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Morphological and Molecular Identification of *Globodera pallida* Associated with Potato in Idaho

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Abstract: The identity of a newly discovered population of pale potato cyst nematode *Globodera pallida* associated with potato in eastern Idaho was established by morphological and molecular methods. Morphometrics of cysts and second-stage juveniles were generally within the expected ranges for *G. pallida* with some variations noted. The Idaho population and paratype material from Epworth, Lincolnshire, England, both showed variations in tail shape, with bluntly rounded to finely pointed tail termini. Compared to literature values for the paratypes, second-stage juveniles of the Idaho population had a somewhat shorter mean body length, and cysts had a slightly higher mean distance from the anus to the nearest edge of the fenestra. PCR-RFLP of the rDNA ITS region, sequence-specific multiplex PCR and DNA sequence comparisons all confirmed the identity of the Idaho population as *G. pallida*. The ITS rDNA sequence of the Idaho isolate was identical to those from York, England, and the Netherlands. Species-specific primers that can positively identify the tobacco cyst nematode *Globodera tabacum* were also developed, providing a new assay for distinguishing this species from *G. pallida* and the golden potato cyst nematode *Globodera rostochiensis*.

Key words: *Globodera*, detection, diagnosis, molecular biology, morphology, *Nicotiana tabacum*, PCR, potato, rDNA, RFLP, *Solanum tuberosum*, taxonomy.

Like the golden potato cyst nematode *Globodera rostochiensis* (Wollenweber, 1923) Behrens, 1975, the pale potato cyst nematode *G. pallida* (Stone, 1973) Behrens, 1975 is a regulated pathogen of potato (*Solanum tuberosum*) in the United States and many other countries. Within the Americas, *G. pallida* has been found in South America, Panama and Newfoundland, Canada (Marks and Brodie, 1998; EPPO, 2004), but had not previously been reported in the U.S. In March 2006, cyst nematodes were discovered in tare soil from a potato processing facility in eastern Idaho. The nematodes were found during a routine survey conducted jointly by the Idaho State Department of Agriculture and the USDA Animal and Plant Health Inspection Service through the Cooperative Agricultural Pest Survey program. Six cysts from the new detection were received by the USDA-ARS Nematology Laboratory for species identification. Subsequent extensive sampling traced the nematode to two fields in northern Bingham County, Idaho (Hafez et al., 2007). Morphological and molecular methods that were used to identify and characterize this population as *G. pallida* are described herein. The new discovery of *G. pallida* in Idaho constitutes a significant threat to the U.S. potato industry, which is valued at over $2.6 billion (Agricultural Statistics Board, 2006). The availability of germplasm resistant to *G. pallida* is currently limited. Thus, the ability to distinguish *G. pallida* from other cyst nematodes that may be present is critical for the application of rational regulatory decisions aimed at preventing further infestation.

A few females of *G. pallida* have been experimentally demonstrated to reproduce on tobacco (*Nicotiana tabacum*) (Parrot and Miller, 1977). Similarly, only a few females of morphologically similar *G. tabacum* (Lownsbery and Lownsbery, 1954) Behrens, 1975 have been shown to reproduce on potato (Stone and Miller, 1974; Baldwin and Mundo-Ocampo, 1991). *Globodera tabacum* has been found in the states of Virginia, Connecticut and North Carolina, as well as in Mexico and various countries in Europe and northern Africa (Marché et al., 2001; Syracuse et al., 2004). Most recently, *G. tabacum* was detected in tobacco from Ontario, Canada, although the subspecies was not reported (Bélair and Miller, 2006). The *G. tabacum* complex contains three subspecies that in nature parasitize solanaceous weeds (*G. tabacum virginiae*) and tobacco (*G. tabacum tabacum* and *G. tabacum solanacearum*) (Baldwin and Mundo-Ocampo, 1991; Syracuse et al., 2004).

The morphometrics from juveniles or females cannot clearly differentiate *G. pallida* from the three subspecies of *G. tabacum* (Baldwin and Mundo-Ocampo, 1991; EPPO, 2004). Neither light microscopy (Stone, 1983; Baldwin and Mundo-Ocampo, 1991; Mota and Eisenback, 1993a-c; EPPO, 2004) nor scanning electron microscopy (Othman et al., 1988) of juveniles or females can clearly distinguish *G. pallida* from the three subspecies of *G. tabacum*. While a number of immunological, protein and DNA-based techniques can separate *G. rostochiensis* from *G. pallida* (reviewed in Fleming and Powers, 1998 and Ibrahim et al., 2001), only a few have included both European and non-European populations (Subbotin et al., 2000; Grenier et al., 2001) or included related species such as *G. tabacum*, *"G. mexicana"* (Campos-Vela, 1967), or *G. achilliae* (Thiéry and Mugniéry, 1996). With the appearance of *G. pallida* now confirmed in the U.S. (Hafez et al., 2007), there is an urgent need for new molecular diagnostics capable of differentiating *G. pallida* from species other than *G.*

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rostochiensis that occur in the Americas. A modified multiplex PCR assay that can distinguish *G. tabacum* from *G. pallida* and *G. rostochiensis* is described.

Materials and Methods

Populations: *Globodera spp*. populations described and discussed are listed in Table 1. *Heterodera avenae* from Idaho was also included in this investigation because of its occasional occurrence in survey samples and utility as a control for the PCR-RFLP analysis.

Morphological Characterization: Juveniles for morphological observations were hatched from cysts that had been sieved from fresh soil and kept in water in Syracuse watch glasses in the laboratory. Juveniles were then fixed in 3% formaldehyde and processed to glycerine by the formalin-glycerine method (Hooper, 1970; Golden, 1990). Cysts were similarly removed from soil samples by sieving, fixed for 12 hr in 3% formaldehyde and processed to glycerine. Photomicrographs of cyst vulval cones and J2 were made with an automatic 35-mm camera attached to a compound microscope fitted with an interference contrast system. Light microscopic images of fixed nematodes were taken on a Leica Wild MPS48 Leitz DMRB compound microscope, and measurements were made with an ocular micrometer on the same microscope. Specimens were then morphologically identified with recent taxonomic keys and a compendium for identification of *Globodera* spp. (Golden, 1986; Baldwin and Mundo-Ocampo, 1991). All measurements are in micrometers unless otherwise stated. Differential Interference Contrast (DIC) images of live specimens mounted on an agar pad were taken

Table 1. List of nematode species and populations used in this study

Species	Abbreviation	Location	Accession #	Experiment[a]	References
Globodera pallida	Gp A	Argentina	DQ097514	PA	Lax et al. (GenBank unpubl.)
	Gp C	Chile	EF153834	BM, PA	This study
	Gp D375	Netherlands		PA	Blok et al., 1998
	Gp Halton	England, Halton		PA	Blok et al., 1998
	Gp ID	USA, Idaho	EF153835-EF753837	RFLP, BM, PA	This study
	Gp Lancashire	England, Lancashire		PA	Ferris et al., 1995
	Gp Luffness	Scotland		PA	Blok et al., 1998
	Gp P4A	South America		PA	Blok et al., 1998
	Gp P5A	South America		PA	Blok et al., 1998
	Gp Pa1 VB	Scotland		PA	Blok et al., 1998
	Gp Pa1 SS	England, Risby		PA	Subbotin et al., 2000
	Gp U	Ukraine	AJ606687	PA	Pylypenko et al., 2005
	Gp Y	England, York	EF153838	RFLP, BM, PA	This study
G. rostochiensis	Gr Jal	Russia		PA	Subbotin et al., 2000
	Gr J	Japan, Izumi	AB207271	PA	Uehara et al., 2005
	Gr NY	USA, New York	EF153839	RFLP, BM, PA	This study
	Gr Y	England, York	EF153840	RFLP, BM, PA	This study
G. tabacum solanacearum	Gt Sol1	USA via IACR-Rothamsted, L. Miller		PA	Subbotin et al., 2000
	Gts VA1	USA, Virginia, Richmond	EF153841	RFLP, BM, PA	This study
	Gts VA2	USA, Viginia, Nottoway County		RFLP, BM	This study
G. tabacum tabacum	Gtt CT	USA, Connecticut	EF153842	RFLP, BM, PA	This study
	Gt J	Japan, Kochi, Yasu	AB207272	PA	Uehara et al., 2005
	Gtt L	Not given	DQ097515	PA	Lax et al. (GenBank unpubl.)
	Gt Tab1	USA via IACR-Rothamsted, L. Miller		PA	Subbotin et al., 2000
G. tabacum virginiae	Gt Vir1	USA via IACR-Rothamsted, L. Miller		PA	Subbotin et al., 2000
G. achilleae		Slovenia, Zadraga	AY599498	PA	Sirca and Urek, 2004
G. artemisiae		Sweden, Sosdala	AY519126	PA	Manduric et al., 2004
		China	AF274415	PA	Subbotin et al., 2000
G. hypolysi		Japan, Hokkaido, Obihiro	AB207273	PA	Uehara et al., 2005
G. millefolii		Estonia	AF161004	PA	Ferris, unpublished
Heterodera avenae	Ha	USA, Idaho	EF153843	RFLP, BM, PA	This study

[a] RFLP—restriction fragment length polymorphism study; BM—multiplex PCR assay using species-specific rDNA primers; PA—phylogenetic and sequence analysis of the ITS1 and ITS2 rDNA region.

on a Zeiss UltraPhot light microscope, with contrast enhanced in Adobe Photoshop CS v. 8.

Molecular characterization: Nematode juveniles or cysts were mechanically disrupted with an eye-knife or sharp forceps tips in 20 µl nematode extraction buffer as described (Thomas et al., 1997) and stored at −80°C. For preparation of nematode extracts, samples were thawed, an additional 1 µl proteinase K (from 2 mg/ml stock solution) was added, and the tubes were incubated at 60°C for 60 min, followed by 95°C for 15 min to deactivate the proteinase K. Two or five microliters of the extract was used for each PCR reaction (see below). Where noted for some PCR reactions, bulk *G. rostochiensis* genomic DNA was included at 20 ng/reaction.

The rDNA PCR primers used in this study are listed in Table 2. Amplifications were performed on two or more individuals from each nematode population. Negative controls with water instead of template were included with each experiment. PCR reactions contained 0.2 µM each of primers TW81 (Joyce et al., 1994) and AB28 (Howlett et al., 1992), 5 µl nematode extract, 200 µM dNTP, 1 U Eppendorf Hot Master Taq (Brinkmann, Westbury, NY) and supplied enzyme reaction buffer at 1x in a total volume of 50 µl. Cycling conditions included an initial denaturation step of 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec, and finished with one cycle at 72°C for 5 min. A 5µl aliquot of each PCR reaction was analyzed by electrophoresis on 1% agarose/Tris-acetate-EDTA (TAE), and the rest was saved for subsequent restriction enzyme digestion. Gels were stained with ethidium bromide and visualized by UV illumination. Gel images were captured with a Chemi Imager (Alpha Innotech, San Leandro, CA).

Restriction enzymes Alu I, Rsa I and Taq I were purchased from New England Biolabs (Ipswich, MA). Bsh 1236I was purchased from Fermentas (Hanover, MD). Restriction digests contained an 8 µl aliquot from the PCR reaction, 1 U restriction enzyme and 1x restriction enzyme buffer in a 20 µl reaction volume and were incubated overnight at 37°C. Products were separated on 2% agarose-TAE gels, stained with ethidium bromide and visualized by UV illumination. PCR-RFLP tests were performed a minimum of two times for each population.

PCR products were excised from agarose gels, purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into the vector pCR2.1 using the Topo-TA Cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared with the Qiagen miniprep kit and digested with Eco RI to verify the presence of insert. Sequencing was performed at the University of Maryland Center for Biosystems Research. Nine clones representing three juveniles from two cysts from the initial tare soil sample were sequenced. Direct sequencing was used to analyze PCR products from: Field 1 (four J2); Field 2 (two J2); as well as *G. pallida* York, England (one J2, one cyst); *G. pallida* Chile, *G. rostochiensis* NY, *G. rostochiensis* York, England, *G. tabacum solanacearum* Richmond, VA, *G. tabacum tabacum* CT (three J2 each); and *H. avenae* ID (one J2). Sequences were submitted to the GenBank database under accession numbers EF153834 – EF153843.

DNA sequences were assembled using Sequencher 4.7 (Genecodes, Ann Arbor, MI). Alignments were made with Clustal W (Thompson et al., 1994) and checked by eye for consistency of conserved positions among sequences. Alignments were edited in GeneDoc (Nicholas et al., 1997). *Globodera artemesiae, G. achilleae, G. millefolii* and *G. hypolysi* were specified as outgroups. ModelTest (Posada and Crandall, 2001) was used to generate parameters useful for constructing a distance tree. Using hLRT criteria, the HKY85 model (Hasegawa et al., 1985) was selected, involving a time-reversible process, non-uniform nucleotide distribution and different transition and transversion rates (HKY+G). Parameters for this model included gamma shape 0.2972, Ti/Tv 2.2957, proportion invariant 0 and −lnL 2514.7830. AIC criteria gave a transversion model with gamma distributed rates across sites and a fraction of sites assumed to be invariable (TVM + I + G), gamma shape 0.7477, proportion invariant 0.3968, −lnL 2509.4099, AIC 5036.8198. Both were implemented in

TABLE 2. Primers used in this study

Primer name	5′ to 3′ Sequence	Purpose	Reference
TW81	GTTTCCGTAGGTGAACCTGC	PCR, sequencing	Joyce et al., 1994
AB28	ATATGCTTAAGTTCAGCGGGT	PCR, sequencing	Howlett et al., 1992
ribof2	CGATTGCTGTTGTCGTCG	Sequencing	Blok et al., 1998
ribof4	TATCGGTGGATCACTCGG	Sequencing	Blok et al., 1998
ribor2	GATGTCACTCCAATGGCG	Sequencing	Blok et al., 1998
2043	GTCGAGTCACCCATTGGG	Sequencing	Blok et al., 1998
ITS5	GGAAGTAAAAGTCGTAACAAGG	Diagnostic	White et al., 1990
PITSr3	AGCGCAGACATGCCGCAA	Diagnostic	Bulman and Marshall, 1997
PITSt3	AGCGCAGA**T**ATGCCGC**GG**[a]	Diagnostic	This study
PITSp4	ACAACAGCAATCGTCGAG	Diagnostic	Bulman and Marshall, 1997
PITSt4	ACA**G**CAGCAATCGTCG**GC**[a]	Diagnostic	This study

[a] Nucleotides marked in bold denote positions modified from Bulman and Marshall primers to be specific for *Globodera tabacum*.

Neighbor Joining (NJ) trees. For Maximum Parsimony trees involving 85 parsimony-informative characters of 846 total characters, fast, stepwise-addition and slow heuristic searches with 1,000 bootstrap replications employed ACCTRAN (accelerated transformation) character-state optimization and TBR (tree bisection-reconnection) branch-swapping. This was performed during the slow search on 57,200 trees, with 85,373 rearrangements. All procedures were implemented in PAUP*, v. 4.0b4a (Swofford, 1998).

Species-specific PCR was performed as described by Bulman and Marshall (1997). Briefly, 2 µl nematode extract and 250 µM of each primer (ITS5, PITSr3 and PITSp4) were included in a 25 µl multiplex reaction, with 160µM dNTP, 0.6 U HotMaster Taq and reaction buffer, as described above. Reactions were cycled once at 94°C for 2 min, followed by 35 cycles of 94°C (30 sec), 60°C (30 sec) and 72°C (30 sec), and completed by one cycle at 72°C for 5 min. Negative controls with no DNA were included in each experiment. Primers PITSr3 and PITSp4 were adapted to amplify *Globodera tabacum* and were named PITSt3 and PITSt4, respectively (Table 2). The modified multiplex reactions included ITS5 and were performed as described above, but had PITSt3 substituted for PITSr3 or PITSt4 substituted for PITSp4. Reaction products from the multiplex assays were analyzed by gel electrophoresis as described above. The experiment was repeated at least twice for each isolate.

Results and Discussion

Second-stage juveniles (n = 80): Measurements are listed in Table 3 and are in micrometers. The morphological characters of second-stage juveniles of this Idaho population agree with those of *G. pallida*, but exhibited a slightly shorter body mean length (452 ± 36 µm) than the lengths reported by Stone (1972) for the Epworth, Lincolnshire, England, population (486 ± 23 µm) and for the Duddingston, Scotland, population (482 ± 18 µm). Due to the observed variations in tail shape in the Idaho population, additional specimens from Idaho were then compared with cultured specimens of *G. pallida* originating from Epworth that had been deposited in the USDA Nematode Collection at Beltsville in 1972 (Fig. 1, A-W). The same variations in tail shape, not reported in the original description of *G. pallida* from England, were observed in both the Idaho population and the type material from England (Fig. 1J,R-W). Representative Idaho specimens with blunt or pointed tail shapes were also subjected to molecular analysis as described below.

Cysts (n = 80): Measurements are listed in Table 3 and are in micrometers, excluding ratios. The quality of cysts from which measurements were taken was good, and the vulval regions were intact (Fig. 2). The morphological characters of cysts of this population agree with those of *G. pallida* except for slightly higher mean in the distance from anus to the nearest edge of fenestra 53.5 (30–80 µm). Both cyst and J2 morphometrics and J2 tail morphology of the Idaho specimens fit well within ranges observed for *G. pallida*, indicating that the Idaho specimens must represent *G. pallida* (Stone, 1973) Behrens, 1975. Table 4 shows updated diagnostic morphometrics of four *Globodera* species genetically related to *G. pallida*, including typographic corrections to a previous table (EPPO, 2004), and new measurements from *G. achilliae* type material deposited in the USDA Nematode Collection at Beltsville, MD (slide numbers: T-1478p to T-1492p). Most notably for *G. pallida* and *G. achilleae* cysts, the maximum values for the vulva to anus distance and the number of cuticular ridges are expanded.

Molecular identification: To complement the morphological diagnosis of the Idaho population as *G. pallida*, three molecular assays were performed: PCR-RFLP of rDNA (Blok et al. 1998; Subbotin et al., 2000; Radivojevic et al., 2001; Reid and Pickup, 2005); phylogenetic

Table 3. Morphometrics of diagnostic characters of second-stage juveniles and cysts of *Globodera pallida* from Idaho.

Character	*G. pallida* Second-stage juveniles (n = 80)			*G. pallida* Cysts (n = 80)		
	Range	Mean	SD	Range	Mean	SD
Body length	380–533	452.0	36.0	—	—	—
Stylet length	22.5–25.0	23.2	0.7	—	—	—
Tail length	40.0–57.0	49.6	3.0	—	—	—
Hyaline tail terminal length	20.0–31.0	25.9	2.4	—	—	—
Body length excluding neck[a]	—	—	—	420–700	574	85
Body width[a]	—	—	—	400–600	534	80
Distance from anus to nearest edge of fenestra (anus–vulva)	—	—	—	30.0–80.0	53.5	11.8
Fenestra length	—	—	—	17.5–45.0	24.8	5.6
Number of cuticular ridges between vulva-anus	—	—	—	7.0–17.0	12.0	2.3
Granek's ratio[b]	—	—	—	1.2–3.6	2.2	0.5

[a] Based upon measurements of 15 cysts.
[b] As modified by Hesling, 1973.

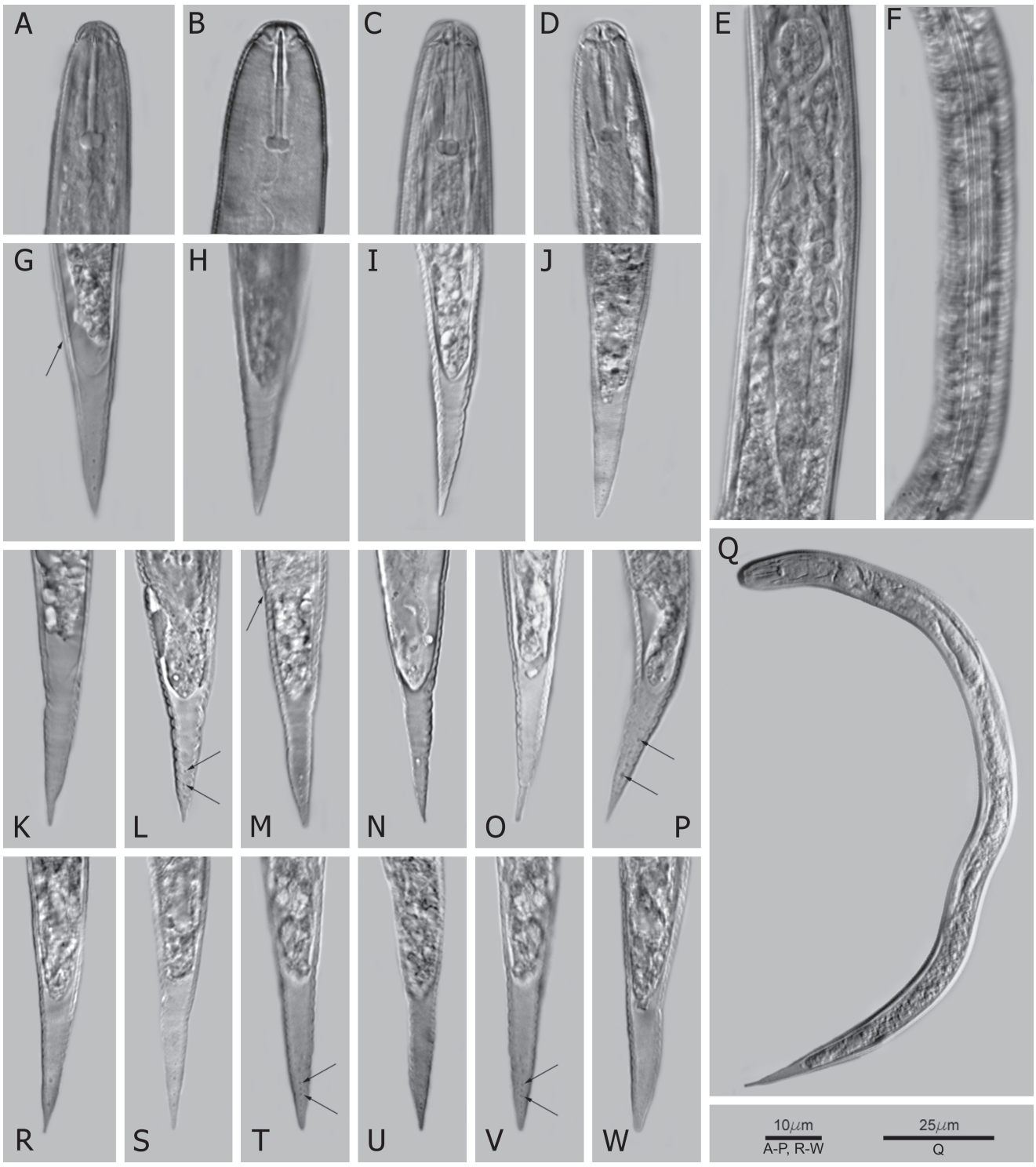

FIG. 1. Photomicrographs of second-stage juveniles of *Globodera pallida* from Idaho and England. Specimens from Idaho: A-C) heads, G-I) tails. Paratypes from England deposited in the USDA Nematode Collection at Beltsville, MD (slides T-1441p, T-1449p): D) head, J) tail. Specimens from Idaho: E) esophageal region, F) lateral field showing four incisures. K-P) Variations in tail shape of Idaho population (bluntly rounded to finely rounded pointed tip) with (arrows) in L, P and M showing refractive bodies and anal area, respectively. R-W) Variations in tail shape (bluntly rounded to finely rounded pointed tip) of paratypes from England deposited in the USDA Nematode Collection at Beltsville, MD (slide T-1441p for R-V, and slide T-1449p for W), with (arrows) in T and V showing refractive bodies. Q) Whole juvenile, Idaho population.

analysis of DNA sequences; and sequence-specific multiplex PCR (Bulman and Marshall, 1997; Pylypenko et al., 2005). The ease and accuracy of these methods for identifying *G. pallida* have been previously verified by a number of laboratories using numerous worldwide populations of *G. pallida* and *G. rostochiensis*. However, few studies (Thiéry and Mugniéry, 1996; Subbotin et al., 2000) included direct comparison of *G. pallida*,

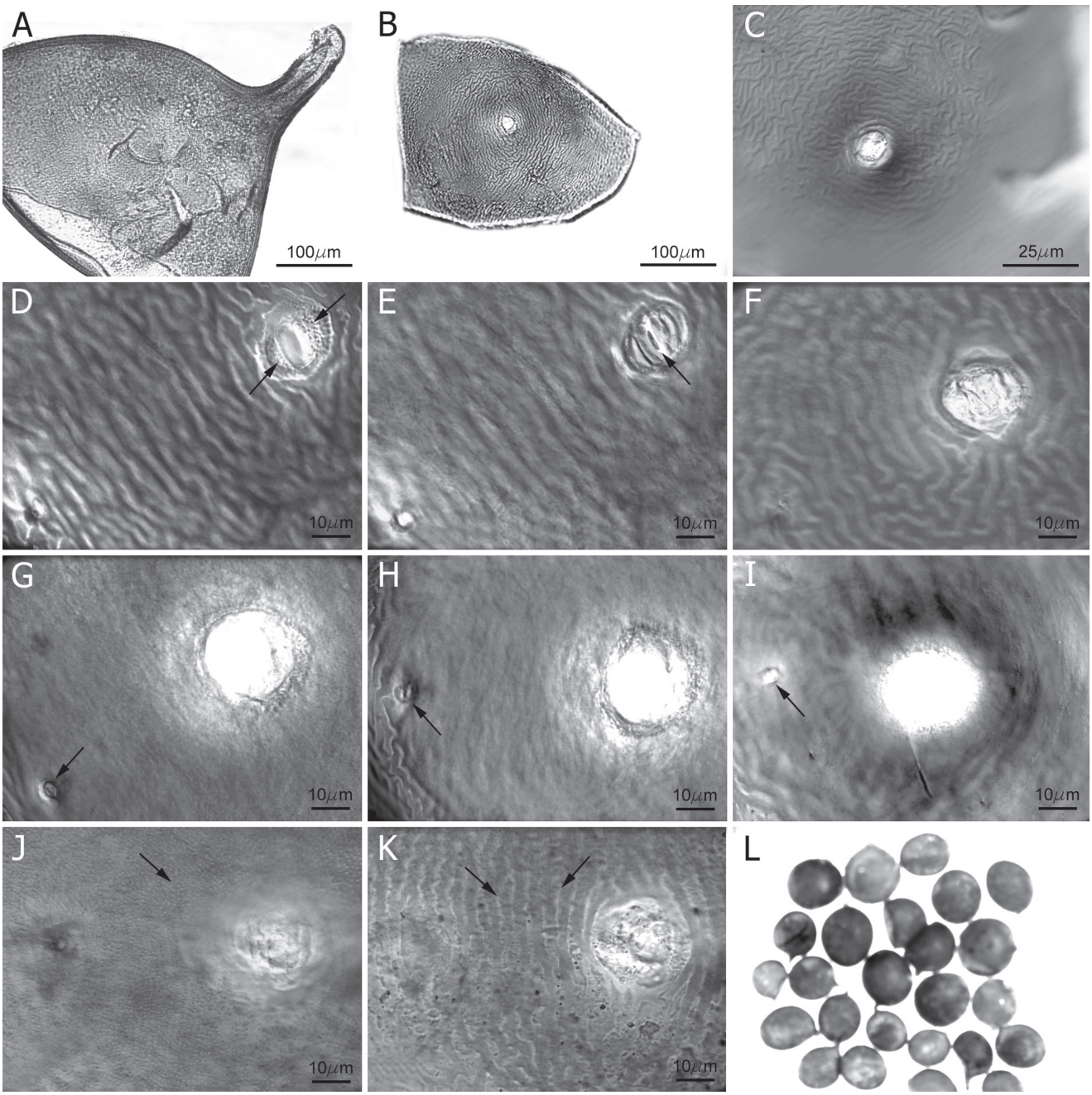

FIG. 2. Photomicrographs of the anterior and terminal areas of *Globodera pallida* cysts from Idaho. A) Anterior region. B-K) Anal-vulval regions with D, E, G-I, J and K (arrows) showing perineal tubercles, vulval-slit, anal areas, punctations and cuticular ridges, respectively. L) Cysts isolated from soil.

G. rostochiensis and *G. tabacum*, prompting us to further validate and extend existing rDNA-based diagnostics to include all three species.

PCR-RFLP: Using primers TW81 and AB28, the isolates from Idaho yielded PCR products of approximately 927 bp, including 18S rDNA (partial 3′) ITS1, 5.8S, ITS2 and 28S (partial 5′). These products appeared similar in size to those from *G. rostochiensis* and *G. pallida* (not shown). Based on diagnostic RFLP profiles described previously (Thiéry and Mugniéry, 1996; Blok et al., 1998; Subbotin et al., 2000; Reid and Pickup, 2005), restriction digestion of ITS PCR products with Alu I, Rsa I, Bsh 1236I or Taq I was used to characterize the Idaho isolates. In addition to known populations of *G. pallida*, *G. rostochiensis* and *Heterodera avenae*, isolates of *G. tabacum solanacearum* and *G. tabacum tabacum* were included for comparison. Alu I digestion (Fig. 3A) showed that the specimens from Idaho (lanes 15–20) were consistent with *G. pallida* (lanes 11–14; fragment sizes 505 and 383 bp) and distinct from *G. rostochiensis* (lane 4; fragment sizes 381, 360 and 148 bp) and *H. avenae* (lanes 2, 3; uncut). However, Alu I digestion

TABLE 4. Morphometrics for *Globodera* Behrens, 1975 amended from EPPO (2004)

Species name from original *Heterodera* description	J2 Stylet (μm)	Cyst, number of cuticular ridges from anus–vulva	Cyst anus–vulva distance (μm)	Granek's ratio
G. rostochiensis				
(Wollenweber, 1923)	19–23 (22)	12–31 (>14)	37–77 (>55)	1.3–9.5 (>3)
(Manduric et al., 2004)	19.3–24	12–28	38–149	1.7–6.7
Composite	19–24	12–31	37–149	1.3–9.5
G. pallida				
(Stone, 1973)	22–24 (23.8)	8–20 (<14)	22–67 (<50)	1.2–3.5 (<3)
(Manduric et al., 2004)	21.1–25.5	9–26	28–88	1.0–6.5
Composite	21.1–25.5	7–26	22–88	1–6.5
G. tabacum				
(Lownsbery and Lownsbery, 1954); (Miller and Gray, 1968); (Miller and Gray, 1972)	19–28 (23–24)	10–14	28–85	1–4.2 (<2.8)
G. achilliae				
(Golden and Klindić, 1973)	24–26 (25)	4–5	22–34 (27)	1.3–1.9 (1.6)
(Brzeski, 1988)	24–26.5 (25)	—	12–42 (24)	0.3–1.9 (1.2)
This study	—	5–11 (8 ± 1.7)	25–60 (21.5 ± 2.6)	1.2–2.1 (1.5 ± 0.3)
G. artemisiae				
(Eroshenko and Kazachenko, 1972)	18–29 (23)	5[a]	25–42 (32)[b]	0.8–1.7 (1.0)

[a] New: Measured from original photograph.
[b] Translated from Russian: "Anus to outer edge of fenestra."

could not discriminate *G. pallida* from *G. tabacum*. The Rsa I digestion (Fig. 3B) clearly identified the Idaho population (lanes 15–20) as *G. pallida* (lanes 11–14; fragment sizes 587 and 385 bp), distinguishing it from *G. tabacum* (lanes 5–10) and *G. rostochiensis* (lane 4), each with fragments of 587, 222 and 162 bp. Restriction patterns from the Bsh 1236I digests clearly differentiated the *G. pallida* isolates (Fig. 3C, lanes 2–6; fragment sizes 503, 347 and 126 bp) from *G. rostochiensis* (lanes 7–10; fragment sizes 842 and 126 bp), *G. tabacum solanacearum* (lanes 11–14; fragment sizes 430, 332, 124 and < 100 bp) and *G. tabacum tabacum* (lanes 15–18; fragment sizes 430, 328 and 114 bp). Taq I digestion also positively identified the Idaho population as *G. pallida*, but did not discriminate between *G. rostochiensis* and *G. tabacum* (not shown). Occasional faint partial digest products and poorly visible fragments less than 100 bp were not included in the analysis and did not preclude detection of diagnostic banding patterns for each species.

The faint products present at approximately 650 bp in *H. avenae* reactions (Fig. 3A, lanes 2, 3; 3B, lanes 2, 3) are non-specific PCR products, as they also appeared in the undigested ITS PCR reactions of several *H. avenae* specimens tested (not shown). Among the *G. pallida* specimens tested from Idaho were some that exhibited unusually blunt or pointed tail shapes (as shown in Fig. 1). These specimens (Fig. 3A,B, lane 16, blunt tail; lane 17, pointed tail) appeared the same in the digests as others having more typical juvenile morphology, thus providing molecular evidence that the tail shape variants were *G. pallida*. While all populations in this study gave rise to the expected banding patterns, it would not be unusual to find populations with polymorphisms in the ITS region that result in missing restriction sites. Therefore, the use of more than one enzyme is generally recommended to achieve a reliable diagnosis of *G. pallida*.

DNA sequence analysis: To further confirm the identity of the Idaho isolates as *G. pallida*, DNA sequences were obtained for the ITS rDNA PCR products obtained with primers TW81 and AB28. A total of 21 point mutations in the amplified region was observed among nine clones representing three J2 from Idaho tare soil. The consensus sequence derived from these clones (EF153837) was identical to the directly sequenced products of specimens from Field 1 (EF153836) and Field 2 (EF153835). Since each base change only appeared in a single clone and none were reflected as heterozygotes in the directly sequenced products, these differences were most likely due to random sequencing errors. No other patterns of variation were observed in the Idaho population that would suggest heterogeneity among different rDNA copies. One overall consensus sequence (designated Gp ID) was used for further phylogenetic comparison to the entire ITS region obtained for other populations of *G. pallida*, *G. rostochiensis* and *G. tabacum*, plus several others obtained from GenBank or published works (Table 1). Relationships among the species and populations inferred from the ITS rDNA are shown in Figure 4. The Gp ID sequence from Idaho was identical to those from Gp Y (York) and Gp D375 (Netherlands) and highly similar to the ITS region from other European populations. With the exception

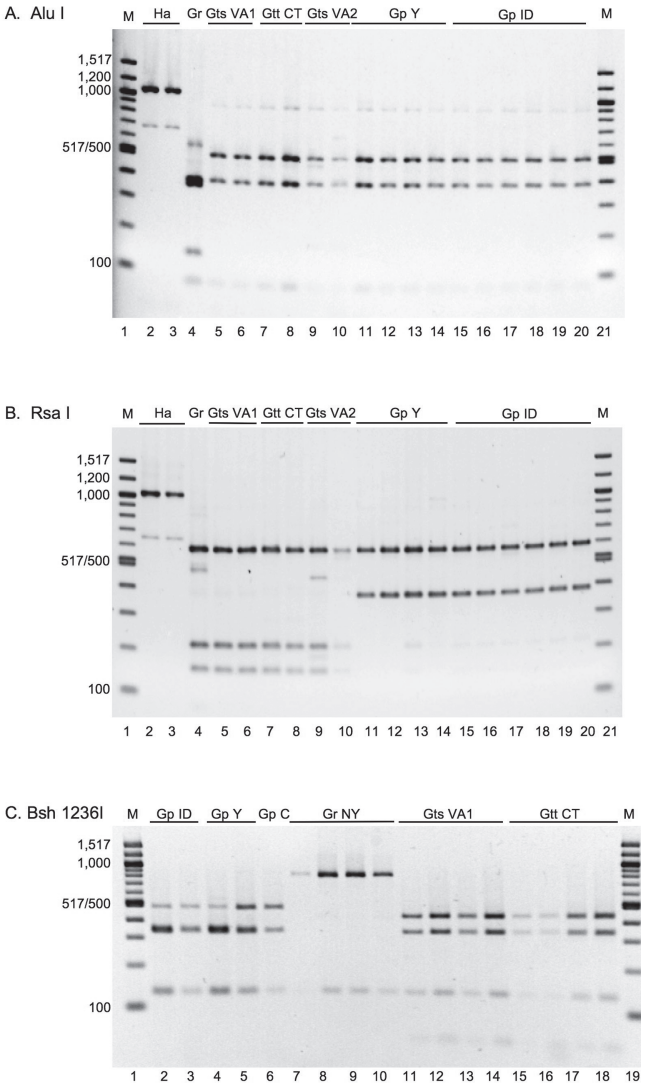

FIG. 3. Amplified PCR products from *Globodera* spp. digested by two enzymes. Panel A, Alu I and Panel B, Rsa I: Lanes 1, 21) 100 bp ladder (New England Biolabs, Ipswich, MA); lanes 2, 3) *H. avenae* Idaho; lane 4) *G. rostochiensis* NY (bulk genomic DNA, 20 ng); lanes 5, 6) *G. tabacum solanacearum* VA; lanes 7, 8) *G. tabacum tabacum* CT; lanes 9, 10) *G. tabacum solanacearum* VA; lanes 11–14) *G. pallida* York; lanes 15–20) *G. pallida* Idaho, tare soil; lane 16) blunt-tailed J2; lane 17) pointed-tailed J2. All PCR reactions included DNA extracts from individual juveniles except for lane 4 (20 ng bulk genomic DNA) and lanes 13, 14 (single cyst extracts). Panel C, Bsh 1236I: Lanes 1, 19) 100 bp ladder; lanes 2, 3) *G. pallida* Idaho field samples; lanes 4, 5) *G. pallida* York; lane 6) *G. pallida* Chile; lanes 7–10) *G. rostochiensis* NY; lanes 11–14) *G. tabacum solanacearum* VA; lanes 15–18) *G. tabacum tabacum* CT. Numbers to the left indicate sizes of selected marker bands in the ladder.

of Gp A from Argentina (DQ097514), all *G. pallida* populations appeared in a single highly supported clade. Using ModelTest parameters estimated on NJ trees, the Gp A population from Argentina grouped with *G. rostochiensis* populations with 71 to 91% bootstrap support; fast MP bootstrapped searches with TBR gave a similar grouping with 57% support (trees not shown). However, slow heuristic searches (318 tree consensus) resulted in a different topology, grouping Gp A with other *G. pallida* populations with 57% bootstrap support, as shown in Figure 4. In considering the equivocal tree position of this population based on ITS, it should be noted that, because many populations of *Globodera* spp. have demonstrated hybridization potential (Miller, 1983; Baldwin and Mundo-Ocampo, 1991), existence of genetically intermediate populations would not be surprising. The Gp A population could therefore be a hybrid or a naturally occurring variant that diverged from the European type *G. pallida* populations.

Based upon ITS rDNA, all *G. tabacum* populations also grouped together in a highly supported clade, with the *G. tabacum tabacum* sequence from Connecticut (Gtt CT) identical to another from the U.S. of unknown specific geographic origin (Gt Tab1) and one from Japan (Gt J). Gtt L (DQ097515) appeared closer to a clade containing both isolates of *G. tabacum solanacearum* (Gt Sol1 and Gts VA) and the population of *G. tabacum virginiae* (Gt Vir1). Likewise, the Gr NY and Gr Y populations appeared together in a clade with populations from Japan (Gr J) and Russia (Gr Ja1). As demonstrated by the head and tail photographs of live cyst nematodes shown in Figure 4, significant variation in qualitative morphological features between individuals makes interspecific comparisons difficult. For instance, while stylet morphology among the cyst nematodes shows considerable overlap (Manduric et al., 2004), the nearly symmetrical mid-tail constriction is a notable characteristic of *G. pallida* individuals within this population.

Additional GenBank entries, including AF016865 to AF016881, allowed further comparison of the Idaho *G. pallida* population with the ITS1 region from several other *Globodera* species and populations (data not shown). Compared to this group of sequences, the Idaho *G. pallida* ITS1 sequence showed closest similarity to isolates from Romania (differing at 2 bp within 549 bp aligned), Spain and Peru (each differing at 4 bp) and Northern Ireland (5 bp). Thus, all of the phylogenetic analyses of Gp ID clearly placed the Idaho population within *G. pallida*.

Sequence-specific PCR: As further confirmation of the species identity, a multiplex PCR assay using species-specific primers was performed according to the method of Bulman and Marshall (1997). *Globodera pallida* positive control specimens from York (Fig. 5, lanes 8, 9), all J2 obtained from tare (lanes 2, 3) or field samples (lanes 4–7) and the isolate from Chile (lanes 14–16) were positive for the expected *G. pallida*-specific 265 bp band. As was done for the restriction digests, nematodes from the Idaho population that exhibited unusually blunt or pointed tail shapes were also subjected to this multiplex assay. These specimens also tested positive for *G. pallida*, adding further confirmation for their identity (not shown). *Globodera rostochiensis* samples from New York (lanes 17, 18) and York,

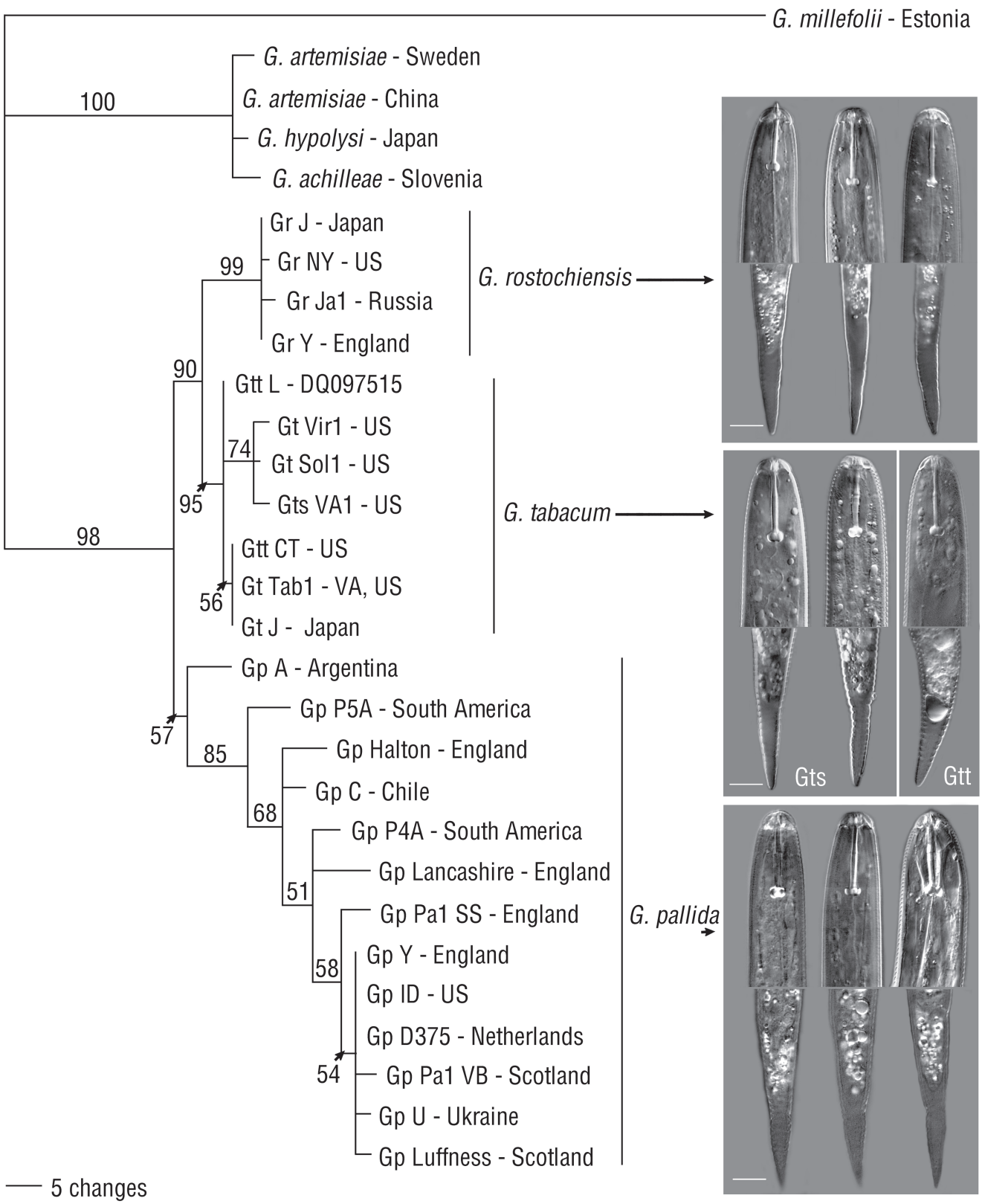

FIG. 4. Maximum Parsimony phylogram of *Globodera* species and populations made from a ClustalW alignment of 29 taxa with 846 nucleotide characters from the ITS1 and ITS2 regions of rDNA. Trees were generated using a heuristic search bootstrapped 1,000 times, employing TBR branch-swapping on 57,200 trees, with 85,373 rearrangements and ACCTRAN character-state optimization as implemented in PAUP*. Bootstrap support values occur above branches. TL = 264, CI = 0.814, RI = 0.889, RC = 0.724, HI = 0.186, goodness of fit = −74.707. GenBank accession numbers and abbreviations for nematode populations are given in Table 1. Differential Interference Contrast images were taken of live specimens with distance bars equivalent to 10 µm.

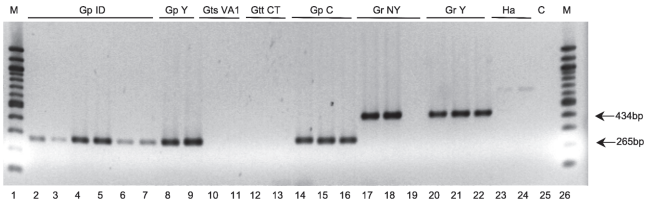

FIG. 5. Multiplex polymerase chain reactions of ITS rDNA from *Globodera* spp. using primers PITSr3, PITSp4 and ITS5. Single juvenile DNA extracts used for these reactions are the same as for Figure 7. Lanes 1) and 26) 100 bp ladder (New England Biolabs, Ipswich, MA); lanes 2, 3) Idaho tare soil; lanes 4, 5) Idaho Field 1; lanes 6, 7) Idaho Field 2; lanes 8, 9) *G. pallida* York; lanes 10, 11) *G. tabacum solanacearum*, VA; lanes 12,13) *G. tabacum tabacum* CT; lanes 14–16) *G. pallida*, Chile; lanes 17–19) *G. rostochiensis* NY; lanes 20–22) *G. rostochiensis* York; lanes 23, 24) *H. avenae* Idaho; lane 25) no DNA control.

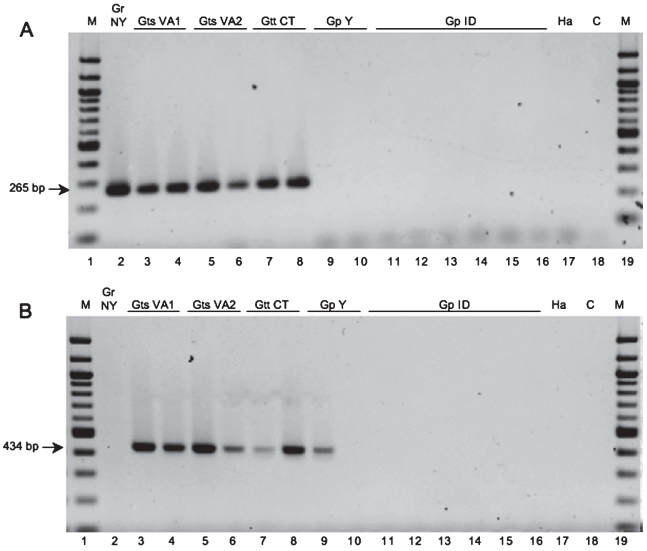

FIG. 6. Conventional PCR reaction of *Globodera* populations using species-specific primers modified from PITSr3 and PITSp4. A. Reactions with primers PITSt4 and ITS5. B. Reactions with primers PITSt3 and ITS5. Lanes 1, 19) 100 bp ladder; lane 2) *G. rostochiensis* NY; lane 3, 4) G. *tabacum solanacearum* Richmond, VA; lane 5, 6) *G. tabacum solanacearum* Nottoway County; lane 7, 8) *G. tabacum tabacum*; lane 9, 10) *G. pallida* York; lanes 11–16) *G. pallida* Idaho, tare soil; lane 17) *H. avenae* Idaho; lane 18) no DNA control.

England (lanes 20–22), gave the 434 bp band expected for this species. One reaction (lane 19) gave no product; further testing showed this was due to a poor quality extract. Because primers ITS5, PITSr3 and PITSp4 had not previously been tested with *G. tabacum*, we examined isolates of *G. tabacum solanacearum* (lanes 10, 11) and *G. tabacum tabacum* (lanes 12, 13). All *G. tabacum* isolates, including *G. tabacum solanacearum* from Nottoway, VA (not shown), and water control (lane 25) reactions were negative for PCR product, confirming the expected results based upon sequence information. The *H. avenae* reactions (lanes 23, 24) had faint products that were most likely due to mismatch priming with these templates, since they were larger than the expected size and did not consistently appear with repeated testing (not shown).

We then used DNA sequence alignments to design new primers that produced a positive PCR reaction for *G. tabacum*. While ITS sequence variation among the species was limited, we were able to modify the sequences of primers PITSr3 and PITSp4. The new primers, named PITSt3 and PITSt4 (Table 2), were each paired separately with ITS5. As predicted from the sequence information, PITSt4 and ITS5 amplified a 265 bp band from both *G. tabacum* and *G. rostochiensis* but not from *G. pallida* or the controls (Fig. 6A). Included among the Idaho nematodes testing negative for *G. tabacum* with this primer set were blunt-tailed (Fig. 6A, lane 12) and pointed-tailed (lane 13) specimens.

Primers PITSt3 and ITS5 amplified a 434 bp band from all three *G. tabacum* isolates but not from *G. rostochiensis* or any of the *G. pallida* specimens from Idaho, including tail shape variants (Fig. 6B, lanes 12, 13). This primer pair occasionally produced a faint PCR band at 434 bp from the *G. pallida* York isolate (Fig. 6B, lane 9). Assuming this was due to contamination of the York specimen, we repeated the assay several times using fresh reagents and template preparations (not shown). This phenomenon appeared only sporadically with the *G. pallida* isolate from York but not with specimens from any other location. It is not clear why the York population was uniquely affected, since the sequence of its ITS region was identical to *G. pallida* from Idaho. Because only two nucleotide changes separate the PITSt3 primer from an exact match to the *G. pallida* sequence, mismatch primer binding on those templates or priming from a rare variant ITS repeat are possible. Raising the annealing temperature slightly from 60°C or otherwise changing the cycling conditions might eliminate this effect, but we did not investigate this any further. Because relatively few nucleotide differences separate all three species and intraspecific variation is known to occur in *Globodera* ITS regions, phenomena such as this highlight the need for caution when applying and interpreting species-specific PCR assays.

Because the PITSt4 primer performed more reliably overall than PITSt3, we then tested this primer in multiplex PCR with ITS5 and PITSp4 (Fig. 7). As expected, PCR of *G. rostochiensis* showed bands at both 434 bp and 265 bp (Fig. 7, lanes 17–22). *Globodera tabacum* isolates showed only the 265 bp band (lanes 10–13); *G. pallida* (lanes 2–9), *H. avenae* (lanes 23, 24) and control reactions (lane 25) were negative. According to these re-

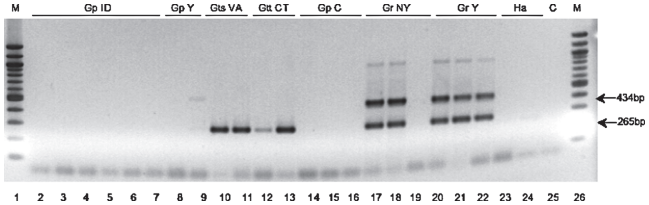

FIG. 7. Multiplex PCR reaction of *Globodera* populations using primers PITSt4, PITSr3 and ITS5. DNA extracts used for these reactions were the same as in Figure 5. See Table 1 for population codes.

sults, the appearance of both bands is diagnostic for *G. rostochiensis*, one band at 265 bp indicates *G. tabacum* and the absence of product is consistent with *G. pallida*, although not definitive. Thus, this multiplex PCR modification of the Bulman and Marshall (1997) assay provides a means to positively identify *G. tabacum* in a similarly simple and straightforward manner. When combined with the original assay, it is now possible to discriminate between *G. pallida*, *G. tabacum* and *G. rostochiensis*. This additional test could be invaluable in diagnostic situations that may arise in the Americas, where the potential for these species to spread across borders has already caused heightened regulatory concern and had significant trade implications.

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