polymerase in a Smartcycler 2 (Cepheid Inc. Sunnyvale, Calif.) real-time PCR thermocycler. Optimal reaction conditions used 0.5 μmol/L of each primer, 135 nmol/L of the Gpall and Gtab probes, 110 nmol/L of the Grost probe, and 2 μL of template DNA. The final optimized thermal cycling profile included an initial activation of *Taq* polymerase at 95 °C for 15 min followed by 35–45 cycles of 20 s at 96 °C and 50 s of extension at 72 °C. Fluorescence was measured at the end of each extension step. The cycle threshold (C_t) of each amplification reaction was reported based on the first PCR cycle at which fluorescence exceeded a manual threshold of 10. To verify that only one PCR product specific to each species was amplified, the size and presence of amplicons were analyzed by gel electrophoresis using Novex 4%–12% precast tris–borate–EDTA (TBE) polyacrylamide gels (Invitrogen, Canada Inc., Burlington, Ont.) in 1× TBE buffer and stained with ethidium bromide.

Efficiency and specificity of the PCR assay

Efficiency of the multiplex real-time TaqMan PCR assay was determined by testing a two-fold dilution series of template DNA for each of the three *Globodera* spp. in real-time TaqMan PCR as described above. The $\log_{10}$ of DNA concentrations of each dilution, based on absorbance readings at 240 nm, were plotted against the C_t values. A regression line was fitted, and the goodness of fit was determined by calculating the regression coefficient (R^2). The slope of the regression line was used in the following equation to calculate reaction efficiency (E): $E = 10^{-1/\text{slope}} - 1$.

A specificity of the primers and probes used in the assay was tested against DNA of the Canadian PCN populations, TCN, *H. schachtii* cysts, and vermiform *D. dipsaci* nema-

Fig. 2. Melting curve profiles of amplicons generated using PCR with primers EvaF and EvaR: (A) DNA templates derived from *Globodera rostochiensis*, *G. pallida*, and *G. tabacum tabacum* separately and (B) a mixed DNA template of *G. rostochiensis*, *G. pallida*, and *Heterodera schachtii*. Melting profile identifications are shown.



todes. Negative controls of nuclease-free water instead of DNA template were included in each PCR run.

To evaluate species discrimination in multiplex PCR, DNA templates were prepared by mixing equal volumes (1.25:1) of DNA from individual cysts and J2 nematodes from *G. rostochiensis*, *G. pallida*, and *G. tabacum* populations. PCR reactions were run for 35 cycles, and the C_t values of fluorescence signals were recorded in each of the three thermocycler channels for each PCR run.

Results

Identification of nematodes and sequence alignments

The identity of the Quebec PCN populations was confirmed as *G. rostochiensis* by PCR using the primers designed by Bulman and Marshall (1997), and both *G. rostochiensis* and *G. pallida* were identified in the cyst populations from the two Newfoundland locations. Amplification of the ITS regions by conventional PCR using the general ITS primers rDNA1 and rDNA2 produced distinct fragments of about 1200 bp from all the PCN populations utilized in this study. The ITS regions of the samples we sequenced (Table 1) aligned with the sequences of homologous *G. rostochiensis* and *G. pallida* in the Genbank database In the region of interest, our *G. rostochiensis* sequences were 98%–99% identical to 33 *G. rostochiensis* sequence accessions in Genbank (eg. EF153839, DQ847120, and AB207271). Similarly, *G. pallida* was 97%–99% identical to 17 Genbank accessions (eg. EF153837 and DQ847109)

The identity of the *G. tabacum* sample obtained from the United States was also confirmed by sequencing the ITS region and by comparison with Genbank sequences using BLASTN. In the region of interest, it was 100% identical to three Genbank accessions of *G. tabacum tabacum* (EF153842, DQ847116, and AB207272) and differed from two *G. tabacum solanacearum* accessions (DQ847114 and EF153841) and two *G. tabacum virginiae* accessions (DQ847113 and DQ84712) in Genbank by two or three nucleotide polymorphisms.

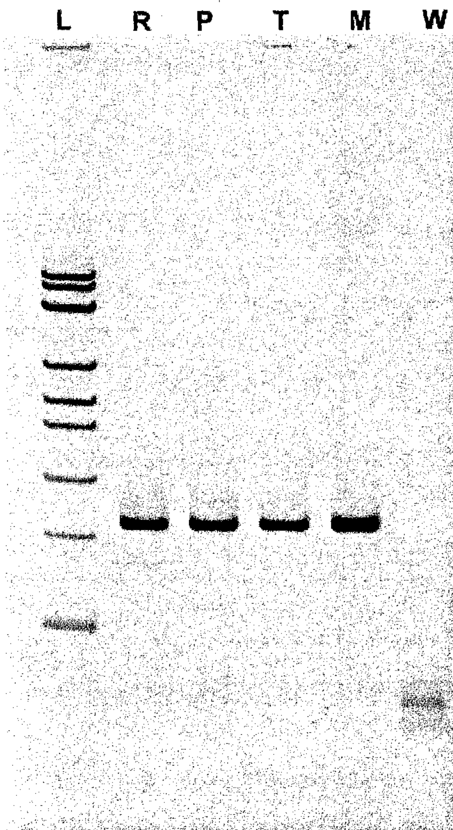
Alignment of the majority consensus sequences of the ITS region from the three *Globodera* species is shown in Fig. 1. Oligonucleotides selected to serve as PCR primers and probes in this study were located within the ITS regions in which no sequence polymorphisms occurred within a species but in which polymorphisms did occur among species. Location of primers and probes within the ITS region are shown in Fig. 1, and actual positive or negative strand sequences are given in Table 2.

The RFLP patterns of the ITS genes for *H. schachtii* and *D. dipsaci* conformed to published patterns (data not shown).

Real-time EvaGreen-based PCR

Primer pair EvaF–EvaR were designed specifically for use in a real-time PCR using the EvaGreen chemistry. In real-

Fig. 3. Electrophoretogram of an ethidium bromide stained 4%–12% precast TBE polyacrylamide gel of PCR products after amplification with primers Gfor, Grev, and PITS-pall for 45 cycles. Lanes R, P, T, and M were loaded with products generated by PCR using template DNA from *Globodera rostochiensis*, *G. pallida*, *G. tabacum tabacum*, and a mixture of all three, respectively. Lanes L and W show the 100 bp DNA ladder and water controls, respectively.



time PCR with EvaGreen, these primers directed the amplification of a 131 bp amplicon from the ITS1 region of *G. pallida*, *G. rostochiensis*, and *G. tabacum*. The amplicons of the three species could be differentiated on the basis of melting curve analysis subsequent to the amplification reaction. The melting temperature peaks were 88.72 ± 0.10 °C (n = 3), 87.77 ± 0.02 °C (n = 3), and 88.09 ± 0.09 °C (n = 5) for the *G. pallida*, *G. rostochiensis*, and *G. tabacum tabacum*, respectively (Fig. 2A). However, when DNA from two or more *Globodera* spp. were mixed, an intermediate melting peak appeared, possibly because of hybrid molecules, and prevented unequivocal species identification (Fig. 2B). Distinct melting curves were not obtained when *D. dipsaci* or *H. schachtii* DNA were used as template for amplification.

Real-time TaqMan PCR

A real-time TaqMan assay was designed as a triplex test in which amplification of each *Globodera* spp. was independently monitored on different channels of the thermocycler. Primer pair Gfor–Grev directed amplification of 232 bp amplicons with template DNA from *G. pallida*, *G. rostochiensis*, and *G. tabacum tabacum*. However, in mixed templates, addition of primer PITS-pall was required to obtain equivalent amplification from the *G. pallida* template (Fig. 3). In the real-time PCR format with the three LNA-modified, labelled TaqMan probes and separate or mixed template DNA from the three *Globodera* spp., fluorescence was detected and recorded in each of the three channels of the SmartCycler thermocycler (Fig. 4). Signals in the Texas Red, Cy5, and FAM channels corresponded consistently with template DNA from *G. pallida*, *G. rostochiensis*, and *G. tabacum tabacum*, respectively, when run separately (Table 3). DNA obtained from PCN occurrences in New

Fig. 4. Fluorescence traces obtained in the three real-time thermocycler channels (FAM, Texas Red 613, and Cy5) which detected amplified DNA from *Globodera tabacum tabacum*, *G. pallida*, and *G. rostochiensis*, respectively, by species-specific hybridization of labelled TaqMan probes in multiplex PCR. PCR template for each sample contained DNA from *Globodera* cysts and second-stage juvenile (J2) worms as follows: (A) *G. rostochiensis* (J2), *G. pallida* (J2), and *G. tabacum tabacum* (J2); (B) *G. tabacum tabacum* (cyst) and *G. rostochiensis* (J2); (C) *G. tabacum tabacum* (cyst) and *G. pallida* (J2); (D) *G. rostochiensis* (J2) and *G. pallida* (cyst); (E) *G. pallida* (J2); (F) *G. pallida* (J2) and *G. rostochiensis* (cyst); and (G) *G. rostochiensis* (cyst).

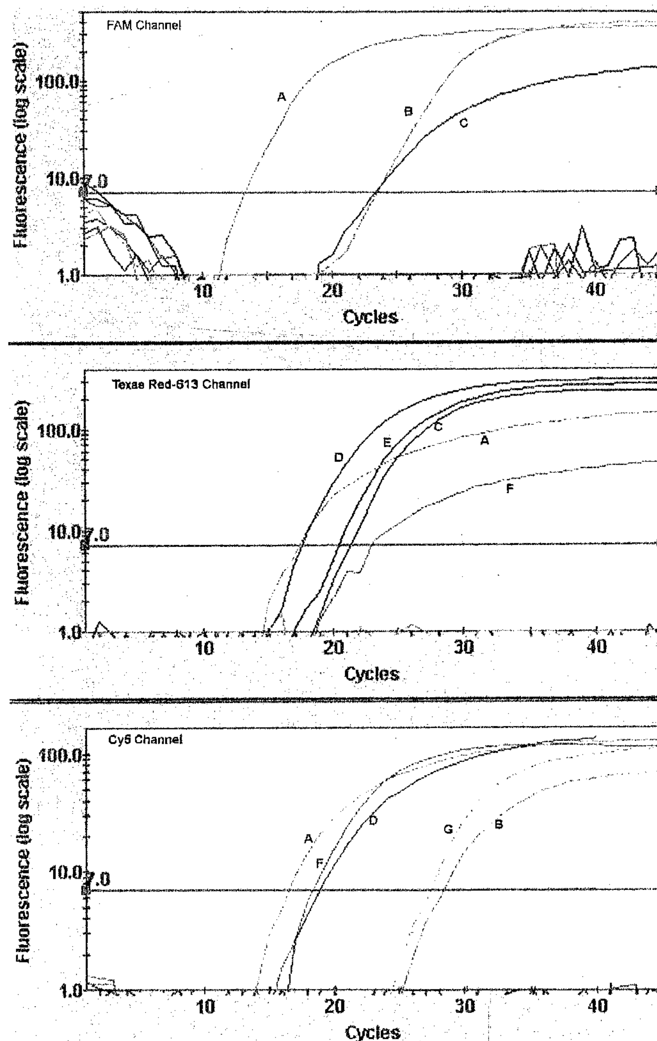


Table 3. *Globodera* spp. identity using the Bulman and Marshall (1997) method and confirmation by real-time TaqMan PCR assay.

	Bulman and Marshall method[b]		TaqMan probe[c]		
Sample identification No.[a]	*G. rostochiensis*	*G. pallida*	Texas Red	Cy5	FAM
BC Saanich	+	–	–	+	–
Quebec F1	+	–	–	+	–
Quebec F2	+	–	–	+	–
NL AD 1	–	+	+	–	–
NL AD 2	–	+	+	–	–
NL AD 3	+	–	–	+	–
NL LP 1	+	–	–	+	–
NL LP 2	+	–	–	+	–
NL LP 3	+	–	–	+	–
NL LP 4	+	–	–	+	–
US-G.t.t.	–	–	–	–	+
Fr-G.r.	+	–	–	+	–
Fr-G.p.	–	+	+	–	–
Fr-G.t.t.	–	–	–	–	+
Dd	–	–	–	–	–
Hs	–	–	–	–	–

[a]BC Saanich, Saanich, British Columbia, sample; Quebec F1 and Quebec F2, Quebec field sample 1 and field sample 2, respectively; NL AD 1, NL AD 2, and NL AD 3, Avondale, Newfoundland, samples; NL LP 1, NL LP 2, NL LP 3, and NL LP 4, Long Pond, Newfoundland samples; US-G.t.t., US sample of *Globodera tabacum tabacum*; Fr-G.r., Fr-G.p., and Fr-G.t.t., French samples of *G. rostochiensis*, *G. pallida*, and *G. tabacum tabacum*, respectively; and Dd and Hs, control samples of *Ditylenchus dipsaci* and *Heterodera schachtii*, respectively.

[b]PCR method of Bulman and Marshall (1997).

[c]Real-time PCR method developed in this study. Detection of fluorescence signals in each channel corresponds to fluorescence of species-specific TaqMan probes.

York and Idaho were identified as being *G. rostochiensis* and *G. pallida*, respectively, which were consistent with known PCN infections in the respective states (data not shown).

Analysis of melting curves of amplicons generated with the inclusion of EvaGreen in the PCR mix did not adequately discriminate among amplicons generated with these primers from DNA templates of the three *Globodera* spp. The melting peak of the *G. pallida* amplicon was 89.5 °C, whereas the amplicons for *G. rostochiensis* and *G. tabacum tabacum* each generated two very similar melting curves with major peaks at 87.5 °C and 87.9 °C and minor peaks at 89.9 °C and 89.6 °C, respectively. As in the real-time PCR with primer pair EvaF–EvaR, no amplification was detected in the TaqMan PCR with DNA templates from *H. schachtii* cysts and vermiform *D. dipsaci* nematodes.

In the real-time TaqMan PCR assays, a direct relationship was observed between the amount of template DNA and C_t values (Fig. 5). Correlation coefficients were 0.9989, 0.9898, and 0.9816 for amplification of DNA from *G. rostochiensis*, *G. pallida*, and *G. tabacum tabacum*, respectively. The efficiencies of the real-time TaqMan PCR assays were 0.91, 1.02, and 0.89 for *G. rostochiensis*, *G. pallida*, and *G. tabacum tabacum*, respectively.

The utility of multiplex PCR for discriminating species was confirmed by assays using mixed cyst and J2 DNA from the three *Globodera* spp. (Table 4, Fig. 4). All fluorescence signals corresponded precisely with presence of cyst or J2 DNA in the template mix as expected. C_t values for cyst DNA were consistently lower than for J2 DNA because DNA prepared from single cysts contained higher genomic copy numbers than DNA prepared from single J2 nematodes.

Discussion

Although populations of *Globodera* spp. are distinguishable on the basis of morphological characteristics and morphometric measurements, characteristics of individual nematodes and cysts from different species may overlap considerably (Manduric et al. 2004). However, the use of multiple characters allows for reproducible designation of species identifications. Among the various technologies that have been evaluated for improving and validating the discrimination among species, those that are based on amplification of genomic DNA sequences have proven to be the most sensitive and robust (Ibrahim et al. 2001). Species identification based on the PCR primers developed by Bulman and Marshall (1997) has been found to be particularly useful by several investigators (Bates et al. 2002; Ibrahim et al. 2001; Minnis et al. 2002; Skantar et al. 2007) and also confirmed the identity of *G. rostochiensis* and *G. pallida* in our study (Table 1). Modifications of these primers were made recently to amplify *G. tabacum* DNA (Skantar et al. 2007).

In our work, we wished to develop a PCR strategy in which individual *Globodera* juveniles or cysts could be identified to species by a single amplification reaction. In such an assay, the same primers would need to amplify DNA from the three species relevant to our work, and the amplicons would need to be distinguishable. In addition to gel electrophoretic mobility, RFLP analysis, and sequencing, amplicons can be distinguished on the basis of differences in melting curve or by differential hybridization to labelled probe. These latter

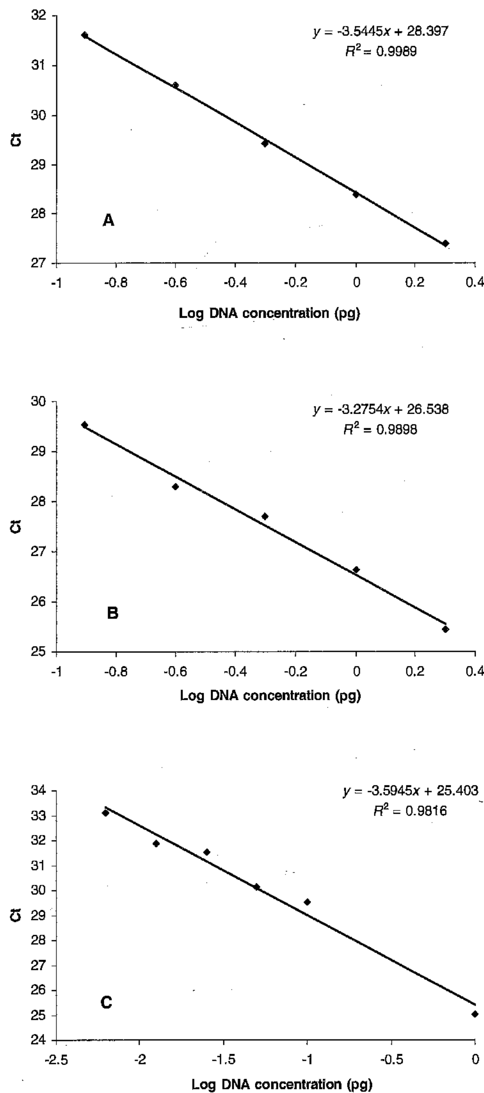


Fig. 5. Relationship between cycle threshold and DNA concentration of (A) *Globodera rostochiensis*, (B) *G. pallida*, and (C) *G. tabacum tabacum* in a three-primer (Gfor, Grev, and PITS-pall), multiplex, real-time PCR with fluorescently labelled TaqMan probes.

two methods can be carried out without the need to remove amplicon from sealed tubes using real-time PCR thermocyclers.

The genomic ITS region has been targeted by others for discriminatory PCR assays, and the sequences are readily available (Bulman and Marshall 1997; Toyota et al. 2008; Uehara et al. 2005). However, polymorphisms in the ITS of *Globodera* spp. are limited. In addition, although selection of primers that would amplify DNA fragments from all *Globodera* spp. was relatively uncomplicated, distinguishing among amplicons was more challenging. Nevertheless, amplicon melting temperature curves differed sufficiently among *Globodera* spp. in the EvaGreen-based assay to allow consistent species identification. However, in the analysis of mixed samples with DNA of two species, hybridization among heterologous strands resulted in intermediate melting curves, which interfered with unequivocal species identification. The use of instrumentation with capacity for high-resolution melting curve analysis would probably have avoided the problem; with such instruments, amplicons with even a single nucleotide polymorphism can be discriminated. The usefulness of melting curve analysis for differentiating between *G. rostochiensis* and *G. pallida* was also confirmed by others who used the primers published by Bulman and Marshall (1997), which amplified a larger ITS fragment than in our study (Bates et al. 2002; Quader et al. 2008). These studies did not include *G. tabacum*, which is closely related to *G. rostochiensis* and has a similar ITS sequence. The melting peak for *G. tabacum tabacum* at 88.1 °C was intermediate between that of *G. rostochiensis* at 87.7 °C and *G. pallida* at 88.7 °C. Populations of *G. tabacum solanacearum* and *G. tabacum virginiae* were not available for this study, but it is known from published sequences that the ITS sequences are identical to *G. tabacum tabacum* in critical regions (Subbotin et al. 2000; Szalanski et al. 1997). Skantar et al. (2007) were also able to identify *G. tabacum* and PCN species using modification of Bulman and Marshall (1997) primers in a conventional PCR assay and amplicon identification based on electrophoretic mobility.

Our multiplex real-time TaqMan PCR assay was developed to differentiate *Globodera* spp. on the basis of specific hybridization of labelled probes. Specificity of the probes was enhanced by including LNAs in probe design. Published research has shown that the stability, affinity, and specificity of oligonucleotide hybridization in real-time PCR is enhanced by introduction of LNA residues in primers and probes (Simeonov and Nikiforov 2002). LNA residues have the ribose ring fixed in a locked position by a linkage between the 2′ oxygen and the 4′ carbon atoms. Incorporation of an LNA monomer raises the thermodynamic stability of the probe or primer by 3–5 °C and enhances the affinity for complementary target sequences. For example, they have been used for enhancing stability and discrimination of specific hybridization targets in microarrays, real-time PCR assays, and fluorescent in situ hybridization. (Kubota et al. 2006).

The different fluorescent dye labels used in our study permitted differentiation of the three *Globodera* spp. in the separate channels of the thermocycler. Although it is possible to combine melting point analysis with real-time TaqMan PCR strategies (e.g., Smith et al. 2008), the melting curves of amplicons generated by the Gfor–Grev–PITS-pall primer set from DNA of the different *Globodera* spp. were insufficiently discriminatory with our thermocycler. However, based on the amplicon sequences melting point differences would likely have been adequate for discriminating among species using an instrument capable of high-resolution melting curve analysis. Notwithstanding this shortcoming in our work, the multiplex real-time TaqMan PCR was most useful for its rapid identification of *Globodera* spp. in single and mixed samples (Table 4, Fig. 4).

In our study, DNA was simply prepared by disrupting nematode cells by lyophilization and treatment with the components of a commercially available microlysis kit. However, the efficiency of the PCR reaction was at or near

Table 4. Range of C_t values for fluorescence response (n = 3) in thermocycler channels for real-time PCR detecting TaqMan probe for *Globodera rostochiensis* labelled with Cy5, for *G. pallida* labelled with Texas Red-613, and for *G. tabacum* labelled with FAM.

	Nematode stage included in sample			C_t of fluorescence signal per channel[a]		
PCR run	*G. rostochiensis*	*G. pallida*	*G. tabacum*	Cy5	Texas Red	FAM
1	Cyst	J2	None	17.2–19.0	23.4–25.8	—
2	J2	Cyst	None	24.8–26.3	19.3–20.1	—
3	Cyst	None	J2	17.0–19.4	—	21.4–24.0
4	J2	None	Cyst	23.5–25.0	—	17.6–20.7
5	None	Cyst	J2	—	18.0–20.1	22.2–24.3
6	None	J2	Cyst	—	24.5–26.0	17.5–18.3
7	J2	J2	J2	21.5–23.5	22.5–23.2	21.7–23.9
8	Cyst	Cyst	Cyst	17.0–19.0	17.0–19.3	17.3–19.8

Note: Template DNA for each PCR run was prepared from samples that included DNA from cysts and (or) J2 nematodes as indicated from the three *Globodera* spp.

[a]—, No fluorescence was observed in the channel.

100% as expected for reactions in which DNA copies double during each cycle. Deviations in amplification efficiency below one occur when PCR is inhibited by contaminating compounds in template DNA, and occasionally exceeds one when more than two copies are generated per cycle. Although there was no need in our study to quantify template concentrations, it is noted that the linear regression between C_t values plotted against the logarithm of template DNA concentration was close to one for each reaction, and thus, the assay could be used in a quantitative manner if required.

In conclusion, we took advantage of available ITS sequence data to design assays useful for rapid and accurate identification of *Globodera* juveniles and cysts. In particular, *Globodera* cysts often need to be identified to establish the nature of field infections, which has consequences for regulatory measures and practices to mitigate crop loss. The cysts are usually obtained after an extensive soil processing procedure to isolate a cyst-containing organic fraction. Individual cysts can then be isolated for species identification by morphological characterization and now using PCR. A further challenge would be to identify cysts directly in the organic fraction or even in raw soil extracts. Development of such technologies would provide significant savings in nematode surveys. Preliminary results suggest that detection in soil fractions is possible, but whether the PCR procedures developed in this study are sufficiently robust to permit their use for routine detection in field samples is uncertain. Further genomic analysis may reveal genomic regions with greater discriminatory polymorphisms, but that still remains to be determined.

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