Can. J. Plant Pathol. (2010), **32(2)**: 252–263

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Soilborne pathogens/Agents pathogènes telluriques

Molecular characterization of Canadian populations of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* using ribosomal nuclear RNA and cytochrome *b* genes

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(Accepted 10 January 2010)

Abstract: The mitochondrial cytochrome b gene (*cytb*), the internal transcribed spacer region (ITS1-5.8S-ITS2) of the rRNA gene and D2-D3 expansion segments of the 28S rRNA gene were amplified, sequenced and used to characterize several populations of potato cyst nematodes, *Globodera pallida* and *G. rostochiensis*, collected from different areas in Canada. Diagnostic PCR-ITS-RFLP profiles with three restriction enzymes are provided for identification of both species. Sequences of ITS rRNA and *cytb* genes were compared with those in Genbank of other potato cyst nematode populations originating from Europe, South America, USA, Australia and New Zealand. The ITS rRNA sequences of Canadian *G. rostochiensis* were similar to those of all previously sequenced populations of this species. Sequence divergence of ITS rRNA for *G. rostochiensis* varied from 0 to 1.6%, whereas for *G. pallida* sequence divergence among populations reached 1.95%. Sequence and phylogenetic analysis of *cytb* and ITS rRNA genes using Bayesian inference revealed that Canadian *G. pallida* is almost identical to European and USA populations and formed a large clade with all these populations on the phylogenetic trees. The present molecular result with *cytb* confirmed the hypothesis that there are possibly several sibling species within *G. pallida*. Our study also supports a previously proposed hypothesis regarding introduction of *G. pallida* from a restricted area in Peru, firstly into Europe with subsequent spread to other continents including North America. Our sequence analysis revealed that several newly obtained sequences cannot be translated into functional *cytb* protein, because point indels disrupt the reading frame. Poly(T) variation in mtDNA genes in *G. pallida* might be explained by post-transcriptional editing mechanisms in *Globodera* mitochondria as well as by errors during PCR amplification of mononucleotide repeats within these genes.

Keywords: molecular diagnostics, mitochondrial DNA, potato cyst nematode

Résumé: Le gène mitochondrial cytochrome b (*cytb*), la région de l'espaceur transcrit interne (ITS1-5.8S-ITS2) du gène rARN et les segments du gène rARN 28S ont été amplifiés et utilisés pour caractériser plusieurs populations de nématodes à kystes de la pomme de terre, *Globodera pallida* et *G. rostochiensis*, collectés dans différentes régions du Canada. Les profils de diagnostics obtenus par PCR, IT et RFLP, traités avec trois enzymes de restriction, servent à identifier les deux espèces. Les séquences d'ITS des gènes rARN et *cytb* ont été comparées à celles de la GenBank ainsi qu'avec d'autres populations de nématodes à kystes de la pomme de terre provenant d'Europe, d'Amérique du Sud, des États-Unis, d'Australie et de Nouvelle-Zélande. Les séquences d'ITS du gène rARN de la souche canadienne de *G. rostochiensis* étaient semblables à celles de toutes les populations préalablement séquencées de cette espèce. La divergence dans les séquences de l'ITS rARN pour *G. rostochiensis* a varié de 0 à 1,6 %, tandis que, pour *G. pallida*, la divergence dans les séquences a atteint 1,95 %. L'analyse phylogénétique et celle de la séquence des gènes *cytb* et ITS rARN, basée sur l'inférence bayésienne, ont révélé que la souche canadienne de *G. pallida* est presque identique à l'européenne et à l'américaine, et formait une large variante sur les arbres phylogénétiques. Ces résultats découlant de l'analyse moléculaire avec *cytb* confirment l'hypothèse qu'il existe probablement plusieurs espèces sœurs chez *G. pallida*. Notre étude soutient également une hypothèse précédente qui avait été proposée en ce qui à trait à l'introduction de *G. pallida* provenant d'une région restreinte du Pérou, d'abord en Europe, puis de là, sur les autres continents, y compris l'Amérique du Nord. Notre analyse des séquences a

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ISSN 0706-0661 print/ISSN 1715-2992 online
DOI: 10.1080/07060661003740033

révélé que plusieurs nouvelles séquences ne peuvent être traduites en protéine *cytb* fonctionnelle parce que les indels perturbent le cadre de lecture. La variation Poly(T) dans les gènes de l'ADN mitochondrial de *G. pallida* pourrait être expliquée par les mécanismes d'édition post-transcriptionnelle dans les mitochondries de *Globodera* ainsi que par des erreurs qui se produisent durant l'amplification PCR des microsatellites dans ces gènes.

Mots clés: ADN mitochondrial, diagnostics moléculaires, nématode à kystes de la pomme de terre

Introduction

Potato cyst nematodes (PCN) *Globodera pallida* (pale potato nematode) and *G. rostochiensis* (yellow or golden potato nematode) are considered among the most economically important nematode pests of potatoes and are the subject of strict quarantine regulations in many countries. More than 12% of average worldwide potato yields have been reported as lost due to PCN (Bates *et al*., 2002). Annual costs due to these nematodes were estimated at £50 million in the UK (Jones & Perry, 2004) and €440 million in the European Union (Ryan *et al*., 2000).

It is generally accepted that PCN originated from the Andean region of South America, where these nematodes parasitize wild potatoes and other *Solanum* species (Evans & Stone, 1977). From South America, PCN has spread as the result of human activity into many regions of the world. Although potatoes were introduced into Europe in about 1570, it is thought that PCN probably came with tubers from South America into Europe only around 1850 (Evans *et al*., 1975). In Central and North America, both species of PCN have been reported in Panama, Costa Rica, the USA, Canada, and Mexico, but in these countries, PCN still has a rather restricted distribution with small infested areas compared to the wide infestations found in European countries. In the USA, *G. rostochiensis* was first found in 1941 in a potato field on Long Island, New York (Cannon, 1941), and later in Steuben county, western New York (Spears, 1968) and Delaware (Spears, 1969), whereas *G. pallida* was discovered only recently in Idaho (Hafez & Sundararaj, 2007). In Canada, *G. rostochiensis* was first found in 1962 in Newfoundland, and while it does not occur in significant agricultural areas, *G. rostochiensis* was subsequently shown to be present in home gardens in eastern and southern regions of the province near St. John's and Conception Bay (Farstad, 1962; Olsen & Mulvey, 1962; Morris, 1971). In the western part of the country, an infestation of *G. rostochiensis* was first reported in the Saanich Peninsula, Vancouver Island, British Columbia and identified as pathotype R1A (Stone *et al*., 1977). In addition to the ongoing persistence of *G. rostochiensis* on Vancouver Island (Rott *et al*., 2008), this species was also recently found in potato fields in the Saint Amable area of Quebec (Sun *et al*., 2007) and in Alberta (A. Boucher, personal communication). In Canada, *G. pallida* has only been found in the Botwood area of Newfoundland in 1977 (Stone *et al*., 1977) and appears to be restricted to this area, although a field plot at the Avondale research sub-station of Agriculture and Agri-Food Canada was artificially inoculated from the Botwood sample with this species in order to test resistance of potato accessions to PCN (S. Wood, personal communication).

An integrated approach to PCN management is usually based mainly on chemical control, crop rotation and resistant cultivars (Trudgill *et al*., 1987). However, the two PCN species can react differently to control measures. In field experiments, Whitehead *et al*. (1984) found that oxamyl controlled *G. pallida* less effectively than *G. rostochiensis*. Presently, there is very little potato germplasm available that is fully resistant to *G. pallida*, whereas a high level of germplasm resistance is available for *G. rostochiensis*. Thus, rapid identification of the PCN species is critical for planning control measures and implementing rational regulatory decisions.

DNA-based techniques developed during the last decades provide an attractive solution to problems associated with identification of nematodes. Comparisons of PCR-ITS-RFLP profiles and sequences of the rRNA genes facilitate reliable and rapid identification of PCN and their differentiation from each other and closely related cyst nematode species (Ferris *et al*., 1995; Thiéry & Mugniéry, 1996; Orui, 1997; Blok *et al*., 1998; Subbotin *et al*., 2000; Sirca & Urek, 2004; Madani *et al*., 2005, 2008; Uehara *et al*., 2005; Skantar *et al*., 2007). Due to a high copy number in individual cells, lack of recombination and strict maternal inheritance, mitochondrial genes (mtDNA) can also be used as excellent markers in addition to the ITS rRNA for diagnostics and the study of phylogenetic relationships. Recently, mtDNA has been used to study genetic relationships among Peruvian populations of *G. pallida* and to identify the origin of Western European populations of this species (Picard *et al*., 2007; Plantard *et al*., 2008). Using mtDNA gene, cytochrome *b* (*cytb*) sequences, Plantard *et al*. (2008) showed that *G. pallida* presently distributed in Europe was derived from a single restricted area in the extreme south of Peru, located between the north shore of the Lake Titicaca and Cusco. This could possibly be considered the first case where the

origin of a plant pest has been identified with a high degree of accuracy, even though the introduction occurred more than a century ago. This finding supported the hypothesis of Ayub & Rumpenhorst (2000). Based on a comprehensive PCR-ITS-RFLP study of European and South America populations, they suggested that introduction of *G. pallida* into Europe had occurred only from a restricted area and probably in low numbers.

The goals of the present work were to characterize Canadian populations of potato cyst nematodes, *G. rostochiensis* and *G. pallida*, using sequences of ITS rRNA, the D2-D3 expansion regions of 28S rRNA ribosomal nuclear RNA genes, and the *cytb* gene. We used both ITS rRNA and *cytb* gene sequences for a comparative phylogenetic analysis of populations of PCN collected from different regions in Canada. Sequence analyses of these genes were performed to reveal inter- and intra-specific variation and to compare them with those of potato cyst nematodes from other countries in order to test the possible origin of introduction of PCN into Canada. PCR-ITS-RFLP diagnostic profiles for three enzymes are provided for identification of *G. pallida* and *G. rostochiensis*.

Materials and methods

Nematode samples and identification

Ten populations and three species of *Globodera* were used in this study (Table 1). These included four Canadian populations of *G. rostochiensis* (nine samples collected from potato fields in two towns in Quebec, three samples from Newfoundland, two samples from British Columbia) and one Canadian *G. pallida* population (seven samples). The Newfoundland *G. pallida* samples were obtained from the Charlottetown Laboratory collection (P5a) and from roots of the *G. rostochiensis*-resistant potato cultivar, Atlantic, grown in the experimental Avondale plot (P5b) (Table 1). In addition, two samples of *G. pallida* from France, and one each from Idaho, USA and Belgium and two populations of *G. tabacum* from France and the USA were analyzed. Collected cysts were fixed in 90% alcohol and kept for molecular study. Morphological identification of cysts was confirmed by molecular studies, including PCR with specific primers (Bulman & Marshal, 1997) and real-time PCR with species specific TaqMan probes (Madani *et al*., 2008).

DNA extraction

Total genomic DNA was prepared from individual second stage juveniles as well as from cysts filled with eggs and juveniles. Cysts were soaked in water for 1 h, then transferred into a drop of 10–30 L of distilled water on a glass slide and crushed with a sterile micro-spatula (Fisher Scientific, Ottawa, ON) under a stereo-microscope. Parts of the cyst wall were removed and several juveniles were individually selected and transferred to 20 L of double distilled water and then dissected into two or three segments with a scalpel blade. Individual nematodes were transferred to 0.2 mL PCR tubes containing 10 L of worm lysis buffer (WLB; 500 mM KCl, 100 mM Tris-Cl pH 8.3, 15 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20, 0.1% gelatine) and water was added to a final volume of 20 L. The remaining suspension of eggs and juveniles was transferred to a 0.5 mL Eppendorf tube and the volume of nematode suspension was reduced to 20 L after brief centrifugation and re-suspension. Nematodes were then crushed using the end of a flame modified glass Pasteur pipette attached to a microhomogenizer (Kontes Pellet Pestle Motor, Daigger & Co, Vernon Hills, IL) for at least 1 min. Nematode tissues were further broken up by freezing at −70 C for 10 min. Two L of Proteinase K (20 g L^{-1}) was added to each tube and incubated for 1 h at 60 C and 10 min at 94 C followed by a brief centrifugation to remove debris. From 2–4 L of the supernatant were used directly for PCR and the remainder was stored at −20 C.

PCR, RFLP, cloning and sequencing

Several sets of primers were used for amplification of two fragments of the rRNA gene (ITS1-5.8S-ITS2 and D2-D3 expansion segments of 28S rRNA) and one mitochondrial DNA fragment (*cytb* gene) (Table 2). Positions of these primers are given in Fig. 1. PCR was performed in a final volume of 25 L with a commercial freeze-dried master mix containing dNTPs, an optimized PCR buffer and BD TITANIUM *Taq* polymerase, proof reading enzyme and BD TaqStart Antibody (Clontech, CA, USA), using 250 nM of each primer and 3 L of template. The amplification profile for ITS-rRNA consisted of a preheating step of 5 min at 94 C followed by six cycles of 94 C for 1 min, 55 C for 1 min and 72 C for 1 min and additional 28 cycles of 94 C for 1 min, 57 C for 45 s and 72 C for 1 min, and a final extension at 68 C for 5 min. *Cytb* gene was amplified using a thermal profile of 94 C for 5 min followed by 38 cycles at 94 C for 45 s, 58 C for 30 s and 68 C for 1 min and a final extension at 68 C for 5 min. The D2-D3 fragment of the 28S gene was amplified using a thermal profile of 3 min at 94 C followed by six cycles of 94 C for 1 min, 55 C for 1 min and 72 C for 1 min and additional 28 cycles of 94 C for 1 min, 57 C for 45 s and 72 C for 1 min, and a

Table 1. *Globodera* species and populations used in the present study.

Species (population number)	Country, province, town, sample	GenBank accession number		
		ITS-rRNA	D2-D3 of 28S rRNA	*Cytb*
G. rostochiensis (P1)	Canada, Québec, Saint Amable, sample 1	GQ294512	–	–
G. rostochiensis (P1)	Canada, Québec, Saint Amable, sample 2	GQ294513	GQ294484	–
G. rostochiensis (P1)	Canada, Québec, Saint Amable, sample 3	GQ294514		–
G. rostochiensis (P1)	Canada, Québec, Saint Amable, sample 4	GQ294515	–	–
G. rostochiensis (P1)	Canada, Québec, Saint Amable, sample 5	GQ294516	–	–
G. rostochiensis (P1)	Canada, Québec, Saint Amabl, sample 6	FJ212166	GQ294485	–
G. rostochiensis (P1)	Canada, Québec, Saint Amable, sample 7	FJ212162	–	–
G. rostochiensis (P2)	Canada, Québec, Saint Hyacinth, sample 1	GQ294517	GQ294486	–
G. rostochiensis (P2)	Canada, Québec, Saint Hyacinthe, sample 2	GQ294518	GQ294487	–
G. rostochiensis (P3a)	Canada, Newfoundland, Long pond, sample 1	GQ294519	–	–
G. rostochiensis (P3b)	Canada, Newfoundland, Avondale, sample 1	FJ212164	GQ294488	–
G. rostochiensis (P3b)	Canada, Newfoundland, Avondale, sample 2	FJ212165	–	–
G. rostochiensis (P4)	Canada, British Columbia, Saanich, sample 1	GQ294520	–	–
G. rostochiensis (P4)	Canada, British Columbia, Saanich, sample 2	GQ294521	–	–
G. pallida (P5a)	Canada, Newfoundland, Avondale, sample 1	GQ294522	GQ294489	GQ294493 GQ29449[a]
G. pallida (P5a)	Canada, Newfoundland, Avondale, sample 2	GQ355975	GQ294490	GQ294495 GQ294496
G. pallida (P5b)	Canada, Newfoundland, Avondale, sample 3	GQ294523	GQ294491	GQ294497 GQ294498
G. pallida (P5b)	Canada, Newfoundland, Avondale, sample 4	–	–	GQ294499 GQ294500
G. pallida (P5b)	Canada, Newfoundland, Avondale, sample 5	–	–	GQ294501
G. pallida (P5b)	Canada, Newfoundland, Avondale, sample 6	–	–	GQ294502
G. pallida (P5b)	Canada, Newfoundland, Avondale, sample 7	–	–	GQ294503 GQ294504
G. pallida (P6)	USA, Idaho	–	–	GQ294505
G. pallida (P7)	France, Chavornay, sample 1,	–	–	GQ294506 GQ294507
G. pallida (P7)	France, Chavornay, sample 2,	–	–	GQ294508 GQ294509
G. pallida (P8)	Belgium, Dadizele, sample 1,	–	–	GQ294510 GQ294511
G. tabacum (P9)	France, Agen	GQ294524	–	–
G. tabacum (P10)	USA, Connecticut	GQ294525	GQ294492	–

Note: [a] represents two clones.

final extension at 68 C for 5 min. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON).

Purified PCR products of ITS-rRNA were digested with one of the following restriction endonucleases: *Rsa*I, *Nde*I or *Hinf*I (New England Biolabs, Ipswich, MA) using the manufacturer's supplied buffers and recommendations. Restriction fragments were separated on a 2% agarose gel in TBE buffer, stained with ethidium bromide, visualized on a UV transilluminator and photographed with a video camera.

Gel purified PCR products were ligated into pGEM-T vector and transformed to *Escherichia coli* strain JM 109 according to the manufacturer's instructions (Promega, CA, USA). For each gene, one or two clones from selected samples were sequenced in both directions using vector universal primers at the sequencing facility of York University, Toronto, Canada. All new sequences were submitted to the GenBank database under the accession numbers indicated in Table 1.

Sequence and phylogenetic analyses

Sequence data were visualized and edited using Chromas Lite (version 2.01, Technelysium Pty Ltd, Tewantin, Australia, http://www.technelysium.com.au/chromas_lite.html). The newly obtained sequences for each gene were aligned using ClustalX 1.83 (Thompson *et al*., 1997) with default parameters for corresponding gene sequences for *G. pallida*, *G. rostochiensis* and outgroup taxa (*G. tabacum* for the ITS-rRNA, *G. rostochiensis* with *G.* '*mexicana*' for *cytb* analysis and *G. millefolii and G. artemisiae* for the D2-D3 of 28S-rRNA) available from Genbank or published in the scientific literature (Ferris *et al*., 1995; Bulman & Marshall, 1997; Szalanski *et al*., 1997; Blok *et al*., 1998; Subbotin *et al*., 2000; Sirca & Urek, 2004; Uehara *et al*.,

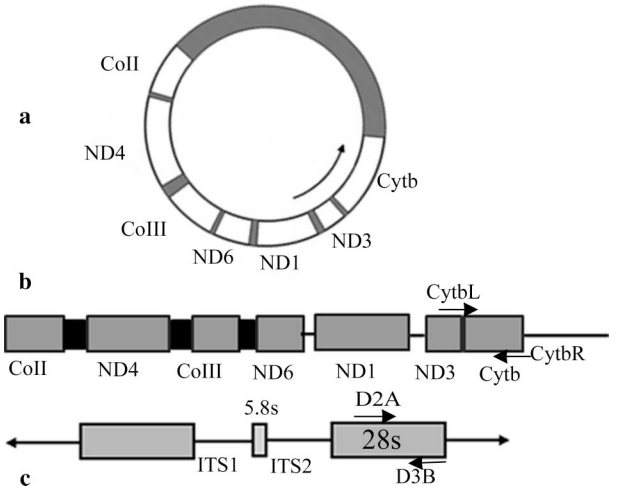

Fig. 1. Genomic organization in *Globodera* species showing **a**, Position of the *cytb* gene in the mitochondrial genome; **b**, Position of the *cytb* primers; and **c**, Position of nuclear rRNA genes and primers.

2005; Knoetze *et al*., 2006; Gibson *et al*., 2007a, 2007b; Skantar *et al*., 2007; Plantard *et al*., 2008; Quader *et al*., 2008; Pylypenko *et al*., 2008; Riepsamen *et al*., 2008). Sequence alignments were manually edited using GeneDoc (version 2.7, Nicholas *et al*., 1997). Pairwise divergence between sequences were computed as the absolute distance values and the percent mean distance values adjusted for missing data with PAUP* 4b4a (Swofford, 2003). Sequence datasets were analyzed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The indels were not considered for the analysis. The best fit model for genetic relatedness was obtained using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion in conjunction with PAUP* 4b4a. BI analysis under GTR+I+G model for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilized after approximately 10^3 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades.

Results

Nematode identification, PCR and RFLP

Identification of all the samples from the 10 *Globodera* populations to species, i.e. *G. rostochiensis* (14 samples), *G. pallida* (7 samples) and *G. tabacum tabacum* (2 samples), were confirmed by conventional multiplex PCR with specific primers and real time PCR with specific TaqMan probes (data not shown).

The amplification of the ITS rRNA region yielded single fragments of 1190–1191 bp for *G. rostochiensis* samples and fragments ranged from 1193–1195 bp for *G. pallida*. Diagnostic PCR-ITS-RFLP profiles for *G. pallida* and *G. rostochiensis* with restriction enzymes, *Rsa*I, *Nde*I and *Hinf*I are given in Fig. 2. PCR product of the D2-D3 region of the 28S gene was 784 bp for each sample of *Globodera* species under study. The *cytb* gene

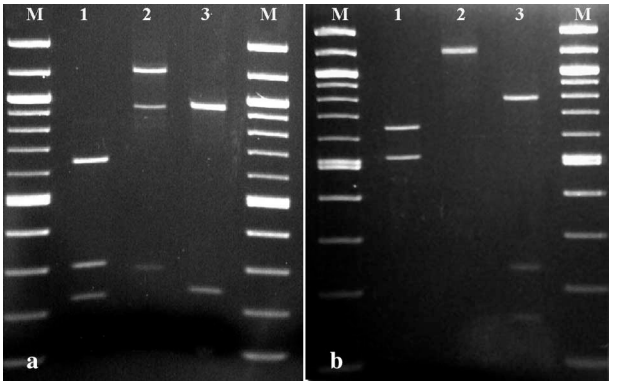

Fig. 2. Restriction fragment length polymorphisms of the ITS region amplified using rDNA1 and rDNA2 primers for **a**, *Globodera pallida* (NF1) and **b**, *G. rostochiensis* (BC) using restriction enzymes (lane 1) *Rsa*I, (lane 2) *Nde*I and (lane 3) *Hinf*I. Lane M is the 100 bp molecular marker.

Table 2. The primers used in the present study.

Primer name	Sequence (5′–3′)	Amplified region	Source
rDNA1	TTGATTACGTCCCTGCCCTTT	ITS1-5.8s-ITS2 rRNA	Vrain *et al*. (1992)
rDNA2	TTTCACTCGCCGTTACTAAGG	ITS1-5.8s-ITS2 rRNA	Vrain *et al*. (1992)
INRAcytbL	GGGTGTGGCCTTGTTATTTC	*cytb* of mtDNA	Picard *et al*. (2007)
INRAcytbR	ACCAGCTAAAACCCCATCCT	*cytb* of mtDNA	Picard *et al*. (2007)
D2A	ACAAGTACCGTGAGGGAAAGTTG	D2-D3 of 28S rRNA	Nunn (1992)
D3B	TCGGAAGGAACCAGCTACTA	D2-D3 of 28S rRNA	Nunn (1992)

was amplified from all *G. pallida* samples. The amplicon sizes for *cytb* gene were 1021 or 1022 bp including primers. No PCR product was obtained in negative controls.

Sequencing analysis

Fourteen new sequences of ITS-rRNA were obtained for *G. rostochiensis*, three for *G. pallida* and two for *G. tabacum*. The ITS-rRNA alignment was 956 positions in length and consisted of 85 accessions including 52 for *G. rostochiensis*, 31 for *G. pallida* and two for *G. tabacum tabacum*. Sequences within *G. rostochiensis* accessions differed in 0–11 nucleotides (0–1.6%), whereas for *G. pallida sensu lato* excluding a population from Argentina (DQ097514) differed in 0–18 nucleotides (0–1.95%). Maximum difference between all studied sequences was 37 nucleotides (4.1%).

Five new sequences of D2-D3 expansion regions of 28S rRNA were obtained for *G. rostochiensis*, three for *G. pallida* and one for *G. tabacum*. The D2-D3 alignment was 635 positions in length and included 18 accessions: eight for *G. rostochiensis*, four for each of *G. tabacum* and *G. pallida* and two for outgroup taxa. Sequences of the D2-D3 of 28S rRNA for Canadian *G. pallida* and *G. rostochiensis* differed in 7–8 nucleotides (0.9–1.0%).

Nineteen new sequences of *cytb* gene were obtained for *G. pallida*, which included 12 sequences for Canadian populations and one for a USA population. Sequence alignment consisted of 73 accessions, i.e. 71 for *G. pallida* and one for each of *G. rostochiensis* and *G.* '*mexicana*' used as outgroup taxa. The *cytb* sequence was 1023 bp in length. Sequence variation of *cytb* for all samples identified as *G. pallida* in the present study reached 1.08% (95 nucleotides). Sequence analysis revealed that several newly obtained sequences cannot be translated into a functional *cytb* protein, because point indels disrupted the reading frame.

Phylogenetic analysis

Phylogenetic relationships within potato cyst nematodes as inferred from the ITS-rRNA gene sequences using Bayesian inference is given in Fig. 3. The ITS data produced two (A and B) major highly supported (PP = 100%) clades separating the two species of *G. pallida* and *G. rostochiensis*. Four subclades (I–IV) included populations identified as *G. pallida* and one large clade (A or V) included all sequences of *G. rostochiensis*. Canadian populations of *G. pallida* formed a highly supported (PP = 100%) subclade I together with all the populations that were included from Europe, New Zealand, USA and several populations from South America. Subclades II and III included only populations from Peru. The Argentinean population (subclade IV) occupied a basal position within clade A.

In the *cytb* based phylogeny analysis, the *G. pallida* populations formed three major highly supported (PP = 100%) clades: I, II and III on the phylogenetic tree (Fig. 4), which corresponded to the subclades I–III on the ITS tree (Fig. 3). Clade I was subdivided into six (1–6) subclades with the largest one (subclade 1) consisting of *G. pallida* from Europe, North America and a sample from Colca, Peru. All Canadian and one USA sample belonged to subclade 1 of clade I, whereas European populations included in the phylogeny were distributed among four subclades (1, 3, 4 and 5) of clade I. Clades II and III contained only populations from Peru.

In the D2–D3 phylogeny, two weekly supported clades (PP = 54 and 72%), which included *G. pallida* and *G. tabacum* with unresolved positions of *G. rostochiensis* samples were observed (data not shown). The D2–D3 sequences did not contain enough informative sites to reconstruct robust phylogenetic relationships within the genus *Globodera*.

Discussion

Due to the limited area infested with PCN in Canada, our *G. pallida* samples were restricted to those from a small area in Newfoundland. This is the only area that *G. pallida* has ever been found and it may have entered the country with early European settlers of this island. However, there is no scientific evidence concerning their origin of entry. Analysis of ITS-rRNA and *cytb* sequences obtained from Canadian and USA populations allowed clarification of the origin of North American populations of *G. pallida*. After studying populations of *G. pallida* from Peru and European countries, Plantard *et al*. (2008) found that 15 Western European populations were distributed within two clades on a phylogenetic tree constructed using *cytb* sequences. Four *cytb* haplotypes were found in Western Europe, one of them being also found in some populations in southern Peru. Thus, *G. pallida* in Europe appears to be derived from a single restricted area in the extreme south of Peru, located between the north shore of Lake Titicaca and Cusco (Plantard *et al*., 2008). However, the question of the number of introductions remains uncertain, because these data could support two alternative hypotheses of origin of Western European populations. Either one unique introduction, if possible intra-population haplotype diversity is taken into account, or double or even multi-introduction, if the results of only phylogenetic analysis are considered. In the present analysis with 19 newly

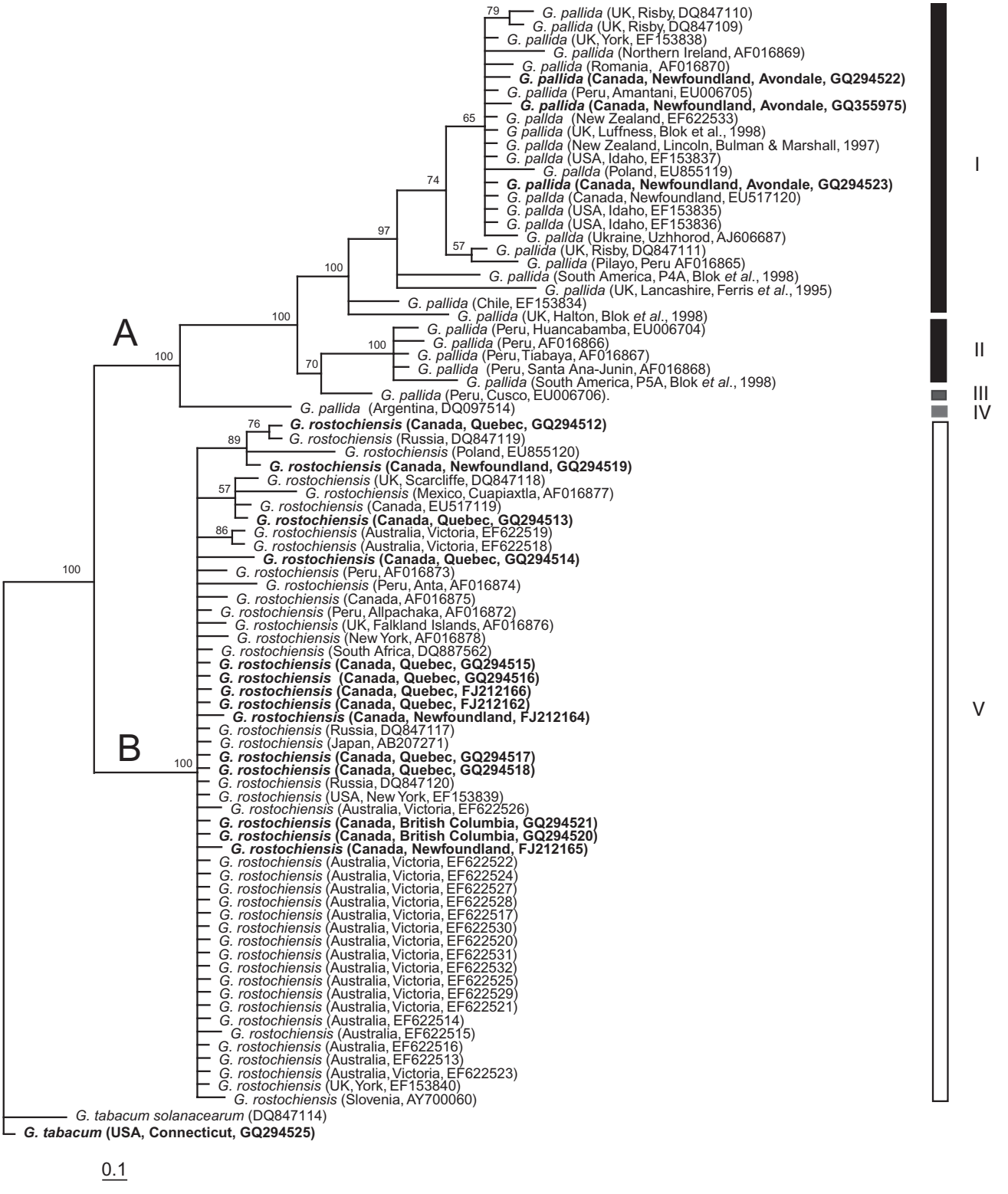

Fig. 3. Phylogenetic relationships between *G. pallida* and *G. rostochiensis* samples: Bayesian 50% majority rule consensus tree from two runs as inferred from ITS1-5.8S-ITS2 sequences of rRNA gene alignment under the GTR+I+G model. Newly obtained sequences are indicated by bold letters.

Fig. 4. Phylogenetic relationships between *G. pallida* samples. Bayesian 50% majority rule consensus tree from two runs as inferred from *cytb* sequence alignment under the GTR+I+G model. *- identified as *G. rostochiensis*. Newly obtained sequences are indicated by bold letters.

obtained sequences and published sequences from Plantard *et al*. (2008) and Pylypenko *et al*. (2008), the Western European populations distributed within four clades (Fig. 4), which might support the hypothesis of multi-introductions of *G. pallida* into Europe from the restricted area in southern Peru. With regard to the introduction of *G. pallida* into North America, a high similarity of *cytb* sequences of populations from Canada and the USA with the sequence of the main European *G. pallida* haplotype (Subclade 1, Fig. 4) was observed in our study. In our ITS analysis, the Canadian and USA *G. pallida* populations were also located in the clade containing samples from Europe and New Zealand and a sample from Amantani, Peru, which is in the Lake Titicaca area (Fig. 3). Thus, the ITS analysis largely corresponds with the phylogeny based on the *cytb* gene, and these analyses suggest that the North American populations are the result of continuing spread of *G. pallida* from Western Europe to other countries and continents, and are unlikely the result of a separate introduction to North America directly from South America.

Although ITS sequence diversity for *G. rostochiensis* populations was only slightly less than that for *G. pallida* (1.6 vs. 1.95), our phylogenetic analysis did not reveal distinct phylogenetic patterns among geographical isolates of this species, and relationships between these *G. rostochiensis* sequences were unresolved. It has been shown that *G. rostochiensis* populations contain a mixture of ITS haplotypes, with a different ratio for different populations, and that sequence diversity of haplotypes within a population could reach 1.02% (Subbotin *et al*., 2000). Thus, this fact together with the relatively low rate of evolution of the ITS-rRNA gene fragment does not allow us to use it as a reliable genetic marker for the study of recent introduction events. The conclusion made by Quader *et al*. (2008) that possibly up to seven introductions of *G. rostochiensis* into Victoria, Australia occurred is not supported by our analysis. Our analysis also does not allow us to determine the origin of Canadian *G. rostochiensis* populations.

After studying PCR-ITS-RFLP patterns generated by 16 restriction enzymes for 30 populations from seven European countries and 24 populations of *G. pallida* from Peru, Ecuador and Colombia, Rumpenhorst & Ayub (2001) concluded that three RFLP profiles represented all their samples: (i) European type, and one population from Peru; (ii) South American type; and (iii) Peruvian/Ecuadorian type with an RFLP pattern that is a combination of the European and South American patterns. Digestion with *Taq*I, *Rsa*I, *Mva*I, *Pst*I, *Nla*III and *Mse*I clearly separated the European populations from the South American one. Grenier *et al*. (2001) also reported heterogeneity in the ITS-rRNA of Peruvian populations and revealed several restriction enzymes distinguishing European and Peruvian populations. In our study, we used only one of the restriction enzymes (*Rsa*I) used by Rumpenhorst & Ayub (2001), but our RFLP patterns combined with predicted restriction profiles of the ITS sequence with other enzymes, showed the identity of the Canadian *G. pallida* population with European ones.

Distinct genetic differences between European populations and South American populations belonging to the P5A pathotype (Phillips & Trudgill, 1998) of *G. pallida* were revealed by ITS-rRNA (Blok *et al*., 1998; Subbotin *et al*., 2000; present study) and *cytb* (Picard *et al*., 2007; Plantard *et al*., 2008; Pylypenko *et al*., 2008; present study) sequences and phylogenetic analyses, simple sequence repeat primer analysis (Blok & Phillips, 1995), PCR-RAPD (Blok *et al*., 1997; Bendezu *et al*., 1988; Grenier *et al*., 2001; Rumpenhorst & Ayub, 2001), IEF of proteins (Rumpenhorst & Ayub, 2001), 2-DGE of proteins (Grenier *et al*., 2001), and satellite DNA analysis (Grenier *et al*., 2001). The sequence and phylogenetic analyses of *Globodera* species parasitizing solanaceous plants made by Subbotin *et al*. (2000) indicated the presence of a separate group of populations closely related to *G. pallida*, which might represent an undescribed *Globodera* species. Based on PCR-RAPD, IEF and ITS-rRNA RFLP and sequence analyses, Rumpenhorst & Ayub (2001) came to the conclusion that a third species might exist within PCN. In our present analysis, the South American populations of *G. pallida*, which formed the subclade II in the ITS tree (Fig. 3) and clade II in the mtDNA tree (Fig. 4), might represent this third PCN species. Moreover, our phylogenetic analysis of *cytb* and ITS-rRNA genes suggests two other candidates for undescribed species (*i* – subclades III in the ITS-rRNA tree and clade III in the mtDNA tree and *ii* – subclade IV in the ITS-rRNA tree). Thus, the pale potato cyst nematode *G. pallida* may indeed represent a species complex. However, we agree with Grenier *et al*. (2001) who emphasized the need to augment these molecular data with biological and morphological studies before addressing the exact taxonomic position of the South American populations.

Mitochondrial DNA has considerable variation in size, structure and gene content amongst organisms. Analysis of mitochondrial DNA from *G. pallida* and *G. rostochiensis* showed an unusual structural organization when compared with other metazoa. This structure consists of six multipartite small circular mtDNA (scmtDNAs) which is unlike most other organisms (Armstrong *et al*., 2007; Gibson *et al*., 2007a, 2007b). Recently, after sequencing multiple clones from DNA extracted from both multiple

individuals and from single cysts of *G. rostochiensis*, Riepsamen *et al*. (2008) revealed a predominant variation in *ND4* and *cytb* in the length of polythymidine tracts. Several nucleotide sequences of these genes can not be conceptually translated into functional proteins, because point indels disrupted the reading frame, or due to presence of premature stop codons in these clones. These authors concluded that the observations were consistent with a hypothesis of post-transcriptional editing in *Globodera* mitochondria, although this was difficult to show conclusively in the presence of intra-individual gene sequence variation. A possible post-transcriptional editing mechanism for tRNA had been previously postulated for mtDNA of different organisms (Hiesel *et al*., 1994; Yokobori & Pääbo, 1995; Kurabayashi & Ueshima, 2000; Masta & Boore, 2004). As an alternative explanation that does not invoke RNA editing, Riepsamen *et al*. (2008) also suggested that the most studied copies were pseudogenes and a small proportion of gene copies that require no editing may be sufficient to maintain mitochondrial function. In our study, after direct sequencing of *cytb* PCR products from several samples, we failed to obtain good sequences and observed numerous double peaks on chromatograms. The sequences obtained from clones showed a variation in polythymidine tracts. In our opinion, PCR errors should also be considered as a possible explanation for the apparent poly(T) variation in mtDNA genes. It has been known that most DNA polymerases introduce error at the rate of approximately 1.1×10^{-4} to 8.9×10^{-5} point mutation/bp/duplication (Cariello *et al*., 1991; Barnes, 1992). In contrast, Clarke *et al*. (2001) found that mononucleotide and dinucleotide repeats were not faithfully reproduced during PCR. Sequencing of individual cloned PCR products using *Taq* polymerase for different genes revealed correct amplification of the $(T)_{11}$ in 90% and $(T)_{13}$ only in 33% of the cases. These data are congruent with the results of our present study and Riepsamen *et al*. (2008), where the highest poly(T) variation was found for copies with lengths of more than $(T)_8$. Although, in our experiments, we used a proof reading enzyme which should minimize errors during amplification, we cannot exclude that observed length variation for polythymidine tracts in mtDNA genes of *G. pallida* might be the result of PCR errors. Additional experiments should be done to resolve this issue.

In summary, the analysis of *cytb*, ITS1-5.8S-ITS2 and D2-D3 of 28S rRNA gene from several *Globodera* samples collected in Canada allowed us to confirm their species identification and suggest that *G. pallida* was introduced in Canada from Europe. However, the data was insufficiently informative to clarify the origin of *G. rostochiensis*. Additional studies of more variable and informative regions of the nematode genome are required on the population structure of *Globodera* species to allow construction of a more complete picture of the distribution of PCN from South America and around the world.

Acknowledgements

The authors thank Drs N. Viaene (ILVO, Belgium), X. Wang (Cornell University, NY, USA), D. Mugniery (INRA, Rennes, France) and J.A. LaMondia (Connecticut Agricultural Experiment Station, Windsor, CT, USA) for providing nematode materials.

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