Eur J Plant Pathol (2011) 130:271–285
DOI 10.1007/s10658-011-9752-z

Hsp90 gene, an additional target for discrimination between the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, and the related species, *G. tabacum tabacum*

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Accepted: 7 February 2011 / Published online: 6 March 2011

Abstract The heat-shock gene, *Hsp90*, was targeted as a new variable genomic region to supplement other DNA-based tests for identification and discrimination of *Globodera pallida, G. rostochiensis* and *G. tabacum tabacum*. Populations of the potato cyst nematodes, *G. pallida* and *G. rostochiensis* (PCN), originating from Canada, France, Belgium and USA, together with two populations of *G. tabacum tabacum* from the USA and France were used for the amplification of a fragment of the *Hsp90* gene. General and specific primers and probes for each species were derived from the consensus and non-consensus regions of the aligned sequences, respectively. A triplex conventional PCR assay, using a general forward and reverse or three specific reverse primers, as well as a real-time PCR using general primers and specific TaqMan probes, were developed. Melting curve analysis and restriction fragment polymorphisms using high resolution electrophoresis were explored for identifying PCR amplicons that characterized and discriminated the three *Globodera* species in both pure and mixed samples. Results from the different molecular assay strategies confirmed the usefulness of *Hsp90* as a new additional gene target and showed that several different test options could be used for discrimination of PCN.

Keywords Diagnosis · Molecular chaperones · TaqMan PCR · Quarantine

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Introduction

Potato cyst nematodes (PCN), *Globodera pallida* and *G. rostochiensis* are plant parasitic nematodes found in potato fields and because of the damage they cause on host plants, are considered to be quarantine pathogens in many countries (Manduric and Andersson 2003; Thiery and Mugniery 1996). In order to apply effective, practical control measures mitigating damage to potato crops, sensitive and rapid detection as well as accurate identification of these species is an absolute requirement. Identification and differentiation of *Globodera* species has traditionally involved microscopic examination of morphological characteristics and morphometric measurements. In many cases, the presence of similar morphological characters among *Globodera* spp. makes precise identification difficult, necessitating the use of protein and/or DNA based diagnostics (Bulman and Marshall 1997; Fleming et al. 1998; Fullaondo et al. 1997; Hockland 2005). Traditionally, DNA-based methods have mainly concentrated on variation in nucleotide

sequences within the internal transcribed spacer region (ITS) of the ribosomal gene operon, either by the generation of unique restriction fragment length polymorphisms (RFLPs) from amplified ITS fragments or the design of species specific primers for use in the polymerase chain reaction (PCR) (Brinby et al. 2000; Feder 1999; Subbotin et al. 2000b). Ribosomal DNA sequences have also been useful for determining relationships among nematode taxa including *Globodera* spp. (Van Megan et al. 2009), and successful application of an ITS-based PCR assay for PCN has been demonstrated in its use for both diagnostic and identification purposes (Pylypenko et al. 2005). The most recent approaches utilize ITS-based real-time multiplex PCR with TaqMan hybridization probes specific to PCN and the tobacco cyst nematode (TCN), *G. tabacum tabacum* (Madani et al. 2008; Nakhla et al. 2010). Despite the high degree of homology within the ITS regions, the ITS has been useful to discriminate between the two PCN species and differentiate them from TCN. However, the possible presence of different haplotypes and ITS heterogeneity that could be due to multiple copies of diverged rRNA (Blok et al. 1998; Subbotin et al. 2000a; Thiery and Mugniery 1996), may limit the sole use of ITS sequences. It has also been shown that results from several methods including iso-electric focusing, enzyme-linked immunosorbent assay, PCR, and morphological based identification for detecting *Globodera* species and subspecies were not fully in agreement in some cases (Ibrahim et al. 2001; Manduric et al. 2004; Skantar et al. 2007).

In most studies in which PCR is used for differentiating *Globodera* spp. only the two PCN species and on occasion one or two other species have been included. However, at least fourteen species of *Globodera* have been reported in the scientific literature and very little genomic sequence data is available for most of them. In fact, many of the non-potato *Globodera* spp. descriptions are based on very few populations, and specimens for many of the species are not readily available. Hence, molecular identification of *Globodera* species in unknown samples, particularly when extracted from soil and not specifically associated with host plant roots, will be tenuous at best if based solely on the relatively homogeneous ITS base sequence. The presence of weed-associated cysts of other *Globodera* sp. such as *G. artemisiae* and *G. achilleae*, in European and Asian agricultural crop land has been noted recently but has not yet been studied extensively (Dobosz et al. 2006; Hockland 2005; Nowaczyk et al. 2008). In any case, it would seem prudent to investigate additional genomic regions for useful discriminatory sequences to supplement ITS-based and other identification methods. Indeed the desirability of basing molecular pathogen detection protocols on at least two independent regions of the genome is generally recognized (Handoo et al. 2005; Skantar et al. 2007; Vincelli and Tisserat 2008).

These considerations led us to investigate the heat-shock protein genes as a possible target for discriminating *Globodera* spp. Heat-shock proteins (Hsps, also called molecular chaperones) are members of a family of cytoplasmic stress response proteins grouped and labelled according to their molecular weight in kilodaltons (Atibalentja and Noel 2007; Feder 1999; Nicola et al. 2008). The most widely studied Hsps are Hsp60, Hsp70 and Hsp90. The function of heat-shock proteins in all living organisms is similar and is triggered by exposure to different environmental stresses such as heat. In addition to the use of Hsp genes for gene expression and human disease studies, Hsp genes have been considered to be useful molecular markers for species identification or phylogenetic analysis of pathogenic bacteria, fungi, and entomopathogenic nematode species (Chianga et al. 2008; Goh et al. 1997; Hashmi et al. 1997; Nicolas et al. 2002). Among plant parasitic nematodes the heat-shock *Hsp90* gene has already been amplified from *Meloidogyne* spp. (root knot nematodes), *Heterodera glycines* (soy bean cyst nematode), and *Pratylenchus* spp. (root lesion nematodes), (Handoo et al. 2005; Ibrahim et al. 2001; Skantar and Carta 2004, 2005), and used to study the phylogeny of selected taxa (Skantar and Carta 2004). Another member of the Hsp family, *Hsp70*, has been used as a molecular target for conventional and real-time PCR detection of the pine wood nematode, *Bursaphelenchus xylophilus* (Leal et al. 2005, 2007). As indicated by Skantar and Carta 2004, a further advantage of using *Hsp90* would be if it occurs as a single copy unlike the ITS operon which occurs in multiple tandem arrays. *Hsp90* is a single copy gene in the *Caenorhabditis elegans* genome (Brinby et al. 2000) as well as in several insect species (Konstantopolou and Scouras 1998: Landais et al. 2001) but copy number of the *Hsp90* gene in other nematodes is not known at this time.

The specific aim of this study was to evaluate the heat-shock gene, *Hsp90,* as a supplementary DNA target to ITS-based molecular methods for the identification and discrimination of PCN and TCN. We explored several molecular strategies using *Hsp90* gene fragment sequences that may prove to be useful additional tools for accurate and reliable detection and differentiation of PCN.

Materials and methods

Nematode populations and DNA preparation

A total of 14 populations of *Globodera* spp. were used in the present study (Table 1). They consisted of *G. pallida* (one population each from Canada, France, Belgium and USA), *G. rostochiensis* (five populations from Canada, one population each from France, Belgium and USA) and two populations of *G. tabacum tabacum* (from USA and France). A population of *Heterodera glycines, H. schachtii* and one *Ditylenchus dipsaci* population were provided by the nematode collection at CFIA Charlottetown Laboratory. Population identity of PCN and TCN was verified to species by a comparison of ITS sequences obtained in this study with those available through GenBank. Further confirmation of population identity was performed using a real-time PCR-based TaqMan assay previously developed (Madani et al. 2008). Identity of *H. glycines* and *H. schachtii* samples was verified by PCR-RFLP analysis as described by Subbotin et al. (2000b); the same approach was used for *D. dipsaci* identification (Wendt et al. 1993).

Nematode DNA was extracted for PCR amplification from both cysts and individual second stage juveniles (J2). For cysts, the cuticle was first crushed and then removed with a needle under a stereomicroscope in a drop of water, the remaining eggs and J2 were transferred to a centrifuge tube. Single J2 or bulk eggs and J2s were crushed using an electric pestle device (Kontes, Vineland, NJ, USA) attached to a microhomogenizer (made by flame-sealing a pasteur pipette) in 15 µl distilled water in a microcentrifuge tube. For DNA extraction, either 5 µl of worm lysis buffer (WLB) (500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM MgCl2, 4.5% Tween 20, 0.1% gelatine) or 5× microlysis buffer (Microlysis Plus, the Gel Company, San Francisco, CA) with 2 µl proteinase K (60 mg/ml) were added to each tube. For both approaches a 35-min fluctuating thermal profile (recommended by Microlysis Plus manufacturer) consisting of 66°C for 30 min, 96°C for 3 min, 65°C for 4 min, 96°C for 2 min, 65°C for 1 min and 96°C for 1 min, or incubation at 65°C, for 60 min and 94°C for 10 min were evaluated to optimize the procedure. After treatment, preparations were

Table 1 Nematode populations used in this study and tests applied to confirm identification of each population

N	Code	Source	Species	Species identity
1	GB1	Quebec-Canada	*Globodera rostochiensis*	[a]ITS 1-TaqMan PCR
2	GB2	Quebec-Canada	*G. rostochiensis*	ITS 1-TaqMan PCR
3	AV1	Newfoundland-Canada	*G. rostochiensis*	ITS 1-TaqMan PCR
4	AV2	Newfoundland-Canada	*G. rostochiensis*	ITS 1-TaqMan PCR
5	BC	British Colombia-Canada	*G. rostochiensis*	ITS 1-TaqMan PCR
6	GR-Fr	Ecosse -France	*G. rostochiensis*	ITS 1-TaqMan PCR
7	GR-Be	Dadizele -Belgium	*G. rostochiensis*	ITS 1-TaqMan PCR
8	GR-US	[b]New-York-USA	*G. rostochiensis*	ITS 1-TaqMan PCR
9	GP-NF	Newfoundland-Canada	*G. pallida*	ITS 1-TaqMan PCR
10	GP-Fr	Chavornay -France	*G. pallida*	ITS 1-TaqMan PCR
11	GP-Be	Dadizele -Belgium	*G. pallida*	ITS 1-TaqMan PCR
12	GP-US	[b]Idaho- USA	*G. pallida*	ITS 1-TaqMan PCR
13	GT-Fr	Agen –France	*G. tabacum tabacum*	ITS 1-TaqMan PCR
14	GT-US	Connecticut-USA	*G. tabacum tabacum*	ITS 1-TaqMan PCR
15	HG	USA	*Heterodera glycines*	ITS-RFLP
16	HS	USA	*H. schachtii*	ITS-RFLP
17	Dit	USA	*Ditylenchus dipsaci*	ITS-RFLP

[a] ITS 1 sequence comparison with GenBank data base and TaqMan assay (Madani et al. 2008)

[b] DNA only

centrifuged for 2 min at 10 000 g and stored at –20°C until use.

Hsp90 gene amplification

DNA from each species of *G. pallida*, *G. rostochiensis* and *G. tabacum tabacum* was amplified by PCR using degenerate primers U831 and L1110 (Skantar and Carta 2000) directed toward the *Hsp90* gene. For controls, DNA from *H. glycines*, *H. schachtii* and *D. dipsaci* as well as tubes without added DNA were used. PCR was performed in a final reaction volume of 25 μl using Sprint Advantage Single Shot PCR Premix (Clontech, Mountain View, CA) to which 0.25 μM of each primer and 2 μl of template DNA were added. The PCR thermal cycling conditions were optimized as follows: 5 min at 95°C (initial denaturation of DNA and enzyme activation), followed by 38 cycles of amplification, 30 s each at 95, 53, and 68°C and a final extension at 68°C for 5 min. PCR products were visualized by 1% agarose gel electrophoresis followed by staining with ethidium bromide (50 μg/ml).

Cloning, primers and probes design

Gel purified (MoBio Laboratories Inc. Carlsbad, CA) PCR amplicons were cloned directly into the pGEM®-T Easy Vector System (Promega, Madison, WA) following the supplier's recommended protocol, and plasmids were purified from positive clones with UltraClean Standard Mini Plasmid Prep Kit (Mo-Bio). Sequencing of several clones from each population using purified plasmids was performed by the Core Molecular Biology Facility of York University (Toronto, ON). Direct sequencing of the amplified *Hsp90* gene from J2 and cysts from some populations was also performed.

Sequence chromatograms were visualized using the computer programs, Chromas Lite (Vesion 2.01, Technelsium Pty Ltd, Twantin, Australia) and edited where needed; final sequence editing was performed with GeneDoc, version 2.7 (Nicholas and Nicholas 1997). Clustal X (Thompson et al. 1997) and BioEdit (Hall 1999) were then used for alignment of raw sequence data and sequence analysis, respectively. Alignment of 60 DNA sequences of fragments generated with the U831/L1110 primer pair were used to design primers and probes that would be useful for discriminating among *G. rostochiensis*, *G. pallida*, and *G. tabacum tabacum*. To ensure consistency in PCR results and to avoid using degenerate primers, several putative primers were designed using the computer program Allele ID (Premier Biosoft Int., Palo Alto, CA). Sets of primers were determined either from non-consensus regions for specific amplification of each species and/or for use as TaqMan probes, and from the consensus regions for general amplification of *Hsp90* gene fragments from all three *Globodera* species containing unique internal species-specific sequences. General forward and reverse primers were selected along with three species-specific forward primers designed to also serve as TaqMan probes. The probes were 5′ labelled with Tex615, Cy5 or 6-carboxyfluorescin (FAM) fluorescent dye, representing specificity to *G. pallida*, *G. rostochiensis* and *G. tabacum tabacum*, respectively. Probes also were labelled with the IBRQ or IBFQ as a quencher dye at the 3′ end. Sequence details for the primers and probes used are listed in Table 2. Although there are limited database entries for *Hsp90* sequences from nematodes and most available published data are from ESTs which lack introns, the primers and probes were assessed for cross-homologies using the BLAST program against the National Center for Biotechnology Information (NCBI) sequence database. Primers and probes used in this study were synthesized by Integrated DNA Technologies (Coralville, IA).

Multiplex detection in conventional PCR

Two sets of general primers Hsp90-F1/Hsp90-R1and Hsp90-F1/Hsp90-R2 were compared for amplifying a part of the *Hsp90* gene from all three *Globodera* species. Primers specific to each of the three *Globodera* species were used in a conventional triplex PCR system using Hsp90-R2 (1 μM) reverse primer in combination with three forward primers: M-GP, M-GR and M-GT (1 μM), each specific to *G. pallida, G. rostochiensis* and *G. tabacum tabacum*, respectively. DNA extracted from a single J2 and cysts as well as DNA extracted from a mixed sample of two or three species was used as template for PCR reactions. To verify detection of a single J2 mixed in with cysts of other species, samples consisting of a single J2 of each species together with a cyst from the other two *Globodera* species used in this study were mixed and DNA was extracted and used in multiplex PCR. The

Table 2 Nucleotide sequence of primers and probes used.

Primer/probes	Sequence (5′–3′)	Reference
U831	AAYAARACMAADCCNTYTGGAC	Skantar and Carta (2000)
L1110	TCRCARTTVTCCATGATRAAVAC	Skantar and Carta (2000)
Hsp90-F1	CCGGACGACATCTCCAACGAG	This study
Hsp90-R2	TTGACTGCCAAATGGTCTTCC	This study
Hsp90-R1	GGCATTCTTGCTCTTCTTGTTCT	This study
M-GP	GCTTTCAGTTATATAAAATGAGTTGAGTTAGCATTTTT	This study
M-GR	TATGGCTTTCGTTTAGGGAAAGGGTTTGATG	This study
M-GT	GAAGGGTTTAGGAATTTAGTTAAATTCTATT	This study
P-GP	TEX615-GCTTTCAGTTATATAAAATGAGTTGAGTTAGCATTTTT-IBRQ	This study
P-GR	CY5-TATGGCTTTCGTTTAGGGAAAGGGTTTGATG-IBRQ2	This study
P-GT	FAM-GAAGGGTTTAGGAATTTAGTTAAATTCTATT-IBFQ	This study

thermal profile consisted of 5 min at 96°C followed by 35 cycles at 94°C for 20 s, 60°C for 30 s and 68°C for 30 s with a final extension of 5 min. The procedure beginning with DNA extraction was repeated two times and the PCR experiments were repeated three times in separate runs. Amplicons were analyzed by gradient polyacrylamide gel electrophoresis or the more sensitive and faster capillary electrophoresis. DNA in the range of 50–70 ng of *H. schachtii*, *H. glycines* and *D. dipsaci* was used as negative controls to confirm the specificity of the primers and probes.

For gel electrophoresis, aliquots (5 μl) of samples were loaded onto a 4–12% precast polyacrylamide TBE gel (Invitrogen, Mississauga, CA) and run for 1 h at 150 V in TBE buffer using an XCell-SureLock apparatus (Invitrogen). The gel was removed from the cassette and stained for 20 min with a 50 μg/ml solution of ethidium bromide. Products were visualized with UV light and documented using a Digital photo-documentation system (Syngene, MD, USA).

For capillary electrophoresis, a 12-capillary QIAxcel instrument (Qiagen, Inc., Mississauga, ON) was used and loaded with a high resolution DNA cartridge. PCR products were diluted 1:1 in DNA dilution buffer (Qiagen, Inc.) and were then separated using the automated protocol specific to the type of cartridge used, and a gel-view of the products in each capillary was constructed automatically.

PCR with melting curve analysis

For the assay with melting curve analysis, two sets of primers Hsp90-F1/Hsp90-R2 and Hsp90-F1/Hsp90-R1 were compared for amplifying the *Hsp90* gene fragment from all three *Globodera* species in real-time PCR, either using DNA of each species separately or with mixed DNA of all species. Amplification was performed in the presence of EvaGreen dye followed by a melt curve analysis of the PCR products at the end of the reaction. PCR was performed in a final reaction volume of 25 μl using Sprint Advantage Single Shot PCR Premix (Clontech, Mountain View, CA) to which was added 0.5 μM of each primer, 2 μl of template DNA, and EvaGreen (20×stock from Biotium). Following a 3 min denaturation/enzyme activation at 95°C, the amplification profile for 35 cycles, using this primer set was: 20 s at 96°C; 25 s at 60°C; and 30 s (with fluorescence acquisition in the FAM channel) at 68°C. Melting curve analysis also was performed in a multiplex PCR reaction using four primers: reverse Hsp90-R2 in combination with M-GT, M-GP and M-GR forward primers, and with the same thermal profile. A final extension at 70°C for 4 min was used to initiate a melting curve analysis from 70–95°C using 0.2°C increments every 4 s. The real-time instrument used was a Rotor-Gene 3000 (Corbett Research, NSW, Australia) with 0.1 ml tubes and a 72 place rotor. To verify the size of PCR products, amplicons were analyzed by capillary gel electrophoresis. Reproducibility and repeatability of the real-time PCR and melt analysis was confirmed by inter (run of the DNA from different population of the same species, i.e. between populations) and intra (run of DNA from the same population, i.e. within populations) assay analyses. Most of the populations in this study were used for inter and

intra assay analysis. For all tests, DNA of *H. schachtii*, *H. glycines* and *D. dipsaci* was used as negative control to confirm the specificity of the reactions.

PCR with RFLP

General primers Hsp90-F1/Hsp90-R2 were used to amplify the *Hsp90* gene fragment from all populations of the three *Globodera* species. Successful amplification of the PCR product was confirmed by agarose gel electrophoresis, and PCR products were purified using a commercial kit (MoBio Laboratories Inc. Carlsbad, CA). Digestion of the amplicons was performed with restriction enzymes *Dra*I and *Eco*RI, selected on the basis of sequence-based virtual RFLP patterns predicted using the program BioEdit (Hall 1999) on sequence information of the *Hsp90* gene obtained in this study. Eight to 10 µl of purified PCR product (approximately 100 to 200 ng) from each species was used for RFLP analysis by separate digestion in a 20 µl total reaction volume containing 5 U/µl of each restriction enzyme and 2 µl of the 10× reaction buffer supplied by the manufacturer. After incubation at 37°C for 2 h, the digested products were analyzed by capillary gel electrophoresis using the Qiaxel instrument and a high resolution cartridge (Qiagen, Inc, Mississauga, ON) as described above.

Multiplex real-time TaqMan PCR

A real-time TaqMan PCR assay with probes P-GP, P-GR and P-GT dual- labelled with Tex615-IBRQ, CY5-IBRQ and FAM-IBFQ, using general primers Hsp90-R2 in combination with two forward primers Hsp90-F1 and U831, respectively, was used to detect 137 to 173 bp fragments of the *Hsp90* gene from all three species containing the unique probe sequences (Table 2). Since results of PCR optimization showed that using the second forward primer (U831) for *G. pallida* amplification was enhanced, we also included this primer for this assay. Real-time PCR with these primers was carried out in a Smartcycler2 thermocycler (Cepheid Inc, CA). PCR reactions in a final volume of 25 µl contained 1 µM of each of the three primers and probe concentrations of 135 nM for P-GR and P-GT and 115 nM for P-GP. Template DNA (2.5 µl) was the final component added to a 25 µl Smartcycler tube containing master mix (Quantitect multiplex PCR-NoRox, Qiagen Inc, Mississauga, ON), primers, and probes. DNA in the range of 50–70 ng of *H. schachtii*, *H. glycines* and *D. dipsaci* was used as negative control. The tubes were centrifuged for 45 s before insertion into the reaction sites of the instrument. The reaction profile consisted of an initial denaturation/activation step for 15 min at 95°C followed by 45 cycles of amplification using a two-step thermal profile of 96°C for 20 s and 64°C for 60 s and a final extension at 72°C for 3 min. Fluorescence in the Tex615, FAM and Cy5 channels was monitored during the extension/annealing step of the amplification phase. Subsequent to TaqMan PCR analysis, melting curve analysis was accomplished by the addition, to each tube, of an equal volume of a 2× concentration of EvaGreen in PCR buffer. The tubes were centrifuged as before and loaded into the instrument. A thermal profile of 95°C for 5 min followed by 64°C for 5 min was followed by a melt curve analysis from 64°C to 97°C ramping at 0.5°C degrees per second. Real-time PCR reactions were repeated using most of the *Globodera* populations available in this study to confirm the reproducibility of results.

Results

Species identity

The ITS1 sequences obtained from the nematode populations used in this study (Genbank accession numbers GQ294512-GQ294525, GQ355975) revealed a 97–99% similarity to those of the expected species derived from GenBank. This, together with the results of a recently developed real-time TaqMan PCR assay used for species characterization (Madani et al. 2008) confirmed species identity. Results of ITS-PCR-RFLP assays on *H. glycines*, *H. schachtii* and *D. dipsaci* were in agreement with the previous published data (Subbotin et al. 2000b; Wendt et al. 1993) and indicative of the correct identity of these species (data not shown).

Hsp90 gene amplification and sequence analysis

Amplification using the degenerate primer set U831 and L1110 (Skantar and Carta 2000) produced amplicons of 448, 483 bp and 474 bp for the *Hsp90*

gene of *G. pallida*, *G. rostochiensis* and *G. tabacum tabacum*, respectively (Fig. 1). For a population of *G. pallida* from France an extra smaller faint band was reproducibly observed in the electrophoretic gel that could be due to the presence of another primer binding site on the genome of this population. An amplicon with an approximate size of 450 bp was also obtained for both *Heterodera* spp. and *D. dipsaci*, (data not shown) due to the degenerate feature of the primers. The optimized thermal profile with an annealing temperature of 53°C produced a better result in PCR compared to the previously published thermal profile (Skantar and Carta 2000). *Hsp90* sequences obtained in this study were deposited in Genbank (accession numbers GQ401343-GQ401353).

Primer sets Hsp90-F1/Hsp90-R1 and Hsp90-F1/Hsp90-R2, positioned as nested primers to U831 and L1110 designed in this study and based on consensus regions of the *Hsp90* gene sequence (Fig. 2), amplified only DNA from *Globodera* species, yielding amplified fragments with electrophoretic mobilities in capillary electrophoresis that matched the fragment sizes predicted from the sequence alignment. Amplicons obtained with primer pair Hsp90-F1/Hsp90-R1 matched the predicted sizes of 363, 398 bp and 389 bp, and amplicons obtained with primer pair Hsp90-F1/Hsp90-R2 primers matched predicted sizes of 213, 250 bp and 248 bp for *G. pallida*, *G. rostochiensis* and *G. tabacum tabacum*, respectively. No PCR products were obtained for *Heterodera* spp. nor *D. dipsaci* DNA with either set of primers. Sequence analyses of products amplified using Hsp90-F1/Hsp90-R1 primers revealed the GC content of 40.99, 43.97% and 40.93% for *G. rostochiensis*, *G. pallida* and *G. tabacum tabacum,* respectively. Sequence identity was 90% between *G. tabacum tabacum* and *G. rostochiensis*, 80% between *G. rostochiensis* and *G. pallida*, and 79% between *G. tabacum tabacum* and *G. pallida*. *Hsp90* sequences among clones of the same species had 99 to 100% identity. The sequence of each of the *Globodera* species also had identity with *H. schachtii* and *D. dipsaci* in the range of 80% and 75%, respectively. Based on the differences in nucleotide sequence observed in the alignment of about 60 *Hsp90* gene sequences from the three *Globodera* species (Fig. 2) obtained in this study, it was possible to design assays dependent on multifunctional oligonucleotides that could be used as specific primers or probes for multiplex conventional and real-time PCR assays.

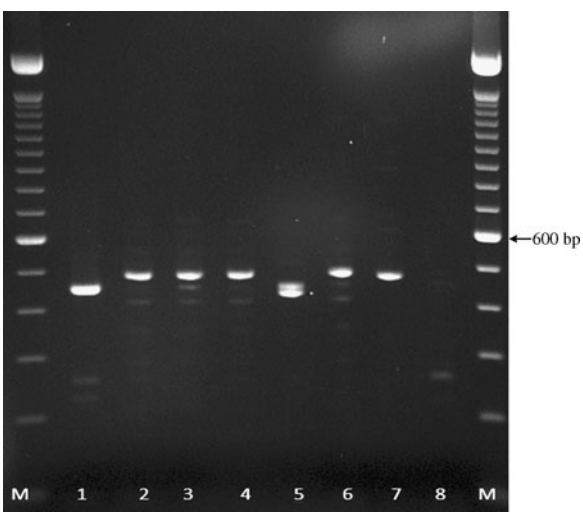

Fig. 1 Agarose gel electrophoresis of the polymerase chain reaction amplified fragment with the U831 and L1110 primers for three species of *Globodera*. Lane 1, *G. pallida*, 448 bp (Canada, GP-NF); lanes 2-3, *G. rostochiensis*, 483 bp (Canada, AV1 and GB1); lane 4, *G. rostochiensis*, (France, GP-Fr); lane 5, *G. pallida*, (France, GP-Fr) and lane 6-7, *G. tabacum*, 474 bp (USA, GT-US and France, GT-Fr), respectively. Lane 8, negative control without DNA template; M, DNA ladder, 100 bp Invitrogen, Canada Inc. Burlington, ON

Triplex conventional PCR

A multiplex primer set consisting of the general reverse primer Hsp90-R2, in combination with the three forward primers of M-GP, M-GR, and M-GT produced unique products with template DNA from both J2 and cysts of the three *Globodera* species. Amplicons of 173, 152 bp and 128 bp were obtained for *G. pallida*, *G. rostochiensis*, and *G. tabacum tabacum*, respectively. PCR products were resolved by high resolution capillary or gradient polyacrylamide gel electrophoresis using single, double, or triple mixes of template DNA from cysts of all three *Globodera* species (Fig. 3a-b). In addition, triplex PCR with DNA extracted from a mixture of a single J2 with a cyst of each of the other two species gave similar results (Fig. 4). A faint band on gels indicated amplification of DNA from J2s of each species as seen in Fig. 4. This represents the lowest amount of DNA used in the study and confirms the sensitivity and specificity of the test.

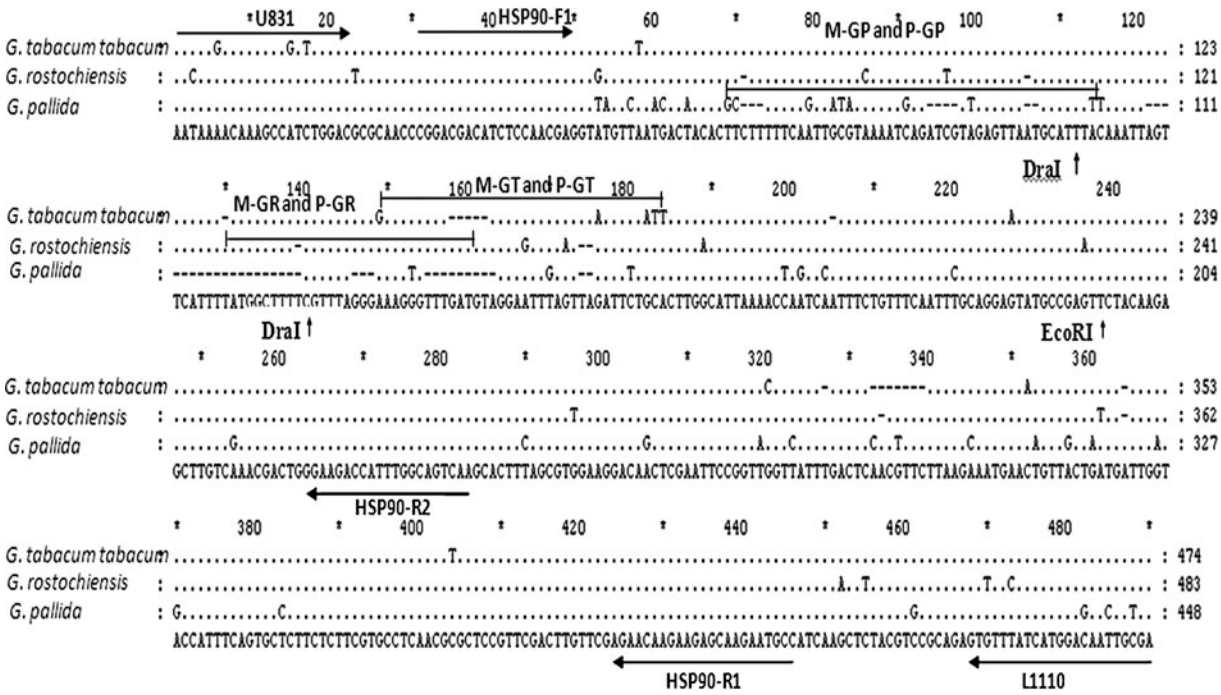

Fig. 2 Position of primers and probes on the aligned consensus sequences of *Hsp90* heat-shock gene for *Globodera pallida*, *G. rostochiensis* and *G. tabacum*. U831 and L1110 degenerate primers (25). Hsp90-F1, Hsp90-R2 and Hsp90-R1 are general primers, M-GP, M-GT and M-GR are specific reverse primers used in triplex PCR designed in this study. P-GT, P-GP and P-GR are the probes used in real-time PCR and are located in the same position as primers. Position of primers and probes are indicated by horizontal arrows and underlined, and restriction site by vertical arrows

Melting curve assay and specificity test

Melting curve analysis using EvaGreen performed at the end of the PCR reaction showed that, unlike primers Hsp90-F1 and Hsp90-R1, the amplicons produced using Hsp90-F1 and Hsp90-R2 could specifically be associated with each of the three *Globodera* spp. based solely on their peak melting temperatures (Fig. 5). A single peak for each species was obtained and no primer-dimers or non specific products were produced. The mean value of peak melting temperature of the amplicons was 82.2°± 0.1°C for *G. tabacum tabacum*, 82.7±0.1°C for *G. rostochiensis*, and 83.5°C±0.1°C for *G. pallida*. Results of inter and intra assay analysis of melting points were not significantly different between runs. However, when a mixture of DNA from the three species was used, the melting point increased relative to the melting point of the individual combinations (data not shown). For example, a mixture of the DNA from *G. pallida* and *G. tabacum* resulted in a higher amplicon melting point than a mixture of *G. pallida* and *G. rostochiensis* DNA. A mix of *G. tabacum tabacum* and *G. rostochiensis* had the same melting point as *G. rostochiensis*.

Melting curve analysis of amplicons produced during the triplex PCR reaction discriminated the three species of *Globodera*. A single peak was obtained for each species at 80.3°C±0.1, 81.7°C± 0.1 and 82.2°C±0.1 for *G. tabacum tabacum*, *G. rostochiensis* and *G. pallida*, respectively (Fig. 6a). However, when mixed DNA samples were used, a shoulder was observed in the melting curve representing a mixture of DNA. When a mix of *G. pallida* and *G. rostochiensis* was used the shoulder was associated with the *G. rostochiensis* curve; when a mix of DNA from *G. rostochiensis* and *G. tabacum tabacum* was used the shoulder was associated with the *G. tabacum tabacum* curve (Fig. 6b). Thus in contrast to multiplex PCR high resolution electrophoresis, the melting curve analysis of mixed DNA samples produced equivocal results.

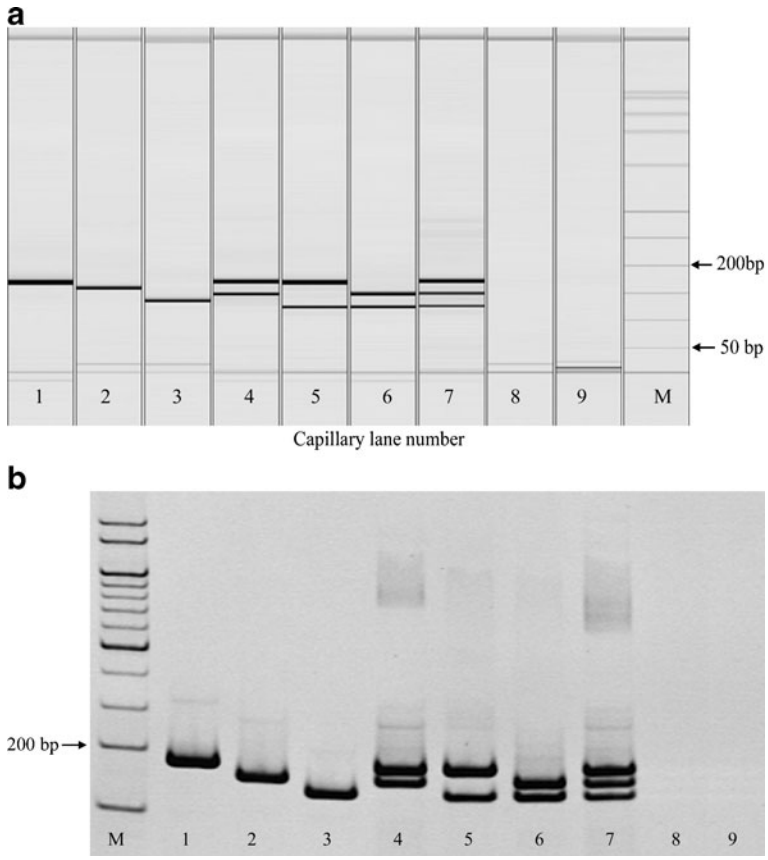

Fig. 3 Comparison between **a** capillary gel and **b** 4-12% gradient acrylamide gel electrophoresis of triplex PCR using reverse primers of Hsp90-R2 and three forward primers of M-GP, M-GR and M-GT specific to each species of *G. pallida* (Canada, GP-NF), *G. rostochiensis* (Canada, BC) and *G. tabacum* (USA, GT-US), respectively. DNA samples were prepared from a full cyst separately from each species or from a mixture of a single cyst from two and three species. Lanes 1, *G. pallida*; 2, *G. rostochiensis*; 3, *G. tabacum*; 4, mixture of *G. pallida* and *G. rostochiensis*; 5, *G. pallida* and *G. tabacum*; 6, *G. rostochiensis* and *G. tabacum*; 7, *G. pallida*, *G. rostochiensis* and *G. tabacum*; 8, negative control without DNA; and 9, DNA of *Heterodera glycines*. M, DNA ladder

RFLP

Enzymatic restriction of the PCR products obtained with primers Hsp90-F1 and Hsp90-R2 produced discriminative band patterns for the three *Globodera* species. Using the BioEdit software, *G. pallida* amplicons were predicted to yield fragment sizes of 126, 68 and 10 bp after restriction by *Dra*I, but not be cut by *Eco*RI. The *G. rostochiensis* amplicon was predicted to produce fragment sizes of 205 and 46 bp after restriction by *Eco*RI and not be cut by *Dra*I, whilst the *G. tabacum tabacum* amplicon would not be cut by either enzyme. The size of the predicted fragments, except for the smallest fragment (10 bp) that could not be resolved, was confirmed by analysis of restriction products by capillary electrophoresis (Fig. 7).

Multiplex real-time TaqMan PCR

A multiplex TaqMan assay was developed using general primers from the consensus regions of the *Hsp90* gene and converting the specific internal primers used in the triplex conventional PCR assay to dual labelled TaqMan probes (Table 2). Initial optimization was carried out using cloned DNA. Subsequent application of the TaqMan PCR was optimized with DNA extracted from individual cysts and J2s with a strong emphasis on ability to run multiplex assays and distinguish *Globodera* spp. in assays with mixed DNA-template samples producing unambiguous results. Results showed that an assay using a combination of two forward primers Hsp90-F1 and U831, and reverse primer Hsp90-R2 was robust and able to provide amplification and discrimination using both mixed and single templates (Table 3). The sensitivity and specificity of the probes were tested against all the populations available in this study. Detection of DNA from a single J2 was the highest level of sensitivity required for practical application, and it was indeed possible to detect a single J2 from all populations under study (e.g. Fig. 4). Also the ability to detect DNA from a single J2 of each species mixed with the DNA

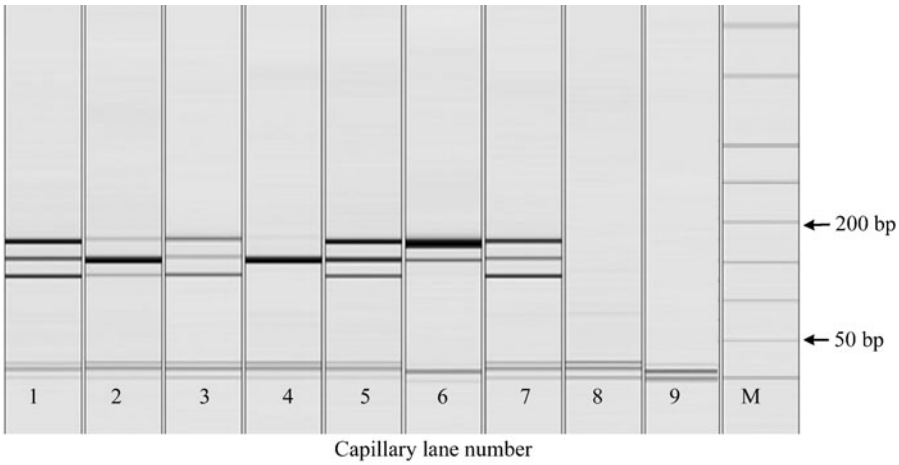

Fig. 4 Single J2 detection of each of *G. pallida* (Canada, GP-NF), *G. rostochiensis* (France, GR-Fr) and *G. tabacum* (USA, GT-US) species in a mixed sample of three species. Capillary gel electrophoresis of triplex PCR with the reverse primer Hsp90-R2, in combination with the three forward primers of M-GP, M-GR and M-GT specific to *G. pallida, G. rostochiensis* and *G. tabacum*, respectively. Lane 1, single J2 of *G. pallida*, cyst of each of *G. rostochiensis* and *G. tabacum*; 2, single J2 of each of *G. pallida* and *G. tabacum* with cyst of *G. rostochiensis*; 3, single J2 of each species; 4, cyst of *G. rostochiensis* and single J2 of *G. pallida*; 5, single J2 of *G. tabacum* and cyst of each of *G. rostochiensis* and *G. pallida*; 6, single J2 of *G. rostochiensis* and cyst of *G. pallida*; 7, single J2 of *G. rostochiensis* and cyst of each of *G. pallida* and *G. tabacum;* 8, negative control without DNA; 9, *Heterodera glycines* negative control; and M, capillary molecular weight

extracted from cysts of the two other species and vice versa was carried out using the TaqMan PCR assay. In multiplex reactions with a mixture of DNA from a cyst of each species, Ct values differed by only one to two cycles compared to using DNA of each species separately as template (Table 3). While a single J2 from one of the *Globodera* species could be detected in a background of DNA extracted from mixtures of single cysts from the two other species, melting curve analysis of products from the TaqMan assay for the three *Globodera* spp. using EvaGreen gave the same relative curves produced using the general primers Hsp90-F1 and Hsp90-R2 with only slight differences in the absolute value of melting points. Capillary gel electrophoresis confirmed the presence of a single PCR product with both single

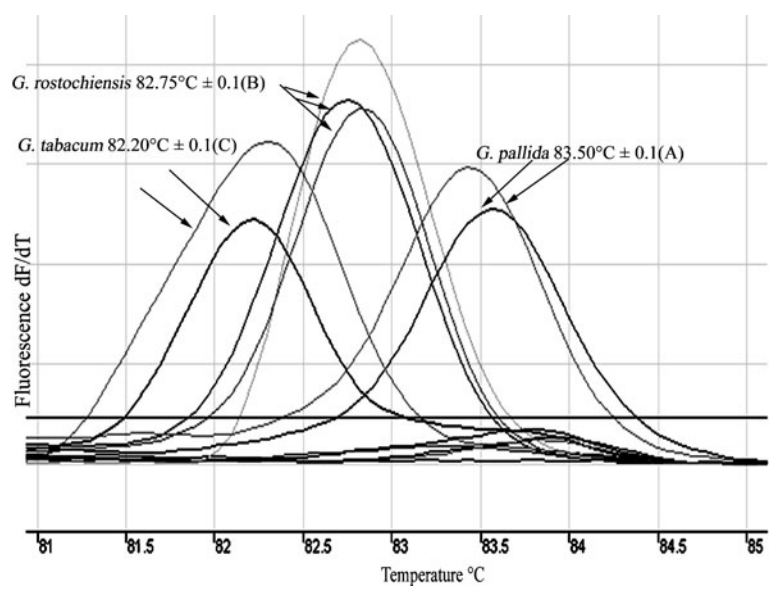

Fig. 5 Melting curves of three *Globodera* species after real-time PCR with forward primer Hsp90-F1and reverse Hsp90-R2. Mean and standard deviation are indicated on the curves. Temperature data are from three separate PCR runs

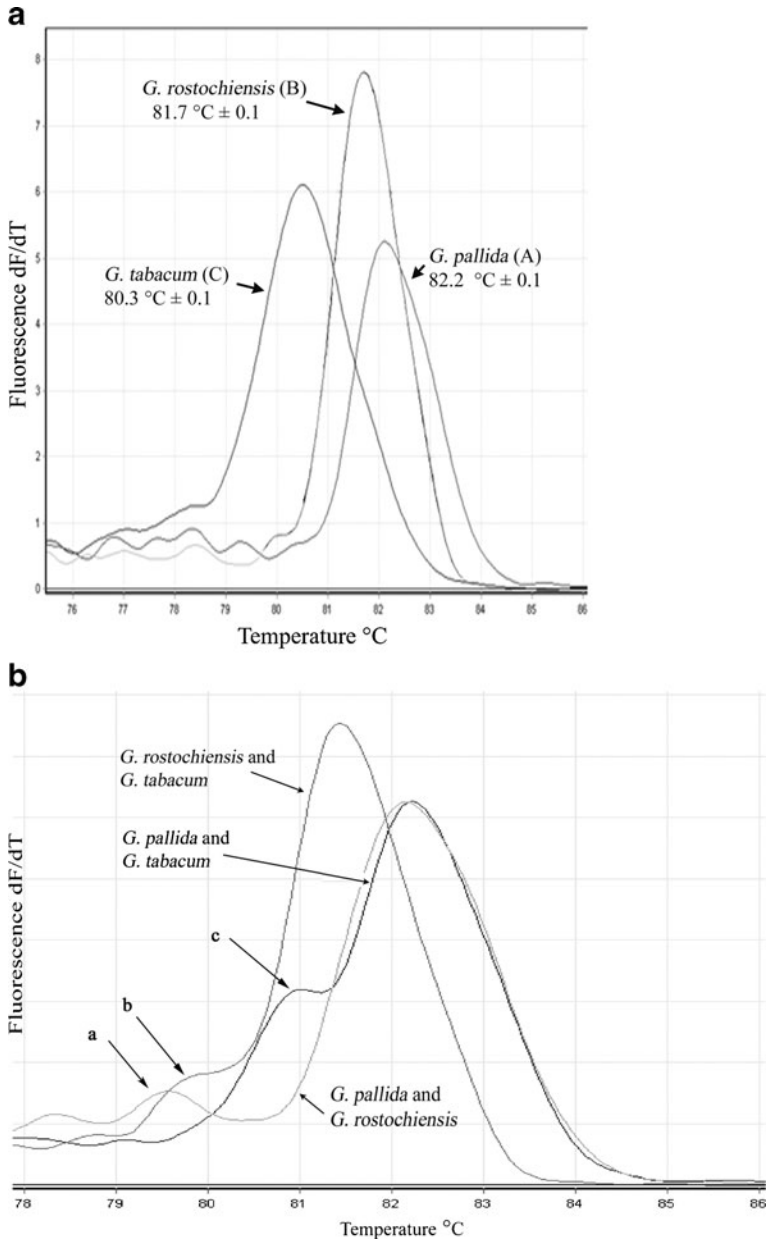

Fig. 6 Melting curves of three *Globodera* species. Triplex PCR reaction with reverse primers of Hsp90-R2, and three forward primers of M-GP, M-GR and M-GT specific to each of *G. pallida, G. rostochiensis* and *G. tabacum*. **a** PCR using pure DNA of each species **b** DNA of mixed species of *Globodera.* Presence of a shoulder is indicated by *arrow* (a-c)

and mixed templates, however, the composition of the samples could only be resolved by the analysis of probe hydrolyses in the TaqMan PCR assay.

Discussion

In this study a fragment of the *Hsp90* gene was amplified and sequenced from *Globodera* populations originating from Canada, Europe and the USA. Unlike the ITS sequence, the *Hsp 90* gene sequence had considerable variability (<90% sequence similarity) among the three *Globodera* species available to us, while it was highly conserved (>99% sequence similarity) within each species. Sequence similarity with the closely related cyst nematode *Heterodera* was <80%. Consequently, we were able to develop a diverse set of approaches for the molecular detection

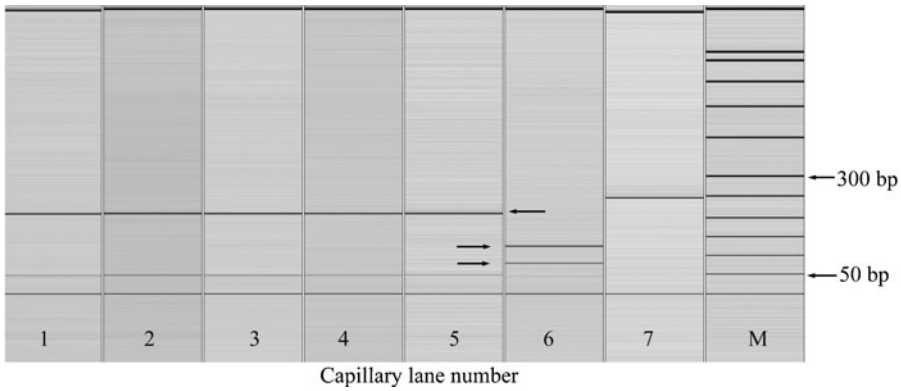

Fig. 7 An example of restriction fragment length polymorphisms (RFLP) of *Hsp90* gene PCR product amplified by primer Hsp90-F1and Hsp90-R2, restricted by *Eco*RI and *Dra*I, generated by capillary gel electrophoresis, for species discrimination of PCN and TCN. Lanes 1 to 5, *Globodera rostochiensis*, populations AV1, BC, GR-Fr, AV2, and GB1, respectively; Lane 6, *G. pallida* (GP-NF); lane 7, *G. tabacum* (GT-US). Lane M, capillary molecular weight. Restriction fragments are indicated by *arrows*

and discrimination of *Globodera* species, which will be useful as additional, supplementary tools to those developed mainly on the basis of ITS gene sequences for accurate identification of PCN samples.

Mindful of the fact that different laboratories do not all have access to the same equipment and may have different preferences for diagnostic tests, we evaluated performance of several *Hsp90*-based molecular test strategies. General oligonucleotides (from conserved regions) were designed to act as external primers for the amplification of a region of *Hsp90* containing species-specific sequences. Internal oligonucleotides specific to each species were designed from non-conserved regions to act in conjunction with general primers as both primers for multiplex conventional PCR and probes for multiplex TaqMan-based assays. This enabled the development of several different assays: a conventional PCR assay using general primers, a triplex PCR using a general and three specific primers, and a real-time PCR assay using specific TaqMan probes. Because confirmation of amplicon identity is important when DNA amplification is used for diagnostic work, we used gel electrophoresis, melting curve analysis, and RFLP patterns to show that they could all be applied to *Hsp90*-amplified products.

The melting curve analysis of our products produced in TaqMan assays, or conventional PCR with two general or one general and three specific primers proved useful using single templates but produced ambiguous data with mixed species of *Globodera*. In contrast, the three channel fluorescence data obtained via probe hydrolysis in the multiplex TaqMan assay, the high resolution electrophoresis of products from the triplex conventional PCR with one general primer and three specific primers, and the restriction fragmentation patterns of products from conventional PCR using general primers, consistently produced unambiguous results using both single and mixed templates. Unlike the conventional PCR

Table 3 Cycle threshold (C_T) values in a real-time PCR assay using a combination of two forward primers Hsp90-F1 and U831, and reverse primers Hsp90-R2 for both mixed and single templates

Species	Fluorescence channel	PCR Template		
		Single J2	Single cyst	[b]Mixture of cysts
Globodera pallida	Texas Red	[a]30.81 (0.83)	19.93 (0.74)	20.87 (1.25)
G. rostochiensis	Cy5	27.71 (0.83)	14.83 (0.66)	17.03 (0.63)
G. tabacum tabacum	FAM	28.99 (1.73)	14.97 (0.21)	13.89 (0.27)

[a] C_T data are from three separate PCR runs, standard deviation in brackets

[b] Mixture of equal volumes of DNA extracts from cysts of *G. pallida*, *G. rostochiensis*, and *G. tabacum tabacum*

assays, the TaqMan assay has the advantage of a true single tube test requiring no further processing but requires the use of specialized instrumentation which may not be available to all laboratories. Results from each of these approaches revealed the reproducibility and robustness of *Hsp90* gene as a target for discrimination between *Globodera* species.

Similar to melting curve analysis, the analysis of restriction fragments was a reliable method of differentiating *Hsp90* amplicons of the three *Globodera* spp. used in this study. Only two restriction enzymes were required to provide discriminating data. Perhaps additional restriction enzymes will be required to distinguish the PCN and TCN species from other *Globodera* spp. not used in this study. Because of the relatively rare and recent finding of *Globodera* spp. in Canada, a concerted effort to determine whether other *Globodera* spp. occur in Canada has not been undertaken, and specimens of weed-associated *Globodera* cysts are not readily available. Development of an *Hsp90* sequence database for *Globodera* spp. worldwide, regardless of host association, would be helpful in order to ascertain the diversity of the genus and the confidence with which *Hsp90* polymorphisms can be used to differentiate and identify species unequivocally. Such a database would complement the sequence database being developed by Helder et al. (2008) for the small subunit ribosomal DNA of European terrestrial and freshwater nematode fauna. With the greater sequence diversity in the *Hsp90* gene compared to the ITS gene region, *Hsp90* fragments may become the target region of choice for rapid identification of nematode cysts.

Mixtures of individual cysts and juveniles of the three *Globodera* spp. available were used to evaluate sensitivity and specificity of the molecular tests. While such mixtures are not likely to be common in agricultural field soil, there are reports of the co-existence of both PCN species in field samples examined in laboratories. Ibrahim et al. (2001) reported a 25% mixture of PCNs, and T.H. Been (*personal communication*, PRI, Wageningen, NL) a 6% mixture. Moreover, there are reports indicating that changes in proportion of the two species can occur over time and lead to a new species becoming dominant. For example, in some areas of the United Kingdom the *Globodera* population shifted from being predominantly *G. rostochiensis* to *G. pallida* upon planting of *G. rostochiensis* resistant cultivars where only a trace of *G. pallida* previously existed (Trudgill et al. 2003). Thus the assay herein developed may be very important for detecting even a low incidence of *G. pallida* if present together with *G. rostochiensis*. However, the objective of our experiments was to illustrate that the molecular tests have the capacity to accurately identify correctly the *Globodera* composition of samples. The results also clearly show that the tests have adequate sensitivity to detect a single J2 even if it is in a background of much higher concentrations of closely related DNA such as that from heterologous cysts.

The corresponding Ct values in the real-time TaqMan assay when using DNA of single J2 or single cyst were higher for *G. pallida*, than for *G. rostochiensis* and *G. tabacum tabacum*. This is an indication of either a lower efficiency of the P-GP probe in PCR reactions or representative of greater complexity in the *G. pallida* genome in terms of haplotype. This observation is in agreement with our previous ITS-based TaqMan assay for the detection of PCNs and TCN (Madani et al. 2008), where detection of *G. pallida* was also less efficient than the two other species. The multiplex assay for *G. rostochiensis* and *G. pallida*, but not *G. tabacum tabacum*, had slightly higher Ct values when using a mixture of cyst DNA from all three species compared to PCR with DNA from only a single species as template, probably because of the dilution factor. The lower Ct value for *G. tabacum tabacum* in the mixture compared to the single cyst could be due to the effect of dilution of any inhibitors that might have been present in this extract. Although this difference is not significant for the purpose of a qualitative test, i.e. for detection, in a quantitative test this could have a significant effect on results.

In summary, we report here on the first triplex conventional PCR assay in which three distinct amplicons are produced for discrimination of three *Globodera* species. The multiplex PCR described by Bulman and Marshal 1997, discriminated only the PCN species and not TCN, whereas the modification of the PCR assay developed by Skantar et al. 2007, did not amplify *G. pallida*. It also produced a faint band from *H. avenae* samples. Sequence analysis showed that compared to the ITS region, the *Hsp90* heat shock gene contained more sequence variations among the three *Globodera* species making it

possible to design multifunctional oligonucleotides for the specific detection of each species using either conventional or real-time PCR assays. These techniques can be used independently based on the availability of the instrumentation required and/or in conjunction with other published conventional and real-time PCR assays thus increasing the overall power of *Globodera* diagnostics. Although we could verify identity of all populations used in this study, access to more PCN populations, the other *G. tabacum* subspecies (i.e. *G. tabacum solanacearum* and *G. tabacum virginiea*), or other *Globodera* spp. will provide more confirmatory data on usefulness of this approach.

Acknowledgements We thank Steve Wood, Guy Belair, and Michael Rott for providing *Globodera* populations from Newfoundland, Quebec, and British Columbia in Canada, respectively, and Xiaohong Wang for providing DNA from *Globodera* populations in New York and Idaho, USA. We also thank J.A. LaMondia for providing cysts of *G. tabacum tabacum* and D. Mugniery and Nicole Viaene from INRA (France) and ILVO (Belgium) for providing cysts of *Globodera* spp.

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