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# Morphological and Molecular Characterization of *Globodera* Populations from Oregon and Idaho

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## ABSTRACT

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An unusual population of cyst nematode was found in soils collected from a Powell Butte, OR field with a cropping history including potato, wheat, other crops, and significant weed presence. These nematodes could not be placed with certainty into any known species and exhibited some unique morphological features in some specimens. Compared with *Globodera pallida*, the cyst body length was slightly longer and the second-stage juvenile stylet length was slightly shorter. In some individuals, the J2 stylet knob height was greater and the tail annules were more prominent than in *G. pallida*, and the tail abruptly narrowed, with a slight constriction near the posterior third of the hyaline terminus. Compared with *G. rostochiensis,* the hyaline tail terminus had a larger number of refractive bodies, and cysts of this population had a smaller Granek's ratio and fewer cuticular ridges between the anus and vulva. In some individuals, the tail termini of second-stage juveniles were more bluntly pointed, and the stylet knobs were more anteriorly directed with greater height. Unlike *G. tabacum*, the cyst wall often lacked a network-like pattern and, in some individuals, the juvenile tail terminus distinctly narrowed after a constriction. Molecularly, the population was distinct from *G. pallida*, *G. rostochiensis*, and *G. tabacum*. Multiplex polymerase chain reaction of the internal transcribed spacer (ITS) rDNA region gave results similar to *G. tabacum*; however, ITS restriction fragment length polymorphism patterns were observed to have individual bands in common with *G. rostochiensis* and *G. pallida*. Phylogenetic analysis based on ITS1 and -2 rDNA sequences showed greatest similarity to populations from Argentina and Chile; together, they form a moderately supported clade, distinct from *G. rostochiensis*, *G. tabacum, G. "mexicana,"* European type *G. pallida*, and several *G. pallida* populations from South America.

*Additional keywords*: detection, diagnosis, morphology, PCR, potato, RFLP, *Solanum tuberosum*, taxonomy.

The pale potato cyst nematode *Globodera pallida* (Stone 1973) Behrens 1975 and the golden potato cyst nematode *G. rostochiensis* (Wollenweber 1923) Behrens 1975 are regulated pathogens of potato, with a United States market valued at nearly $3.9 billion (3). Collectively, these species are referred to as potato cyst nematodes (PCNs). Within the United States, *G. rostochiensis* was first found in New York in 1941 and has been the subject of quarantine restriction for decades (11). The pale potato cyst nematode *G. pallida* was first discovered in Idaho in 2006 (17). Since then, an extensive survey of the major potato-growing acreage has been carried out to determine the extent of PCN distribution and to ensure that the appropriate steps are taken to prevent further infestation. Material suspected to contain PCN cysts has been analyzed by morphological and molecular means at the United States Department of Agriculture (USDA)–Agricultural Research Service Nematology Laboratory in Beltsville, MD.

Comprehensive descriptions of reference populations are key for accurate diagnosis of PCN and, ultimately, for preventing the spread of these nematodes. In real-world diagnostic scenarios, morphological and molecular approaches are essential, serving complementary diagnostic roles. Despite the increasing reliance upon molecular diagnostics, traditional morphological identification is an invaluable triage tool. Molecular assays for PCN that have been validated for use by state and federal diagnostic labs are primarily based upon amplification of the ribosomal internal transcribed spacer region (ITS rDNA). In addition to polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of the ITS region (15,41,44) and multiplex PCR (12,39), real-time PCR methods are emerging for rapid and sensitive detection of PCN (23,36). However, these methods are subject to complications due to the presence of intraspecific or intra-population sequence variation. Bulman and Marshall (12) recognized the presence of ITS haplotypes in both *G. pallida* and *G. rostochiensis* and discussed the possible impact they might have on the effectiveness of species-specific multiplex PCR. Anthoine et al. (4) reported that molecular variation in *Globodera* spp. affected the reliability of several published molecular diagnostic tests. Although time consuming, sequence analysis is sometimes needed to provide additional information for PCN identification. Therefore, it is increasingly important to investigate new occurrences of *Globodera* spp. that do not fit the typical profiles, and to determine their potential impact on existing diagnostic procedures.

In 2008, cyst nematodes were found in soils collected from a Powell Butte, OR field with a cropping history including potato, wheat, other crops, and significant weed presence, and from two fields within Idaho. Herein, we describe the morphological and molecular characteristics of these populations. The significance of this research on the molecular methods used for diagnosis of PCN is discussed.

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## MATERIALS AND METHODS

**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 glycerin by the formalin-glycerin method (14,20). Cysts were similarly removed from soil samples by sieving, fixed for 12 h in 3% formaldehyde, and processed to glycerin. Photomicrographs of cyst vulval cones and fixed J2 were made with an automatic 35-mm camera attached to a Leica Wild MPS48 Leitz DMRB compound microscope, and measurements were made with an ocular micrometer on the same microscope. Some J2 heads and tail photomicrographs were taken on a Zeiss Ultraphot with DIC (differential interference contrast) and a Q-imaging Micropublisher 5 camera. Specimens were then morphologically identified with recent taxonomic keys and a compendium for identification of *Globodera* spp. (5,13).

**Molecular characterization.** Molecular characterization of the populations included sequence-specific multiplex PCR (12,39), PCR-RFLP of ITS1 and -2 rDNA (7,37,38), and phylogenetic analysis of ITS1 and -2 rDNA sequences (41). Nematode juveniles or cysts were mechanically disrupted with a microscopy knife in 20 µl of nematode extraction buffer (47) and stored at –80°C. Template preparation, PCR, restriction digests (RFLP), and purification of amplicon DNA were carried out as described previously (39). PCR products were cloned using the Strataclone PCR Cloning Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Plasmid clone DNA was prepared with the QiaPrep Spin Miniprep Kit and digested with *Eco*RI to verify the presence of the insert. Sequencing was performed at the University of Maryland Center for Biosystems Research. DNA from 14 ITS rDNA clones representing juveniles from three separate cysts from the Oregon soil sample were sequenced. Direct sequencing was performed on four ITS rDNA amplicons, representing four juveniles from the single available cyst of Idaho sample no. 167. DNA from 11 clones from Idaho sample no. 347, representing juveniles from two cysts, was sequenced. New sequences were submitted to the GenBank database at the National Center for Biotechnology Information (NCBI) under accession numbers GQ896542 to GQ896547.

DNA sequences were assembled using Sequencher 4.9 (Genecodes, Ann Arbor, MI). Alignments were made with Clustal W (48), with minor corrections made in GeneDoc (27). All phylogenetic procedures were implemented in PAUP* version 4.0b4a (42). *G. achilleae* was specified as the outgroup. JModelTest (16,33,34) was used to generate parameters for constructing a distance tree. Using AIC criteria, the HKY85 model (18) was selected, involving a time-reversible process, nonuniform nucleotide distribution, and different transition and transversion rates (HKY+I+G). The gamma shape parameter of 0.4440 for this model was implemented with the neighbor-joining (BioNJ) algorithm employing a minimum evolution objective function to produce a distance tree bootstrapped with 1,000 replications. A maximum parsimony tree was also constructed with 103 parsimony informative characters of 848 total characters, with fast, stepwise-addition and slow heuristic searches of 1,000 bootstrap replications with ACCTRAN (accelerated transormation) character-state optimization and TBR (tree bisection-reconnection) branch-swapping.

## RESULTS

**Morphological descriptions.** Measurements of cysts and second-stage juveniles are shown in Table 1 and listed in micrometers unless stated otherwise.

*Oregon population cysts.* ($n$ = 12). Cysts light brown to slightly dark brown in color, spherical to subspherical with a protruding neck. Anus prominent and visible in all specimens, often with a V-shaped mark (Fig. 1A and B). Subcrystalline layer absent. Vulval region intact or fenestrated with a single circumfenestrate opening occupying all part of vulval basin. Vulval bridge, underbridge, and bullae absent. However, occasionally bullae-like structures were noted in a few specimens (Fig. 1C and D). Cyst wall patterns were ridge-like with heavy punctuations (Fig. 1E and F).

*Oregon population second-stage juveniles.* ($n$ = 57). Body vermiform, tapering at both extremities, more so posteriorly (Fig. 2A). Some specimens were folded four times within egg. Head rounded, slightly set off with prominent cephalic sclerotization, bearing four to five annules (Fig. 2B to E). Stylet short, well developed; basal knobs rounded with a greater height and with a forward projection on anterior side (Fig. 2C). Excretory pore located near the beginning of the basal esophageal bulb. Phasmids not observed. Tail tapering uniformly but abruptly narrows with a slight constriction near the posterior third of the hyaline portion, ending with a finely rounded to pointed terminus (Fig. 2F to J). Hyaline tail terminus with four to seven refractive bodies. Cuticular annulations distinct with more prominent annules near the tail terminus. Lateral field with occasional areolation, consisting of four incisures.

*Oregon population males.* Not detected.

*Idaho population no. 167 cysts.* ($n$ = 2). Light brown color, spherical with a protruding neck (Fig. 3A). Cyst wall pattern ridge-like with heavy punctuations (Fig. 3B). Vulval region intact or fenestrated, with a single circumfenestrate opening that occupies all part of vulval basin (Fig. 3C and D). Whole fenestra length = 22.5 to 25 µm; distance from nearest edge of fenestra to anus = 40 to 52 µm; number of ridges between anus and nearest edge of fenestra = 10 to 14; Granek's ratio = 1.8 to 2.1.

TABLE 1. Morphometrics of diagnostic characters of second-stage juveniles and cysts of *Globodera* sp. from Oregon[a]

| | Oregon population of *Globodera* spp. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Second-stage juveniles | | | | Cysts | | | |
| Character | *n* | Range | Mean | SD | *n* | Range | Mean | SD |
| Body length | 56 | 390–515 | 446 | 29 | … | … | … | … |
| Stylet length | 60 | 20.0–22.5 | 21.3 | 0.81 | … | … | … | … |
| Tail length | 45 | 42.5–55.0 | 48.3 | 2.84 | … | … | … | … |
| Hyaline tail terminal length | 57 | 21.0–32.5 | 25.8 | 2.28 | … | … | … | … |
| Body length excluding neck | … | … | … | … | 10 | 470–737 | 602 | 88 |
| Body width | … | … | … | … | 10 | 445–693 | 591 | 97 |
| Distance from anus to nearest edge of fenestra (anus – vulva) | … | … | … | … | 13 | 50–80 | 62.6 | 9.49 |
| Fenestra length | … | … | … | … | 12 | 20–35 | 25.0 | 3.95 |
| No. of cuticular ridges between vulva-anus | … | … | … | … | 13 | 11–18 | 13.2 | 2.20 |
| Granek's ratio[b] | … | … | … | … | 11 | 1.85–3.00 | 2.41 | 0.33 |

[a] SD = standard deviation.
[b] As modified by Hesling (19).

*Idaho population no. 167* s*econd-stage juveniles.* (*n* = 10). Body vermiform, specimens were folded four times within egg when cracked open. Tail tapering uniformly and usually bluntly rounded to pointed at the terminus (Figs. 3E to I and 4A). Tail abruptly narrowed, with a slight constriction near the posterior third of the hyaline portion, ending with a finely rounded to pointed terminus (Fig. 3J, M, and I). Lateral field with four incisures. Head rounded, slightly set off, with prominent cephalic sclerotization, bearing four annules. Stylet well developed; basal knobs rounded and with a forward projection on anterior side. Excretory pore located near the beginning of the basal esophageal bulb. Phasmids not observed. Length = 425 to 485 µm (451.7; SD 23.74); stylet = 21 to 23 µm (22.3; SD 0.52); tail = 42 to 50 µm (48; SD 3.09); hyaline tail terminus = 20 to 30 µm (24.6; SD 3.26).

*Idaho population no. 347 cysts.* (*n* = 2). Light brown color, spherical with a protruding neck. Cyst wall pattern ridge-like with heavy punctations. Vulval region intact or fenestrated, with a

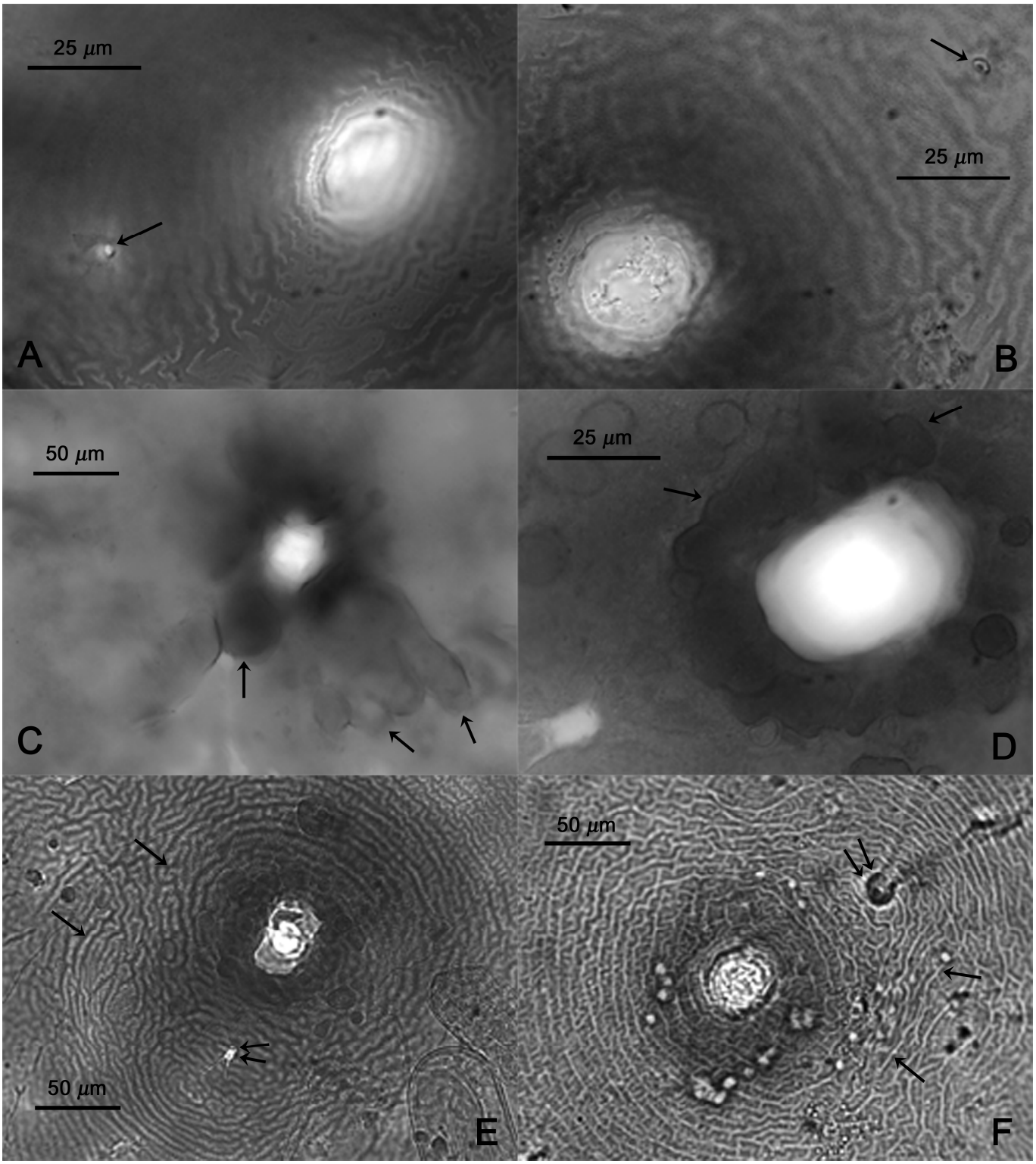


**Fig. 1.** Photomicrographs of terminal areas of cysts of the *Globodera* population from Oregon. Anal-vulval regions with arrows showing **A and B,** anal areas; **C and D,** bullae-like structures; and **E and F,** cyst wall patterns; and **E and F,** double arrows showing anal areas.

single circumfenestrate opening occupying all part of vulval basin. Whole fenestra length = 25 µm; distance from nearest edge of fenestra to anus = 30 to 55 µm; number of ridges between anus and nearest edge of fenestra = 8 to 14; Granek's ratio = 1.2 to 2.2.

*Idaho population no. 347 second-stage juveniles.* ($n$ = 8). Body vermiform, specimens were degraded and folded four times within egg when cracked open. Tail tapering uniformly and usually bluntly rounded to pointed at the terminus (Fig. 4B).

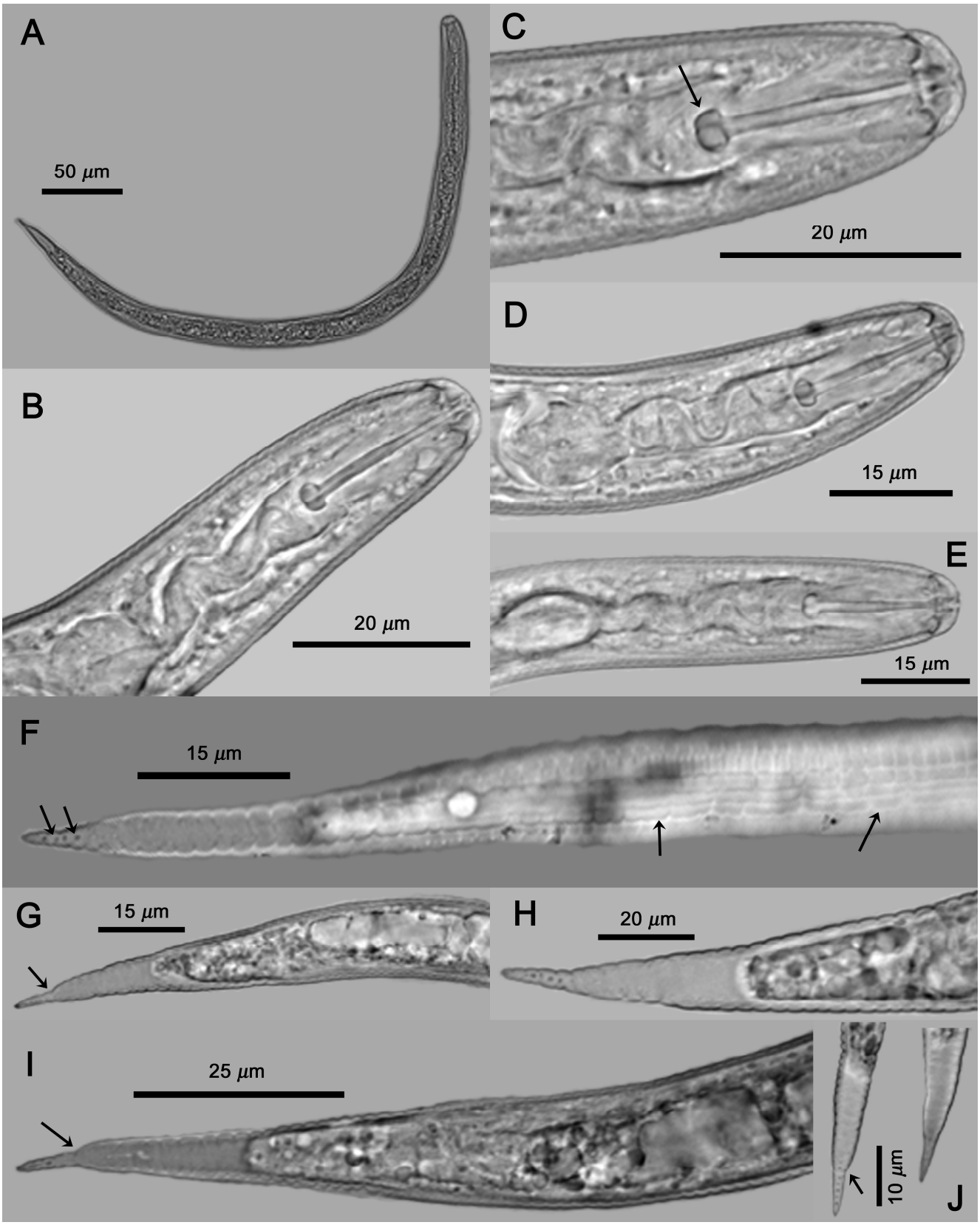


**Fig. 2.** Photomicrographs of second-stage juveniles of the *Globodera* population from Oregon. **A,** Whole juvenile; **B to E,** anterior regions showing stylet and part of esophagus; **C,** high stylet knobs (arrow). **F,** Tail showing areolated nature of lateral field with four lines (right arrows) and refractive bodies in tail terminus (left arrows). **G to J,** Tail variations; **G, I, and J,** arrows showing abrupt narrowing of tail with slight constriction.

Lateral field with four incisures. Head rounded, slightly set off with prominent cephalic sclerotization, bearing four annules. Stylet well developed; basal knobs rounded and with a forward projection on anterior side. Length 421 to 435 µm (428; SD 9.89); stylet = 22 to 23 µm (22.5; SD 0.32); tail = 48 µm; hyaline tail terminus = 22.5 to 30 µm (25.7; SD 2.52).

**Sample locality and repository.** The Oregon population was collected in May 2008 during a mandated survey of a Powell

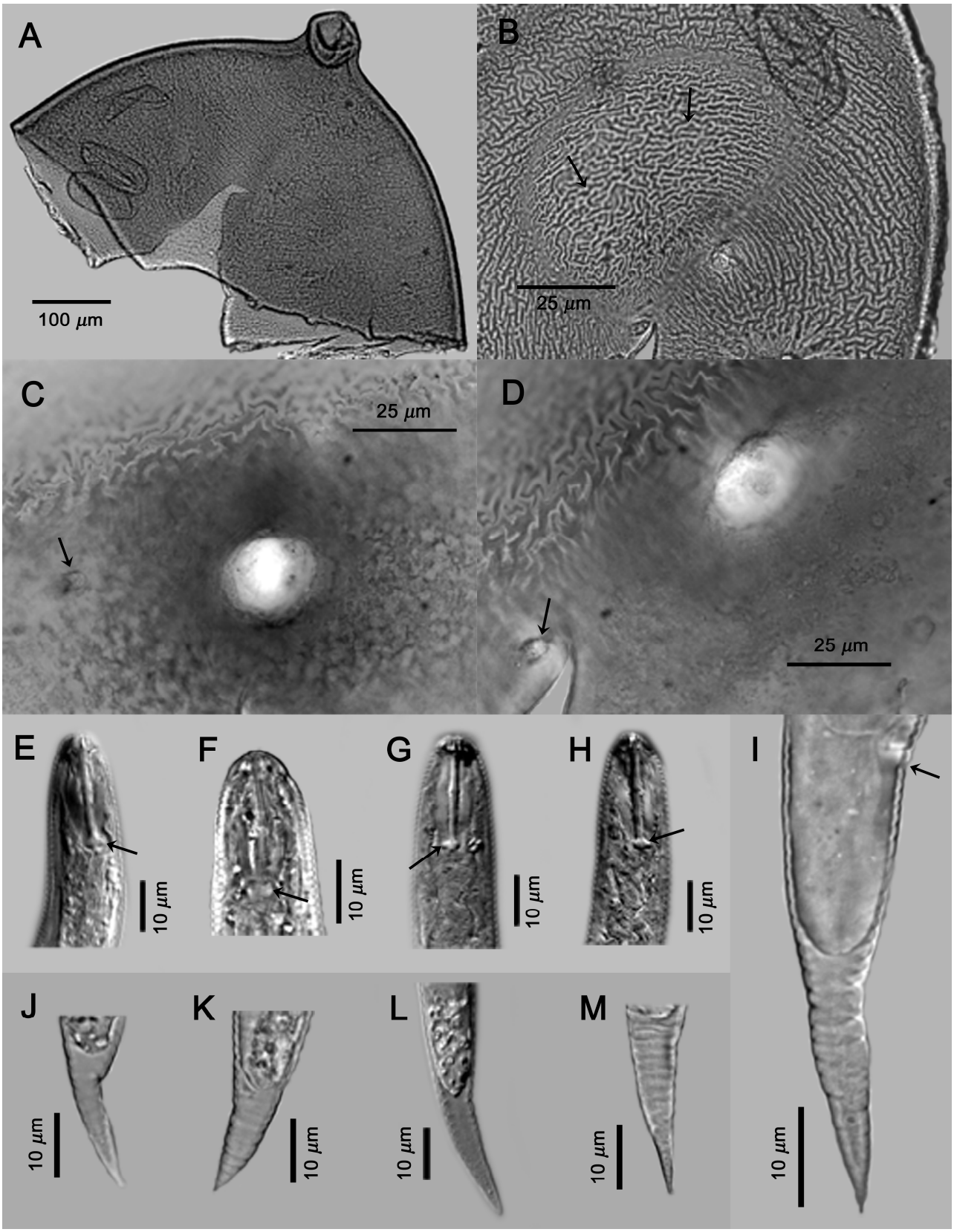


**Fig. 3.** Photomicrographs of cysts and second-stage juveniles of the *Globodera* population from Idaho. **A,** Anterior region. **B to D,** Anal-vulval regions: **B,** arrows showing cyst wall pattern; **C and D,** arrows showing anal areas. **E to H,** Anterior and posterior regions of second-stage juveniles; **E to G,** arrows showing stylet knobs, with DIC images of E, G, and H. **I to M,** Tails with variable termini; DIC image of **L**. Tail in I shows anal area (arrow).

Butte, OR field with a history of potato, wheat, other crops, and weeds. Two additional populations, Idaho no. 167 and Idaho no. 347, were also detected during these surveys in August and October 2008. Idaho no. 167 came from a nonregulated field (location unknown) and Idaho no. 347 came from a field in Teton County.

Specimens were deposited in the USDA Nematode Collection, Beltsville, MD. For the Oregon population, second-stage juveniles, slide numbers were T-5847p to T-5862p and vial numbers T535p to T-541p; for cysts, slides numbers were T5863p to T-5870p. For Idaho populations, second-stage juveniles, slide numbers were T-5871p to T-5873p and vial numbers T-542p to T-543p; for cysts, slide numbers were T-5874p to T-5879p.

**Morphological summary.** These unusual populations are characterized as belonging to the genus *Globodera* and having second-stage juveniles with a body length of 446 µm (390 to 515); stylet length = 21.3 µm (20 to 22.5), with rounded to high basal knobs with a forward projection on anterior side; areolated lateral field with four incisures; tail = 48 µm (43 to 55) long, tapering uniformly but sometimes abruptly narrowing, with a pronounced to slight constriction near the posterior third of the hyaline portion, ending with a finely rounded to pointed terminus; hyaline tail terminus = 26 µm (21 to 33); cysts spherical to subspherical; light to dark brown; circumfenestrate; number of cuticular ridges between vulva and anus = 13 (11 to 18); and Granek's ratio = 2.4 (1.9 to 3).

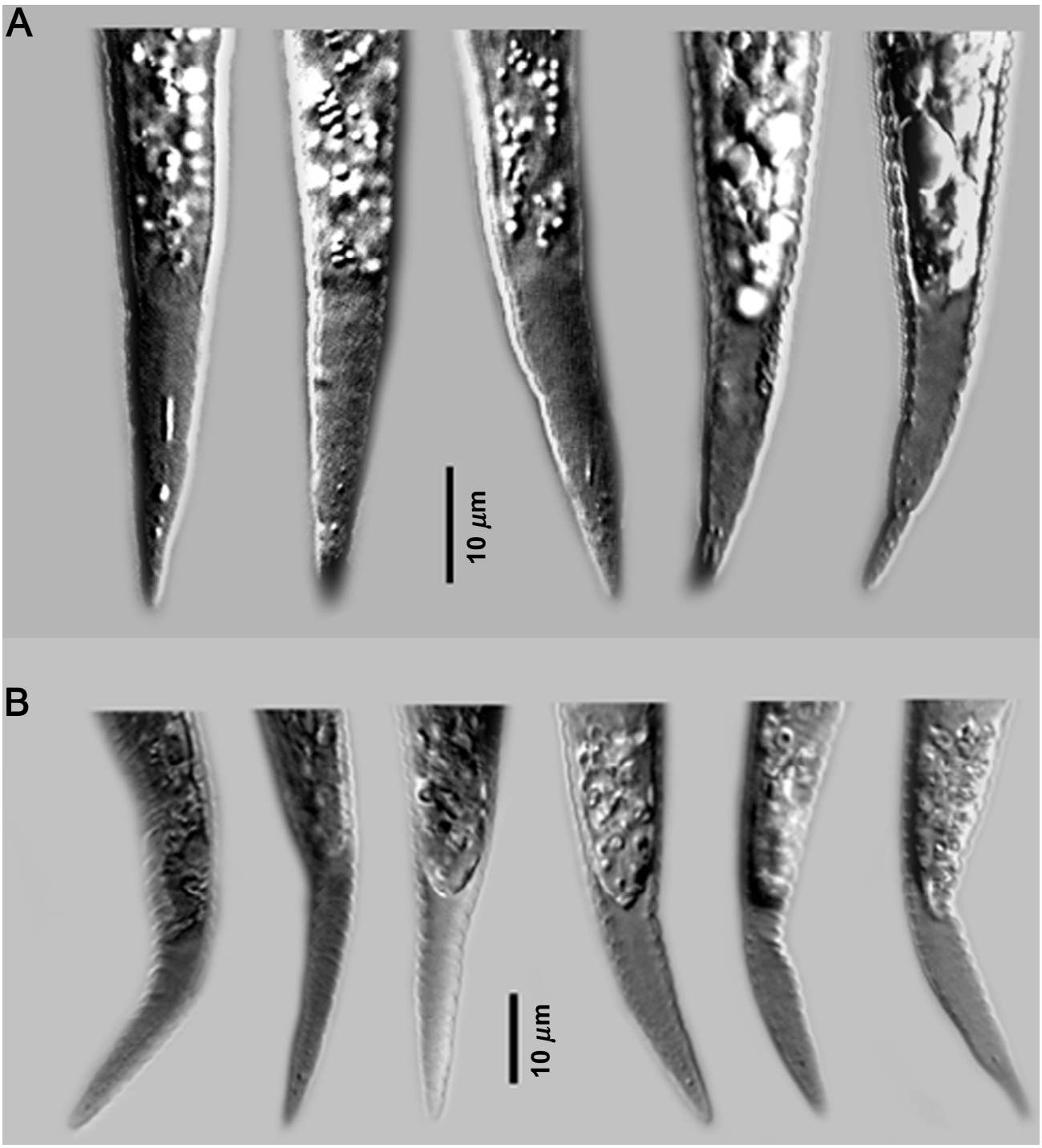


**Fig. 4.** Photomicrographs of DIC images of second-stage juvenile tails of the *Globodera* populations from **A,** Oregon and **B,** Idaho, showing morphological variation between unique distal constriction and bluntly pointed tails in both populations.

**ITS PCR and RFLP.** *Globodera* spp. populations included in this study are listed in Table 2. DNA extracted from single juveniles of the Oregon population was subjected to conventional and multiplex PCR amplification of the ITS1 and -2 region. Amplifications with general ITS primers TW81 and AB28 resulted in PCR products of the expected size (≈1,000 bp), consistent with those observed for *G. pallida*, *G. rostochiensis*, and *G. tabacum* (not shown). However, multiplex primers pITSp4, pITSr3, and pITS5 failed to amplify any product from the Oregon samples (Fig. 5A), while the control DNA from *G. rostochiensis* or *G. pallida* gave the expected products of 464 or 265 bp, respectively. When modified multiplex primer pITSt4 was included with pITS5 and pITSr3 (37), amplification from the Oregon DNA resulted in a 265-bp amplicon that was consistent with *G. tabacum* and *G. rostochiensis* (Fig. 5B); the 434-bp band that is typical of *G. rostochiensis* was absent.

Discrimination of *G. pallida* and *G. rostochiensis* is typically accomplished by digestion of ITS PCR products with restriction enzymes *Alu*I, *Rsa*I, Sau3AI, or *Taq*I; the enzyme *Bsh*1236I is known to distinguish these species plus *G. tabacum*. When the ITS amplicons from the Oregon population of a *Globodera* sp. were subjected to *Bsh*1236I digestion, the banding pattern differed from those of *G. pallida*, *G. rostochiensis*, and *G. tabacum,* appearing instead as a mixed pattern with individual bands in common with each species (Fig. 5C). Digestion with *Rsa*I also gave a distinct pattern for this population (data not shown). PCR-RFLP of the two Idaho samples gave results similar to those of the Oregon specimens, in that the standard ITS PCR products were of the expected size (≈1 kb), the multiplex PCR results were similar to *G. tabacum*, and the RFLP patterns were mixed (data not shown).

**DNA sequence analysis.** Direct sequencing of purified ITS amplicons from the Oregon population resulted in numerous ambiguous base calls; therefore, 14 PCR product clones were selected for sequencing. Sequence divergence among these clones was as high as 2.8%, with one clone (GQ896543) containing a unique 27-bp deletion. The few available J2 from Idaho sample no. 167 yielded PCR products with identical sequences that were obtained directly from the purified amplicons. For Idaho no. 347, 11 ITS rDNA clones were sequenced. Out of 977 bp, the clones exhibited a maximum divergence of 0.61%. Alignment of sequences from all three populations revealed variation of 0 to 27 bp, with several identical clones and most deviating <5 bp from the consensus. Sequence divergence between *G. pallida* populations was previously reported to be 1.02%, and divergence between clones within a population was 0.44% (41). Excluding the Oregon clone with the deletion, the degree of sequence divergence within these populations was similar to those reported for *G. tabacum, G. rostochiensis,* or *G. pallida* (12,41).

**Phylogenetic analysis.** Compared with the NCBI database, sequences from the Oregon and Idaho populations were 98.7% identical to DQ097514, representing a *G. pallida* population from Argentina. DQ097514 differed from the individual clone sequences of the Oregon or Idaho populations at 5 to 15 bp. Representative ITS sequences from the Oregon or Idaho no. 347 clones and the consensus from Idaho no. 167 were aligned with several sequences from GenBank and unpublished data, with *G. achilleae* as the outgroup (Table 2). A neighbor-joining tree

TABLE 2. List of nematode species and populations used in this study

| Species | Location | Accession no. | Experiment[a] |
|---|---|---|---|
| *Globodera* sp. | Idaho no. 167, United States | GQ896546 | R, BM, PA |
| | Idaho no. 347, United States | GQ896547 | R, BM, PA |
| | Oregon, United States | GQ896542–GQ896545 | R, BM, PA |
| | Argentina | DQ097514 | PA |
| | Chile (cystA3) | Grenier et al. (*personal communication*) | PA |
| | Antofagasta, Chile | GU084807-GU084808 | PA |
| *Globodera pallida* | Chile | EF153834 | BM, PA |
| | Chile | GU084801 | PA |
| | Halton, England | Blok et al. 1998 | PA |
| | York, England | EF153838 | R, BM |
| | D375, Netherlands | Blok et al. 1998 | PA |
| | Luffness, Scotland | Blok et al. 1998 | PA |
| | Pa1, Scotland | Blok et al. 1998 | PA |
| | Amantani, Peru | EU006705 | PA |
| | Cusco, Peru | EU006706 | PA |
| | Huamachuco, Peru | Grenier et al. (*personal communication*) | PA |
| | Huancabamba, Peru | EU006704 | PA |
| | Huanuco, Peru | Grenier et al. (*personal communication*) | PA |
| | Taquile, Peru | GU084797 | PA |
| | P4A, Peru | Blok et al. 1998 | PA |
| | P5A, Peru | Blok et al. 1998 | PA |
| | Idaho, United States | EF153835 | R, BM, PA |
| | Ukraine | AJ606687 | PA |
| *G. "mexicana"* | Mexico | EU006707-EU006709 | PA |
| *G. rostochiensis* | New York, United States | EF153839 | R, BM, PA |
| | York, England | EF153840 | R, BM, PA |
| | Bolivia | GU084809, GU084810 | PA |
| | Russia | DQ847117, DQ847120 | PA |
| *G. tabacum solanacearum* | Virginia, United States | EF153841 | R, BM, PA |
| *G. tabacum tabacum* | Connecticut, United States | EF153842 | R, BM, PA |
| | Argentina | DQ097515 | PA |
| | Japan | AB207272 | PA |
| *G. tabacum virginiae* | Virginia, United States | DQ847112 | PA |
| *G. achilleae* | Slovenia | AY599498 | PA |
| *G. artemisiae* | China | AF274415 | PA |
| | Estonia | AF161003 | PA |
| *G. millefolii* | Estonia | AF161004 | PA |

[a] R = restriction fragment length polymorphism study, BM = multiplex polymerase chain reaction assay using species-specific rDNA primers, and PA = phylogenetic and sequence analysis of the internal transcribed spacer (ITS)1 and ITS2 rDNA region.

showing relationships inferred from the ITS-rDNA gene is shown in Figure 6. The Oregon and Idaho populations fit into a highly supported clade (93%) that included the Argentina sequence and unpublished sequences from a Chilean population (E. Grenier, *personal communication*). Within the *Globodera* sp. clade, two subclades of ITS haplotypes are apparent. The *Globodera* sp. clade formed a sister group to the clade containing *G. rostochiensis* and *G. tabacum* complex, although bootstrap support for

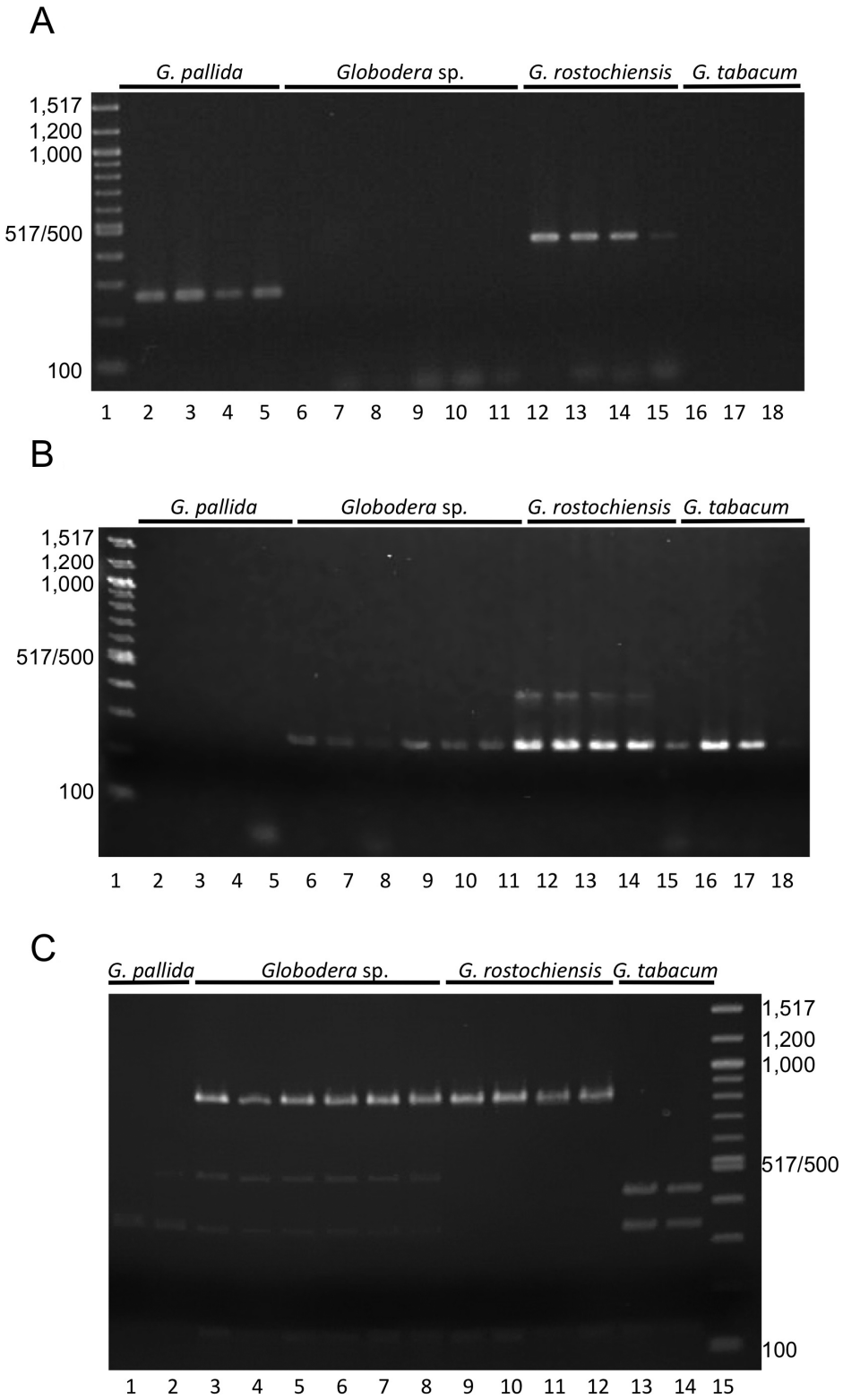


**Fig. 5.** Molecular analysis of the *Globodera* population. **A,** Multiplex polymerase chain reactions of internal transcribed spacer (ITS) rDNA from *Globodera* spp. using primers PITSr3, PITSp4, and ITS5. **B,** Multiplex polymerase chain reaction (PCR) of *Globodera* populations using primers PITSt4, PITSr3, and ITS5. **A and B,** Lane 1, 100-bp ladder (New England Biolabs, Ipswich, MA); lanes 2 and 3, *Globodera pallida*, Idaho; lanes 4 and 5, *G. pallida*, York, UK; lanes 6 to 11, *G.* sp., Oregon; lanes 12 and 13, *G. rostochiensis*, New York; lanes 14 and 15, *G. rostochiensis*, York, United Kingdom; lanes 16 and 17, *G. tabacum tabacum* Connecticut; lane 18, *G. tabacum solanacearum*, Virginia. Numbers to the left indicate sizes of selected marker bands in the ladder. **C,** Amplified ITS PCR products from *Globodera* spp. digested by *Bsh*1236I. Lanes 1 and 2, *G. pallida*, York, United Kingdom; lanes 3 to 8, *Globodera* sp., Oregon; lanes 9 and 10, *G. rostochiensis,* NY; lanes 11 and 12, *G. rostochiensis,* United Kingdom; lanes 13 and 14, *G. tabacum tabacum,* CT; and lane 15, 100-bp ladder; sizes to the right.

this position was modest (64%). Bootstrap support was 99% for the *G. tabacum* clade and 80% for *G. rostochiensis.* Bootstrap support for the *G. "mexicana"* group was also high (100%), although support for the overall grouping of *G. pallida* was weaker (68%). The latter situation most likely reflects the molecular diversity of South American *G. pallida* populations that have been described in recent years. Maximum parsimony analysis (not shown) gave similar overall tree topologies, positioning the *Globodera* sp. clade as a sister group to the clade containing *G. tabacum* and *G. rostochiensis*, with relatively weak bootstrap support (60%).

**Fig. 6.** Neighbor-joining phylogram of internal transcribed spacer (ITS) rDNA sequences from Oregon and Idaho populations and other *Globodera* spp. with outgroups *Globodera achilleae, G. artemisiae*, and *G. hypolysi,* and with bootstrap values from 1,000 replicates as implemented in PAUP* 4.0b10 (42). Bootstrap values >50% are shown above branches. Roman numerals in parentheses indicate clade designations determined from cytochrome b data (32).

## DISCUSSION

The described *Globodera* populations are morphologically variable, with some features outside the ranges expected for *G. pallida*, *G. rostochiensis*, or *G. tabacum* complex. Compared with *G. pallida*, the cyst body length is slightly longer, and the second-stage juvenile stylet length is slightly shorter. In some individuals, the stylet knob height is greater, the tail shape abruptly narrows, with a slight constriction near the posterior third of the hyaline tail terminus, and tail annules are more prominent. Compared with *G. rostochiensis,* the hyaline tail terminus has a large number of refractive bodies (four to seven). Cysts of the new populations have a smaller Granek's ratio and fewer cuticular ridges between the anus and vulva. In some individuals, the tail termini of second-stage juveniles are more bluntly pointed, and the stylet knobs are more anteriorly directed, with greater height. Unlike *G. tabacum* (Lownsbery & Lownsbery 1954) Skarbilovich 1959, the cyst wall often lacks a network-like pattern and, in some individuals, the juvenile tail terminus distinctly narrows after a constriction. Approximately two-thirds of juvenile tails in the Oregon population and few from the Idaho population possessed the unique distal tail constriction and attenuation (Fig. 4).

Results of the multiplex PCR (Fig. 5A and B) and RFLP studies (Fig. 5C) can be explained in light of the DNA sequences obtained from the Oregon and Idaho populations. For instance, the 265-bp amplicon observed for the *Globodera* sp. populations (Fig. 5B) can be explained by the exact match between the ITS sequences for the Oregon and Idaho populations and the *G. tabacum* pITSt4 primer. Also, in silico analysis of the ITS clone sequences revealed the existence of restriction site polymorphisms that are predicted to result in either three or four bands upon digestion with *Bsh*1236I. In comparison, European-type *G. pallida* sequences would be expected to give five fragments, *G. rostochiensis*, three; and *G. tabacum*, five (with fragments <75 not visible on a gel). As reflected in the different clone sequences recovered, loss of one or two *Bsh*1236I restriction sites should result in three or four fragments. The restriction patterns observed in Figure 5C were due to the presence of a mixture of ITS haplotypes within the single J2 used for each lane of the RFLP experiment. These results underscore the need for caution in interpreting the results of multiplex PCR or RFLP in a diagnostic setting, especially in circumstances where the population does not fit a "typical" profile. The development of a multiple marker, molecular diagnostic key for *Globodera* spp. may ultimately be necessary to compensate for molecular complexities encountered in any one marker (1).

Other than the DNA sequence in GenBank, there is little information available for the Argentina population. Cysts of this nematode were found on *Solanum tuberosum* subsp. *andigena* (also known as *S. andigenum*) in the Salta province of Northern Argentina, and the morphometrics and ITS rDNA reportedly fit best with *G. pallida* (21,22). Unfortunately, no morphological comparisons with the Argentina population were possible, because there are no published photographs or morphometrics and this particular isolate was not maintained in culture. It should also be noted that the Chilean sequences (Antofagasta 1 and 2, and Chile cyst A3) are distinct from the Chilean *G. pallida* sequence EF153834 which, instead, groups with European populations and *G. pallida* from Idaho (EF153835). Further information about the Antofagasta population is expected in another study (E. Grenier, *personal communication*). However, it is interesting to note that Antofagasta, Chile is similar in latitude and within 500 km of Salta Province, Argentina, the presumptive source of the *Globodera* population represented by sequence DQ097514. Additional sampling of *Globodera* spp. from the entire Andean region would provide valuable clues to the relationship between populations from that diverse ecosystem and those from the United States.

The morphological and molecular plasticity observed in these populations has been previously described for the genus *Globodera* (5,32,45,46). In one instance, a potato cyst nematode found in a soil sample from Scotland did not clearly fit with typical descriptions of either *G. pallida* or *G. rostochiensis* (38). Both long and short stylet lengths were found in juveniles from a single cyst. PCR-RFLP of the ITS region revealed unexpected restriction patterns, some of which appeared like *G. pallida* and some like *G. rostochiensis*. This unusual result was ultimately explained by sequence analysis, which revealed a 67-bp deletion in one clone and a restriction site-changing point mutation in another. Despite this variation, the population was not considered to represent a new species, because the phylogenetic analysis of ITS rDNA sequences placed them solidly in a clade with other European populations of *G. pallida*.

The existence of atypical PCNs has been demonstrated by a number of earlier molecular studies comparing *G. pallida* populations from South America with those from Europe. The uniquely virulent Peruvian isolate of pathotype P5A (29) was distinguished from European isolates by simple sequence repeat (SSR) analysis (8) and random amplified polymorphic DNA (RAPD) PCR (6,9) and by digestion of ITS amplicons with *Mbo*I or *Taq*I (7). These results led to speculation that this population could represent a new species. In a phylogenetic study of worldwide *Globodera* populations inferred from ITS rDNA, Subbotin et al. (41) had found that P5A, along with Peruvian populations Huamachuco P6A, Huancabamba, and Cusco (GenBank accession numbers AF016866 to AF016868), formed a sister group to the major European clade, later found to also include the U.S. population from Idaho (39). Thus, Subbotin et al. (41) extended the earlier presumption about P5A by presenting that group as an unnamed new species. Taken in context with more recent information on these South American populations (32), P5A groups within the Vc subclade of *G. pallida* (Fig. 6). Therefore, the genetic diversity within the accepted species boundary of *G. pallida* appears to be expanding as more populations are characterized.

A comprehensive study, including RAPD analysis, ITS-RFLP, two-dimensional gel electrophoresis, and analysis of satellite DNA, confirmed the molecular separation of European *G. pallida* from several Peruvian populations, although the latter were not assigned unambiguously to any *Globodera* spp. (15). More recently, relationships of a larger assemblage of Peruvian isolates were established through phylogenetic analysis of microsatellite markers and mitochondrial cytochrome b (cyt b) sequences, revealing five major clades (30). Further analysis of microsatellite loci and cyt b data localized the geographic origin of European populations to a small area in southern Peru, and revealed a south-to-north decrease in genetic diversity among these distinct clades (31,32). Based on cyt b phylogeny, Pylypenko et al. (35) concluded that P5A was closely related to Peru populations Huancayo, Cajabama, and Otuzco (clade IVc) and most likely had a different geographic origin than the major group of European populations (clade Ic). Additional analysis of cyt b and other markers from the Oregon population and nematodes from Argentina should shed more light on their genetic relationship to these Peruvian isolates.

The morphological and molecular variation observed in the Oregon and Idaho populations, together with the molecular separation of some South American *Globodera* populations, raises important philosophical and practical questions about precisely what constitutes a species and on the criteria used to define species boundaries (2). *Globodera* spp. have been historically classified on the basis of morphology, host specificity, and reproductive isolation, although some species assignments in the Heteroderidae that were based on these criteria have failed to remain valid, especially after evaluation by molecular methods. For example, *G. solanacearum* (Miller & Gray 1972) Behrens

1975 and *G. virginiae* (Miller & Gray 1968) Stone 1973, once considered separate species, are now regarded as subspecies of *G. tabacum*, due largely to the genetic similarity demonstrated therein (10,24,42,43). The morphological variation we observed within and among the Oregon and Idaho populations is unfortunately common for many species within the genus. The conclusion of Stone (40) that "No absolute criterion distinguishes even the most well-characterized species of the *Globodera* complex" thus remains true within the current morphological context.

Although some stress the importance of demonstrating reproductive isolation as a decisive factor for species delimitation, this presents a particular challenge for passively dispersing plant-parasitic nematodes and sometimes conflicts with other criteria. Although viable hybrid populations have been created in the laboratory from crosses of *G. rostochiensis*, *G. pallida*, and *G. tabacum* (25), such hybridization may not happen in naturally occurring mixtures; for example, of *G. pallida and G. rostochiensis* (28). Moreover, mating experiments have not resolved the taxonomic status of *G.* "*mexicana*" Campos-Vela 1967. Viable and fertile hybrids were obtained from crosses of *G.* "*mexicana*" and *G. pallida* (26), consistent with their molecular similarity (10,15) but despite their differences in host range (45). Therefore, reluctance to separate some Peruvian clades of *Globodera* into distinct species is understandable in light of the viable offspring obtained from crosses between individuals from northern and southern Peruvian populations, and on the apparent lack of morphological diversity among them (30). However, the *Globodera* sp. clade appears genetically more distant from *G. pallida* than the Peruvian and European *G. pallida* clades are to each other, and more distant than *G. pallida* is from *G.* "*mexicana*" (Fig. 6).

Analyses of many Peruvian isolates have not included detailed morphological descriptions; therefore, the extent of anatomical variation they have compared with type specimens of *G. pallida* has not been demonstrated. Although the pathogenicity of some Peruvian populations on potato was previously described (15, 29,35), no clear correlation between molecular markers and virulence has been found. Perhaps morphological descriptions of the South American populations would help to further define the taxonomic criteria by which populations such as those from Oregon and Idaho should be evaluated.

Ultimately, the ability to reproduce on potato is the most important characteristic of *Globodera* populations with respect to the potential for economic impact. Preliminary greenhouse and laboratory studies comprising multiple observations indicate that the Oregon population reproduces on potato (*S. tuberosum*). Further investigation of the morphology, molecular taxonomy, and pathogenicity of these *Globodera* populations is needed. Until alternative diagnostics markers that are unaffected by polymorphisms can be developed, ITS-based identification methods for *Globodera* spp. must be used with extreme caution. Sequence analysis of ITS rDNA or other markers may be necessary to resolve unclear or conflicting results obtained by existing ITS-based methods.

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