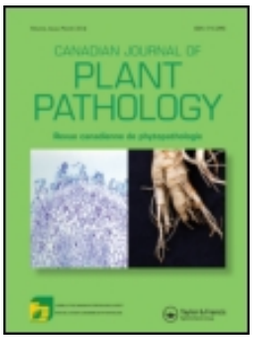

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Characterization of *Globodera rostochiensis* (Tylenchida: Heteroderidae) associated with potato in Quebec, Canada†

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Abstract: In August 2006, a ware potato field of 19 ha, in the Saint-Amable region, Quebec, Canada showed patches of poor growth. Roots of infested plants showed golden spherical shaped cysts. Cysts and second stage juveniles were extracted from soil and root samples. The cyst nematodes were subsequently identified as *Globodera rostochiensis* Wollenweber, 1923 (Behrens, 1975) by morphological and molecular methods. The cysts had the general characteristics of *Globodera* genus with a Granek's ratio of 4.4 (1.8–6.0). The second stage juvenile stylets were 21 (18–23) µm long with rounded knobs, the dorsal one sloping posteriorly. Polymerase chain reaction of the nematodes with the species-specific primers yielded a fragment of about 315 bp, consistent with the fragment from a known *G. rostochiensis*. The phylogenetic analysis inferred by the sequence of the rDNA ITS region confirmed the identity of the cyst nematode from Quebec, Canada as *G. rostochiensis* with 100% match to the *G. rostochiensis* from Russia, Peru, Japan and UK.

Keywords: detection, diagnosis, *Globodera rostochiensis*, molecular biology, morphology, PCN, PCR, phylogeny, potato, rDNA, RFLP

Résumé: Au mois d'août 2006, un champ de pommes de terre de consommation de 19 ha de la région de Saint-Amable au Québec, Canada, affichait des portions où la croissance laissait à désirer. Les racines des plantes infestées étaient couvertes de kystes sphériques dorés. Des kystes et des jeunes de deuxième stade ont été extraits du sol et des échantillons de racines. À la suite d'analyses morphologiques et moléculaires, les kystes des nématodes ont été identifiés en tant que *Globodera rostochiensis* Wollenweber, 1923 (Behrens, 1975). Les kystes affichaient les caractéristiques du genre *Globodera* avec un ratio de Granek de 4,4 (1,8–6,0). Les stylets des jeunes de deuxième stade mesuraient 21 µm (18–23) de long et possédaient des tubercules arrondis, le tubercule dorsal étant incliné vers l'arrière. La réaction des nématodes aux amorces spécifiques d'espèces de la PCR a produit un fragment d'environ 315 bp, correspondant au fragment d'un *G. rostochiensis* connu. L'analyse phylogénétique déduite de la séquence de la région du rADN ITS a confirmé l'identité du nématode à kyste provenant du Québec en tant que *G. rostochiensis*, et la compatibilité entre celui-ci et les *G. rostochiensis* de Russie, du Pérou, du Japon et du Royaume-Uni était totale.

Mots clés: biologie moléculaire, détection, diagnose, *Globodera rostochiensis*, morphologie, PCN, PCR, phylogénie, pomme de terre, rADN, RFLP

Introduction

Two species of the potato cyst nematode (PCN) which include the potato golden cyst nematode, *Globodera rostochiensis* (Wollenweber 1923) Behren 1975, and the potato pale nematode, *G. pallida* (Stones 1973) Behren 1975, are serious pests of potatoes worldwide and are quarantined internationally, subject to stringent regulatory measures wherever either one or both occur. Potato cyst nematode can be devastating pests of potatoes in temperate regions if not controlled (Baldwin & Mundo-Ocampo, 1991; Marks & Brodie, 1998).

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Table 1. Morphometrics of *Globodera rostochiensis* associated with potato in Quebec, Canada compared with neotypes.

Stage	Character	Neotypes by Golden & Ellington (1972)	Manduric *et al.* (2004)	Quebec population
J2				
	n	50	36	60
	L (mm)	0.43 (0.37–0.47)[a]	0.44 (0.35–0.57)	0.41 (0.36–0.47)
	Tail length (µm)	51 (44–57)	54 (40–61)	50 (42–59)
	Stylet (µm)	22 (21–23)	23 (19–24)	21 (18–23)
	Hyaline tail terminal (µm)	24(18–30)	25 (15–33)	24 (17–31)
	a	19 (16–23)	19 (15–26)	18 (15–25)
	b	2.3 (2.2–2.5)	2.4 (2.1–2.7)	2.3 (2.1–2.7)
	c	8 (7–9)	9 (6–10)	8 (7–9)
Cyst				
	n	50	35	35
	Number of cuticular ridges	21 (12–31)	20 (12–28)	21 (12–31)
	Anus–vulva distance (µm)	60 (35–77)	74 (38–149)	81 (29–165)
	Granek's ratio	4.1 (1.3–9.5)	4.5 (1.7–6.7)	4.4 (1.8–6.0)

Note: [a]Data are in the format of average (range).

Globodera rostochiensis has been found in 75 countries with the most recent report from Iran (Gitty & Maafi, 2009). In North America, this species occurs in Mexico, USA and Canada (Brodie, 1998). In Canada, *G. rostochiensis* has previously been found in the Saanich Peninsula of Vancouver Island, British Columbia (Orchard, 1965), and both *G. rostochiensis* and *G. pallida* were found on the island of Newfoundland (Morris, 1971). Recently, another species of this genus *G. tabaccum* (Lownsbery & Lownsbery) was detected in tobacco in Quebec (Bélair & Miller, 2006).

In August 2006, potato cyst nematodes were discovered in a potato field in Saint-Amable, Quebec and were diagnosed as *G. rostochiensis* (Sun *et al*., 2007). After an extensive survey, the infested area was defined and strict regulatory measures were implemented to prevent the further spread of *G. rostochiensis.* National surveys of PCN and other comprehensive collaborative research projects on pathotypes, genetic populations and integrated management have been implemented by scientists from the Canadian Food Inspection Agency (CFIA), Agriculture and Agri-Food Canada (AAFC) and Le Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ).

Due to their huge economic and trade impacts, accurate and rapid identification of *G. rostochiense* and *G. pallida* is extremely important. Morphological identification based on a few characters of the second stage juvenile (J2) and of the perineal of the cyst have been quite successful but always with some uncertainty, since the morphometrics of these characters are overlapping. Molecular methods such as Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP) and DNA sequence comparison have been successfully applied to differentiate cyst nematode species, especially *G. rostochiensis* and *G. pallida*. PCR was used for identifying PCN in Australia (Bulman & Marshall, 1997) and Ukraine (Pylypenko *et al*., 2005), and species-specific primers for PCR have been developed for identifying *G. rostochinesis* and *G. pallida* (Fullaondo *et al*., 1999). Amplified fragment length polymorphism (AFLP) was used for identifying *G. tabacum* (Marché *et al*., 2001), and DNA sequencing for *G. pallida* in Idaho, USA (Skantar *et al*., 2007).

The objective of this study was to characterize *G. rostochiensis* from Quebec, Canada using morphological and molecular methods.

Materials and methods

Nematode samples

Voucher specimens of *G. rostochiensis* and *G. pallida* previously collected from infested fields in Newfoundland and deposited in the Canadian National Collection of Nematodes in Ottawa were used as the reference materials for the molecular comparisons. Soil samples were collected from infested fields in Saint-Amabel, Quebec, Canada. The cysts were extracted from the soil samples using the Fenwick Can method (Fenwick, 1940). The second stage juveniles (J2) were hatched from the cysts with the presence of potato root exudates (Evans, 1983). Eight isolates of *G. rostochiensis* from Quebec and one isolate of *G. pallida* from Newfoundland were used for molecular studies.

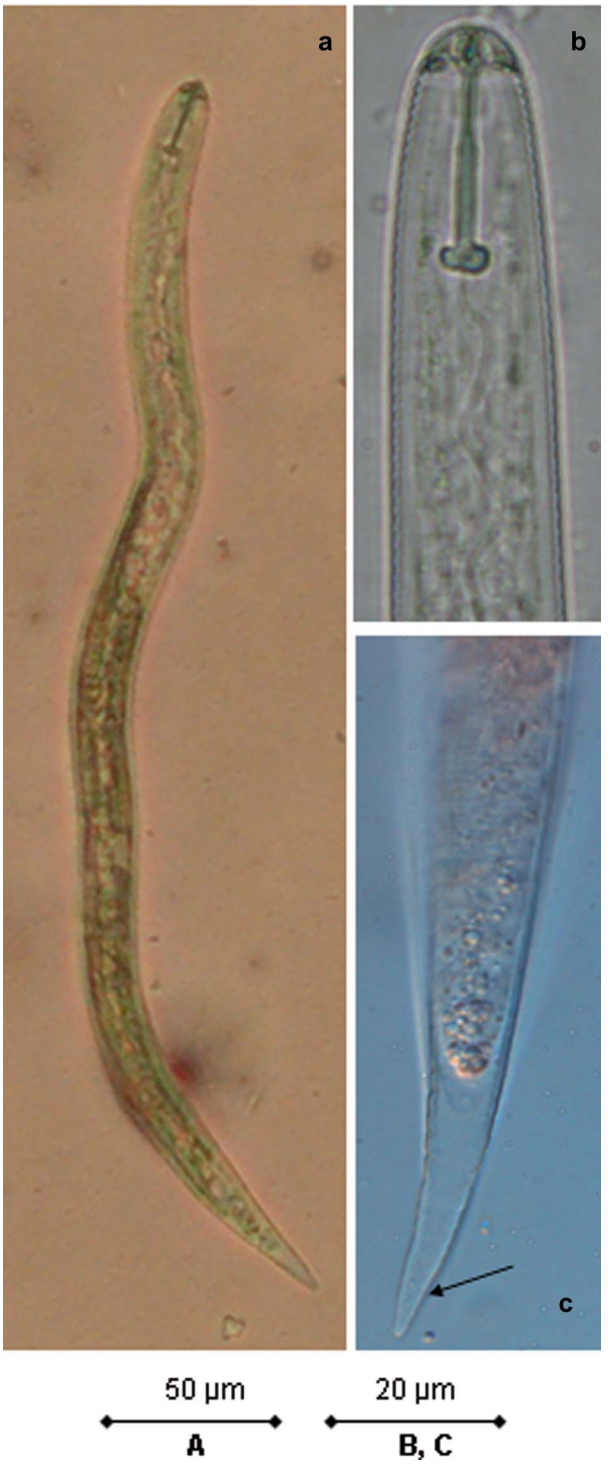

Fig. 1. (Colour online). Photomicrographs of the second stage juveniles of *G. rostochiensis* associated with potato in Quebec, Canada: **a**, entire body; **b**, head; **c**, tail.

Morphological characterization

Vulva cones of cysts were cut from cysts and mounted in Canada balsam (Hooper, 1970). Juveniles were fixed in TAF (formalin, triethanolamine and distilled water at a ratio of 7:2:92) and processed in glycerin. Specimens were examined using a Leica DM5500 B compound microscope with differential interference contrast and pictures were taken with a Leica DFC 420 digital camera. The observed characters of the cysts and J2 were compared with those reference materials and the description of the Neotypes in the literature (Golden & Ellington, 1972; Manduric *et al*., 2004). Measurements were made using a Lexica micro application system on the images, and dimensions are expressed in a formula proposed by de Man (1880).

Nematode DNA

Freshly hatched juveniles were used for DNA extractions. Each juvenile was cut with a sharp forceps and placed into an Eppendorf tube with 20 µL of the prepared lysis buffer (10 mM TRIS pH 8.0, 1mM EDTA, 1% Tergitol-type NP-40 and 100 µg mL^{-1} proteinase K) and incubated at 55 °C for 1 h. Following the lysis reaction, proteinase K was inactivated by incubating the samples at 95 °C for 10 min. Samples were stored in a –20 °C freezer.

PCR by species-specific primers

Species identification for *G. rostochiensis* and *G. pallida* was performed by using the primers and methods developed by Fullaondo *et al*. (1999). The primers for *G. rostochiensis* were: 5′-GCAAGCCAGCGTCAGCAA C-3′ and 5′-GAACATCAACCTCCTATCGG-3′. The primers for *G. pallida* were: 5′-TGTCCATTCCTCTCC ACCAG-3′ and 5′-CCGCTTCCCCATTGCTTTCG-3′.

PCR–RFLP

The rDNA ITS 1 region of the nematodes were PCR amplified using primers: rDNA1 (5′- TTGATTACGTCC CTGCCCTTT-3′) (Vrain *et al*., 1992) and 1.58S (5′-ACGAGCCGAGTCATCCACCG-3′) (Cherry *et al*., 1997). The PCR-amplified products were digested with restriction enzyme *Bst*U I following the protocol provided by the supplier (Sigma, St. Louis, MO). A master mix was prepared for PCR: 1X Native Pfu Ultra Buffer (VWR, Mississauga, ON), 0.2 µM dNTP (Qiagen, Mississauga, ON), 0.6 µM each primer (Invitrogen, Burlington, ON), 0.05 U μL^{-1} native Pfu ultra high fidelity DNA polymerase (VWR), ultra pure distilled and DNase, RNase free water (Invitrogen). The total reaction volume was 100 µL. The PCR cycling conditions were as follow: 35 cycles of

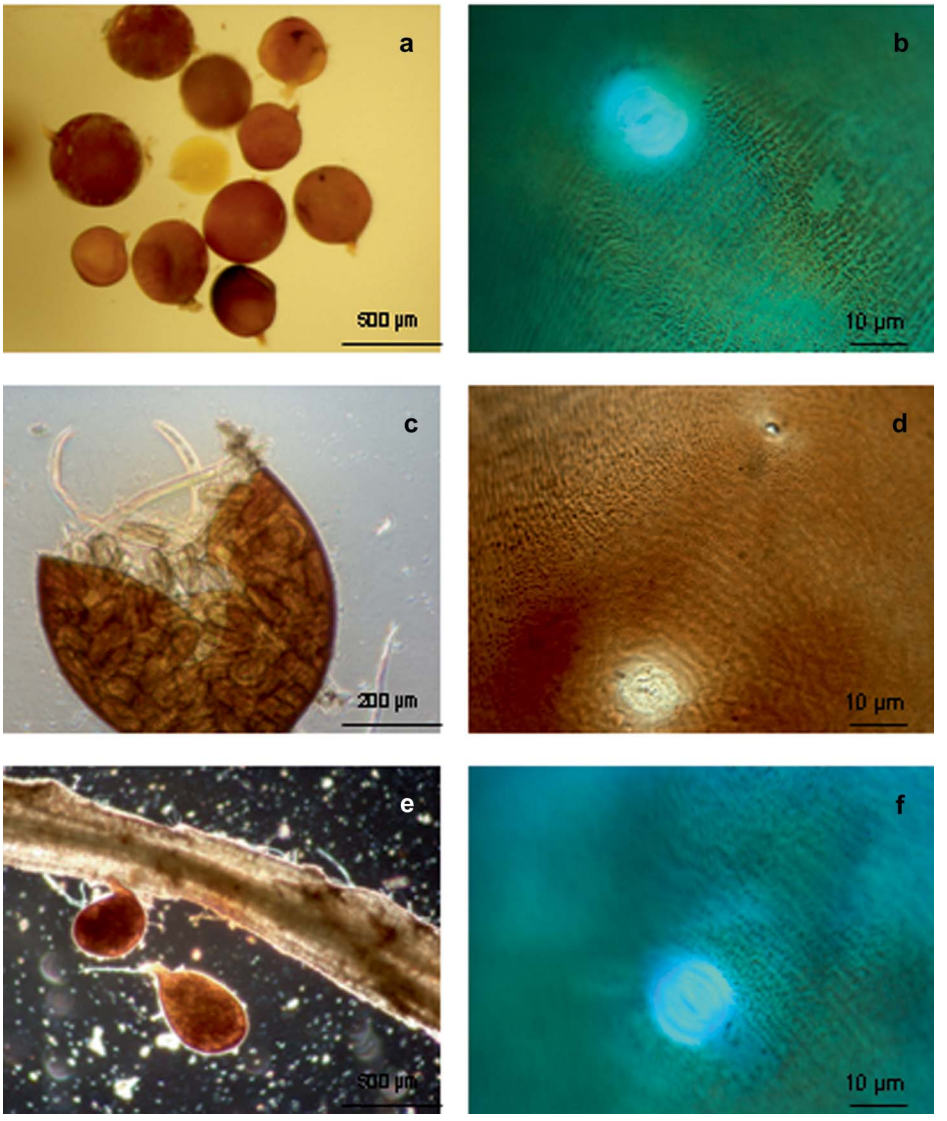

Fig. 2. Photomicrographs of the cysts of *G. rostochiensis* associated with potato in Quebec, Canada: **a**, and **e**, spherical shaped cysts; **c**, crushed cyst to reveal eggs and juveniles; **b**, **d**, and **f**, perineal region of cysts.

95 °C for 30 s, 60 °C for 30 s, 72 °C for 2.5 min. The PCN product was digested with restriction enzyme *BstU I* and revealed by electrophoresis on a 1.5% agarose gel. Ultra pure distilled, water and lysis buffer were used as negative control.

PCR, cloning and DNA sequencing

The segment of rDNA comprised of partial 18S, ITS 1, 5.8S, ITS 2 and partial 28S of the nematode was amplified by PCR. The primers were rDNA1 (5′-TTGAT TACGTCCCTGCCCTTT -3′) and rDNA2 (5′-TTTCAC TCGCCGTTACTAAGG -3′), and the PCR conditions were as specified by Subbotin *et al*. (2000). The amplified products on the gels were cut out, extracted, and purified using the GenElute Gel Extraction kit (Sigma-Aldrich, Oakville, ON), and cloned directly into the pST Blue-1 Blunt vector (Novagen, San Diego, CA) following the protocol provided by the supplies. Fragments were sequenced using Applied Biosystems 3730 DNA Analyzer by the Ottawa Health Research Institute, StemCore Laboratories, DNA Sequencing Facility (Ottawa, ON).

Phylogenetic analysis

DNA sequences were aligned by Clustal W (http://workbench.sdsc.edu, Bioinformatics and Computational Biology Group, Dept. Bioengineering, UC San Diego,

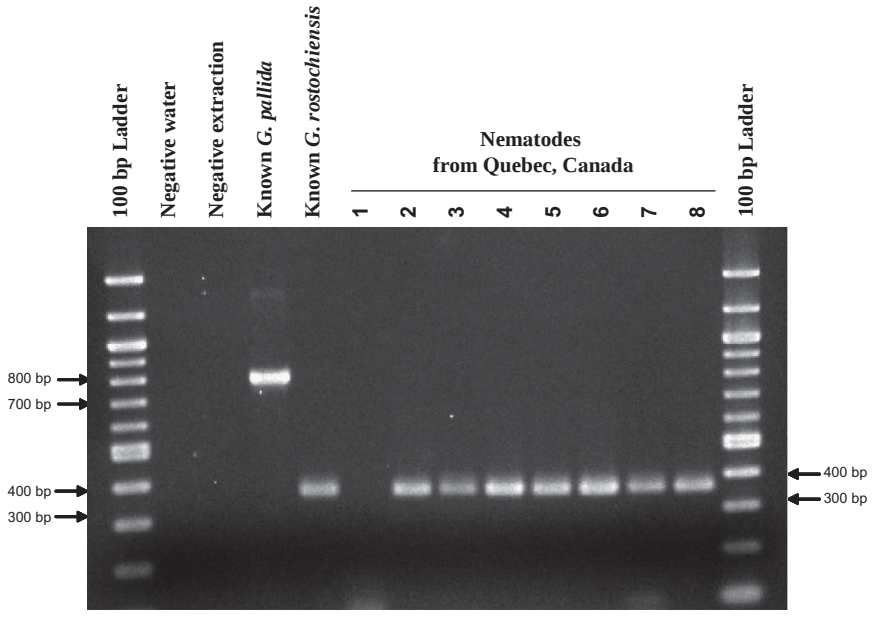

Fig. 3. Agarose gel electrophoresis for PCR using species-specific primers for testing *Globodera* species.

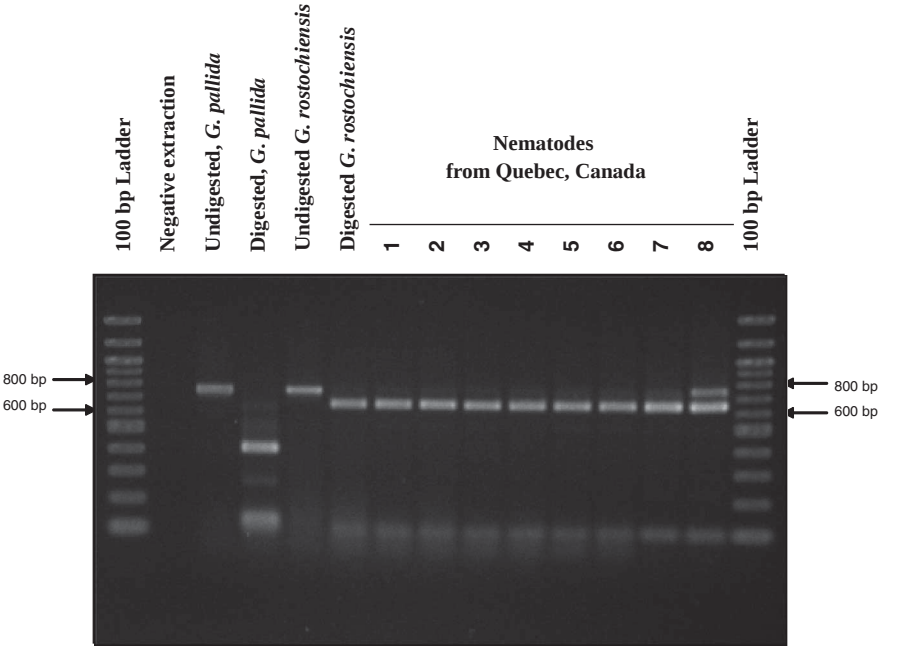

Fig. 4. Agarose gel electrophoresis for PCR-RFLP of rDNA ITS 1 cut by restriction enzyme *Bst*U I for testing *Globodera* species.

CA). The model of base substitution was evaluated using MODELTEST (Posada & Crandall, 1998; Huelsenbeck & Ronquist, 2001). The Akaike-supported model, the base frequencies, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck & Ronquist, 2001) running the chain for 1×10^6 generations and setting the "burnin" at 1000. The MCMC (Markov Chain Monte Carlo) method was used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget & Simon, 1999) using 50% majority-rule.

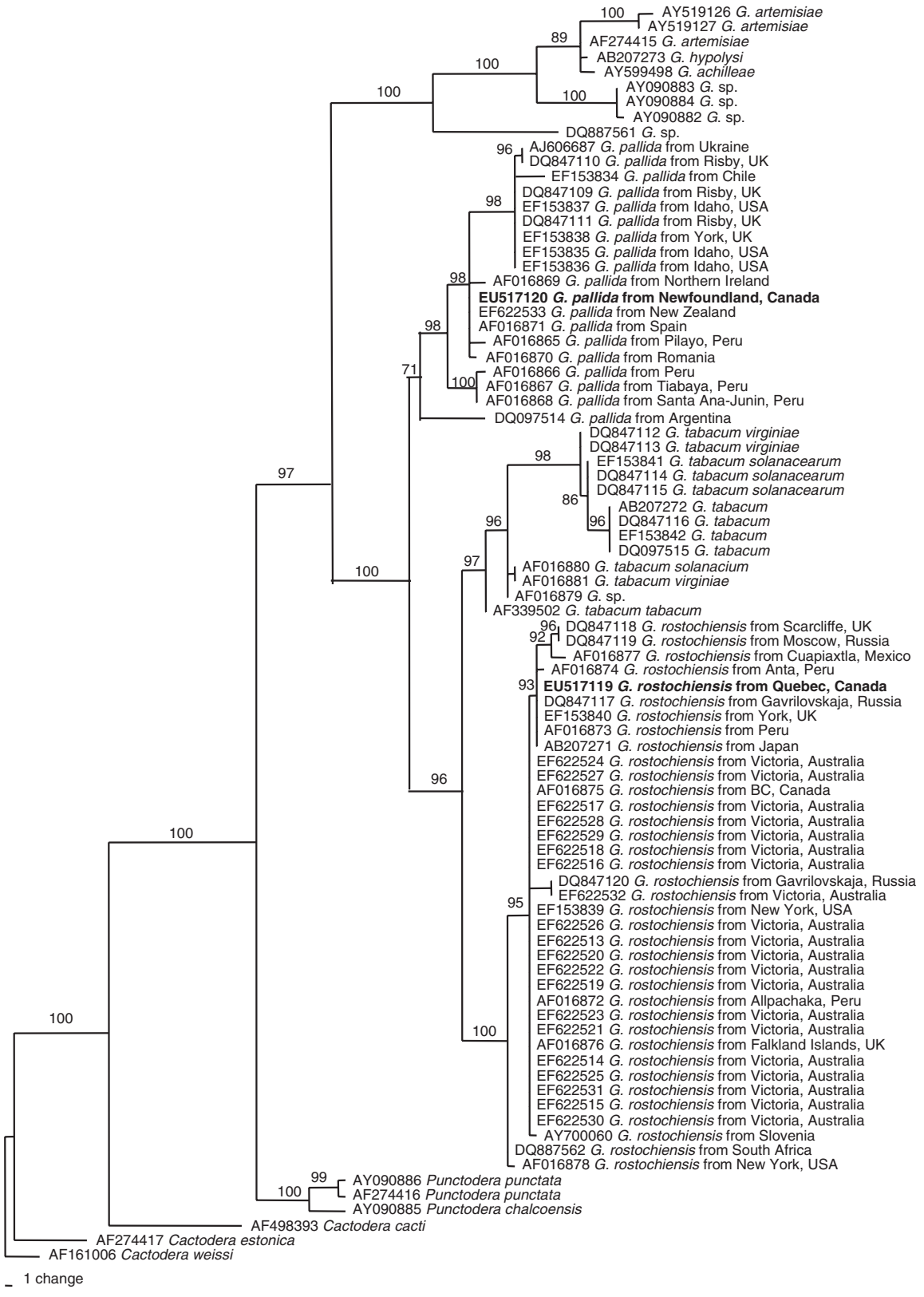

Fig. 5. The 10001st Bayesian tree inferred from ITS1 rDNA sequence under GTR+G model (lnL = 2329.3086; freqA = 0.1895; freqC = 0.2557; freqG = 0.2933; freqT = 0.2614; R(a) = 0.6018; R(b) = 4.0381; R(c) = 1.6271; R(d) = 0.3558; R(e) = 2.5265; R(f) = 1; Pinva = 0; Shape = 0.4198). Posterior probability values exceeding 50% are given on appropriate clades.

Results

Morphology and morphometrics

The morphological characters of the cysts and J2 of the nematodes from Quebec populations matched the descriptions of those of the neotypes of *G. rostochiensis*. The morphometrics were in the range of those of the neotypes (Table 1).

Second stage juvenile. The body tapered off at both ends but much more posteriorly. There was lateral field with four lines for most of the body. The head was offset slightly with five annules. The stylet was well-developed with round knobs, the dorsal knob sloping posteriorly. The excretory pore was posterior and almost adjacent to the hemizonid. The tail tapered to a small, rounded terminus (Fig. 1).

Cysts. Cysts were brown in colour, spherical in shape, with protruding necks. Vulva were fenestrate, much larger than the small but distinct, V-shaped anus. The cyst wall pattern was prominent especially near the mid-body (Fig. 2).

PCR by species-specific primers

All the selected Quebec samples, except one, yielded a single PCR product of about 315 bp, the same size as the one from the known *G. rostochiensis* standard, and the *G. pallida* standard yielded a band at the size of about 800 bp (Fig. 3).

PCR–RFLP

The profiles of the restriction digestion products of the PCR products with *BstU I* of the Quebec samples were consistent with *G. rostochiensis*. All of them had two bands (103, 650 bp), whereas the *G. pallida* had four bands (103, 116, 135, 399 bp) (Fig. 4).

DNA sequencing

Four cloned sequences from the Quebec isolate of *G. rostochiensis* were obtained. One overall consensus sequence was derived from these four cloned sequences, and was used for further phylogenetic analysis. The rDNA sequence of *G. rostochiensis* from Quebec and *G. pallida* from Newfoundland were deposited in Genbank with the accession members EU517119 and EU517120, respectively.

Phylogenetic analysis

Figure 5 presents a phylogenetic tree based on the rDNA ITS 1 from a multiple alignment of 612 total characters. This dataset has 455 constant characters (74.35%). The average nucleotide composition was as follows: 18.95% A, 25.57% C, 29.33% G and 26.14% T. Using *Cactodera weissi* as an out-group taxon, this tree inferred many highly supported monophyletic groups. All species of *Globodera* are in a clade with 97% support, clearly separated from all other genera. All 37 populations of *G. rostochiensis* from different regions of the world were in a 100% supported monophyletic clade, with *G. tabacum* as the closest sister clade. The sequence of the Quebec population was identical to those populations from Gasvarilovakaja, Russia (DQ847117); from York, UK (EF153840); from Peru (AF016873); and from Japan (AB207271). This population has only three nucleotide differences from those populations from BC, Canada (AF016875); from Peru (AF016872); from UK (AF016876); and Victoria, Australia (EF622513 – EF622530). The 19 populations of *G. pallida* from different regions of the world were in one clade, a sister clade to the clade comprised of *G. tabaccum* clade and *G. rostochiensis* clade.

Discussion

The results in this study clearly demonstrated that the cyst nematode associated with potato in Quebec, Canada is the golden cyst nematode, *G. rostochiensis*. The results also demonstrated that the species-specific primers for *G. rostochiensis* for PCR are consistent and reliable. The results revealed that the phylogenetic analysis based on rDNA ITS1 was highly useful for identifying species and examining their phylogenetic relationships from all species/populations around the world. This analysis resolved the key monophyletic groups, including *Globodera*, *G. rostochiensis*, *G. pallida* and *G. tabaccum*. The DNA sequencing is proved to be an accurate tool to identify species with highly similar morphology and morphometrics of the closely related species. The results were in agreement with the results achieved in similar studies by Skantar *et al*. (2007) and Subbotin *et al*. (2000).

The lower level of genetic variation of ITS rDNA of the isolates of *G. rostochiensis* over the genetic variation of ITS rDNA of the isolates of *G. pallida* revealed in this study is in agreement with the finding by Zaheer *et al*. (1996). The higher genetic variation of *G. pallida* over *G. rostochiensis* may suggest the possibility that the former one is a more recently evolved species. Although the ITS sequence of the *G. rostochiensis* from Quebec

was identical to those from Russia, Peru, Japan and UK, this does not necessarily mean that the nematode in Quebec originated from these locations.

Cyst-forming nematodes from Heteroderinae are some of the most important nematode pathogens worldwide (Baldwin & Mundo-Ocampo, 1991). Most species are restricted to cool climates. In Canada, most genera of the family are present, including species of *Dolichodera*, *Globodera*, *Punctodera*, *Heterodera* and *Cactodera* (Ebsary, 1986). In the genus *Globodera*, the three major species are recorded: *G. rostochiensis*, *G. pallida* and *G. tabacum*. As globalization expands, and climate change continues, more resources will be required to combat alien invasive species such as *G. rostochiensis* and *G. pallida* from threatening agriculture in Canada.

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