Guidelines for Isolation and Identification of Regulated Nematodes of Potato (*Solanum tuberosum* L.) in North America

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Abstract. Detailed morphological, molecular and extraction criteria are presented for verification of the identities of quarantined nematode pests of trade concern in North American potatoes judged to be reliable by representatives of Canada, Mexico and the United States. These nematodes include *Globodera rostochiensis*, *G. pallida*, *Meloidogyne chitwoodi*, *Ditylenchus destructor*, and *D. dipsaci*. Information on source material and specimen archive, with discussion of alternative molecular diagnostic methods and primers is included. Guidelines are provided for interpretation of results from diagnostics. This information is referenced in the North American Plant Protection Organization Regional Standard for Phytosanitary Measures No. 3.

Additional keywords: Microscopy, PCR (polymerase chain reaction), regulatory nematology.

Resumen. Detalles morfológicos, moleculares y criterios sobre la extracción se presentan para la verificación de la identidad de nematodos plaga, cuarentenados en la comercialización de papa en Norteamérica, los cuales son considerados confiables por las entidades representativas de Canada, México y los Estados Unidos. Estos nematodos incluyen *Globodera rostochiensis*, *G. pallida*, *Meloidogyne chitwoodi*, *Ditylenchus destructor* y *D. dipsaci*. Se incluye información sobre material de referencia y archivos de especímenes, con discusión sobre métodos y sondas alternativos para diagnósticos moleculares. Se dan pautas para la interpretación de resultados diagnósticos. Esta información es referencia en los estándares regionales de medidas fitosanitarias No. 3, de la Organización de Norteamérica para la Protección Vegetal.

Palabras clave adicionales: Microscopía, PCR (reacción en cadena de la polimerasa), nematología regulatoria.

INTRODUCTION

The most important nematode threat to potato (*Solanum tuberosum* L.) production is undoubtedly caused by two species of cyst nematodes [*Globodera rostochiensis* (Wollenweber) Skarbilovich (the "golden" nematode) and *G. pallida* (Stone) Behrens], which cause severe damage wherever present. The golden nematode disease is known to occur in several countries, especially in cooler areas of subtropical and tropical regions, as well as temperate regions of the world (OEPP/EPPO, 2004b). Yield loss as high as 80% has been reported in some potato growing areas of the tropics where infestation levels due to golden nematode are high (Spears, 1968). The Columbia root-knot nematode, *Meloidogyne chitwoodi* Golden, O'Bannon, Santo, and Finley, decreases potato tuber quality with galls and brown spots that are unacceptable for processing or fresh market sale (Santo *et al*., 2004). Major nematode parasites of potatoes also include other root-knot [*Meloidogyne* spp., *e.g. M. hapla* Chitwood, *M. incognita* (Kofoid and White) Chitwood, *M. javanica* (Treub.) Chitwood, *M. naasi* Franklin], bulb and stem nematode [*Ditylenchus dipsaci* (Kühn) Filipjev], and potato rot (*Ditylenchus destructor* Thorne) nematode (EPPO/CABI, 1997a, b). The potato rot and potato stem nematodes are reported from temperate climates including North and South America. Potato stem nematodes attack leaves, petioles and may injure tubers, while potato rot nematodes mainly damage tubers. Other nematodes damaging potato are false

root-knot, *Nacobbus aberrans* Thorne (Thorne and Allen), and lesion nematodes (*Pratylenchus* spp.); of less importance are sting, dagger, reniform, burrowing and pin nematodes (Stevenson *et al*., 2001). *Globodera rostochiensis*, *G. pallida*, *Meloidogyne chitwoodi*, and *Ditylenchus destructor* are considered by Canada, Mexico, and the United States to be A2-rated quarantine pests of limited distribution under official control (NAPPO, 1998). They are also on the pest list http://nematode.unl.edu/pesttable1.htm, of the U.S. Animal and Plant Health Inspection Service and Society of Nematologists (SON) Regulatory Committee. The history and geographic distribution in North and Central America of *Globodera* is reviewed in Brodie (1998), and CABI/EPPO (2004c); and of *Meloidogyne chitwoodi* in CABI/EPPO (2004d), and Santo *et al*. (2004). History and distribution of *Ditylenchus* of regulatory importance are given in Brzeski (1991), Sturhan and Brzeski (1991), Escuer (1998), and CABI/EPPO (2004a, b). Morphological identification is detailed elsewhere for *Globodera* (Baldwin and Mundo-Ocampo, 1991; Golden, 1986; Mulvey and Golden, 1983; EPPO/CABI, 1997c; OEPP/EPPO, 2004b; and Sosa-Moss, 1997); for *Meloidogyne* (Cuevas, 1997; Eisenback and Triantaphyllou, 1991; Golden *et al*., 1980; Jepson, 1987; Karssen, 2002; and OEPP/EPPO, 2004a); and for *Ditylenchus* spp. (EPPO/CABI, 1997a, b; Escuer, 1998; Subbotin *et al*., 2005; Viscardi and Brzeski, 1993).

PURPOSE

The methods and procedures outlined in this paper were developed by the authors as members of a Technical Advisory Group of the North American Plant Protection Organization (NAPPO). This paper will be referenced in the NAPPO Regional Standard for Phytosanitary Measures No. 3 entitled "Requirements for Importation of Potatoes into a NAPPO Member Country." NAPPO promotes the application of the most up-to-date methods and procedures with other national and regional plant protection organizations of the International Plant Protection Convention, to facilitate safe movement of potatoes. A NAPPO-sponsored Mexico City Nematode Identification Workshop was held September 10 and 11, 2003. Following the workshop, a nematode technical advisory group was appointed by the NAPPO Potato Panel to develop diagnostics protocols for species within *Globodera*, *Meloidogyne*, and *Ditylenchus* of regulatory importance. This document will deal with guidelines for their extraction and identification. Procedures and rationale for alternative protocols can be used as guidelines in evaluating or avoiding potential trade disputes. An overview of these protocols for tubers is given in Figure 1.

Ideal source material for species identification. Possible nematode sources include soil, roots, and tubers. While soil holds the greatest threat of cross contamination with other nematodes or soil organisms, this may be the most readily available source. Roots may harbor the greatest concentration of nematodes of regulatory interest, but tubers may provide the most obvious signs to aid nematode sampling, and represent the sampled trade commodity. Females from roots or tubers are the most important stage for morphological and molecular identification. Soil analysis for extraction of cysts is recommended if this nematode is suspected.

Sample symptoms, size, extraction. Tubers represent typical export samples. Potato rot nematode (*D. destructor*) symptoms on the tuber surface include sunken, dark-colored pits or skin cracks. The potato stem nematode (*D. dipsaci*), mainly a parasite of foliage, causes conical pits and skin splitting on tubers. Within the tubers, *Ditylenchus* may be found in white pockets (EPPO/CABI, 1997a). *G. rostochiensis* and *G. pallida* may stunt tubers (EPPO/CABI, 1997c). *Meloidogyne chitwoodi* forms pimple-like galls, and *M. hapla* makes less distinct swellings on the tuber surface (Santo *et al*., 2004). While sample size often depends on material available, a standard industry sample of 400 tubers from a lot is common and used in diagnostic recommendations for bacterial ring-rot of potato (De Boer, personal communication). A diagnostic sample should include unscrubbed symptomatic and randomly selected tubers. Extract female nematodes by gently washing tubers sliced in different directions, especially in symptomatic areas. Dissect intact post-second generation females from translucent or brown (after egg-laying) infection sites (Santo *et al*., 2004) from slices or from under the tuber surface to about 3-4 cm inward into 0.9% NaCl or M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 M $MgSO_4$, water to 1 L) before microscopy and PCR. Root symptoms are fairly distinct for different nematodes, which may be dissected or extracted in a mist-chamber (Hooper, 1986). Potato rot and stem nematodes, *Ditylenchus* spp., may exhibit necrosis or rotting of rhizomes or roots, but symptoms here are less common than with cyst or root-knot nematodes. *Globodera rostochiensis* appears as small immature female of white or yellow stages, or brown cysts if the plant roots are examined at the flowering stage. Galls on potato roots caused by most root-knot nematodes are easily distinguished from the bead-like galls on roots caused by false root-knot nematode, *Nacobbus aberrans*. However, *Meloidogyne chitwoodi* causes almost no root galling (EPPO/CABI, 1997d), while *M. hapla* produces small galls with lateral root proliferation (Santo *et al*., 2004). Soil harbors juveniles, some males and some eggs, all of which are less diagnostically useful than females. All *D. destructor* stages may overwinter underground (EPPO/CABI, 1997a). Soil sampling strategies for potato cyst nematode (Haydock and Perry, 1998) may also be useful for other nematodes. Soil surrounding tubers or from the rhizosphere should be collected after removing the top 3-5 cm of soil and litter layer. Soil and fine feeder roots should be collected in plastic bags, tied and tagged with a label bearing details of the habitat, host, locality and other data such as soil type, associated vegetation and date of collection. The 1 cup to 1 L sample should be processed as soon as possible, but if necessary can be stored in plastic bags at 4°C.

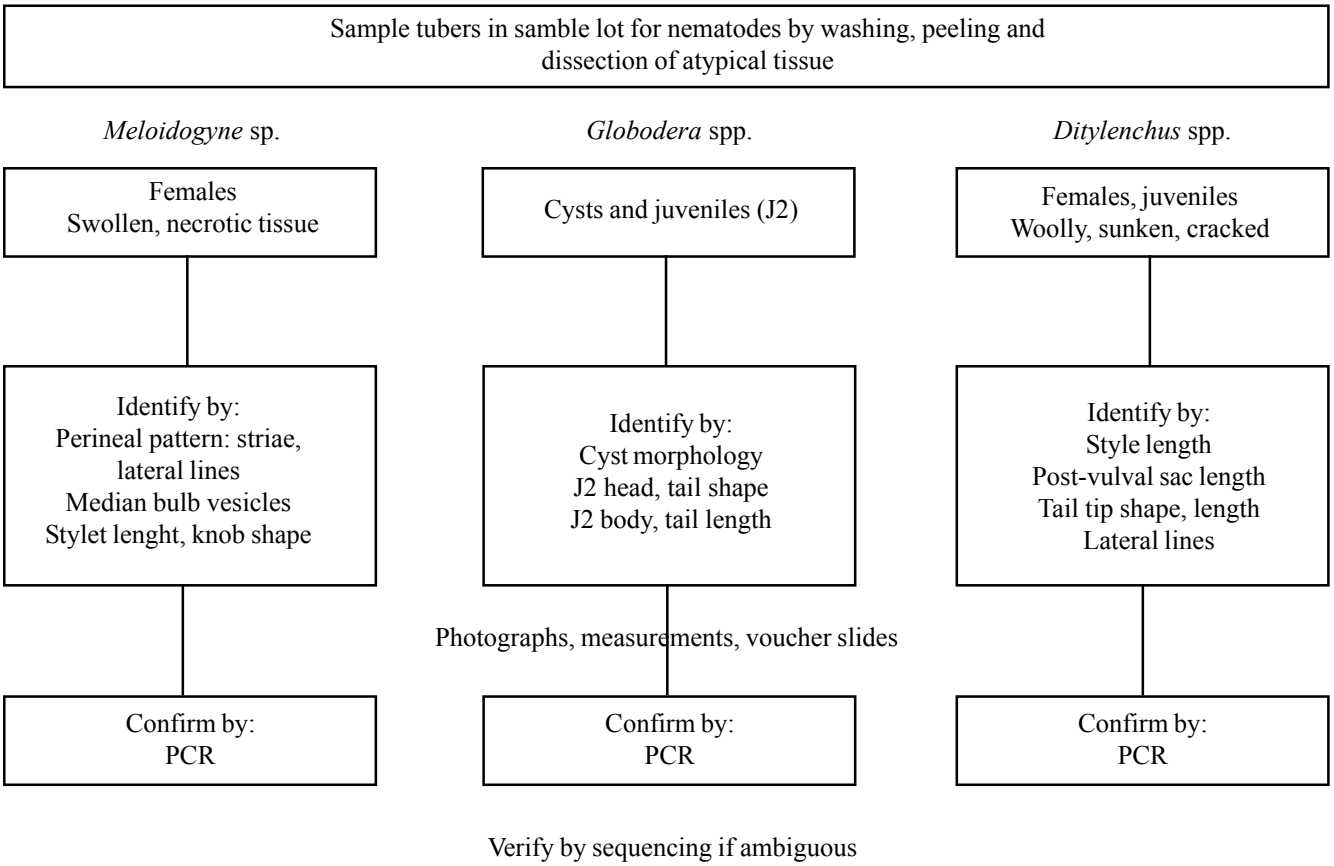

Fig. 1. Determination of *Meloidogyne chitwoodi*, *Globodera rostochiensis*, *Ditylenchus destructor* and *D. dipsaci* from potato tubers.

Maintaining intercepted specimens through avoidance of temperature extremes is especially important in situations of potential quarantine concern. Specimen preservation is often a matter of official policy. See Appendix 1 for nematode extraction protocols.

Microscopy and archival methods: traditional, digital, DNA. It is very important to deposit archived nematodes in a reference collection. For identification and taxonomic study, good, clean specimens showing most anatomical details are essential. Proper killing and fixing of nematodes are important steps in obtaining good results since permanent mounts are essential for long-term preservation (see Appendices 1-3 for protocols and references on extraction, killing-fixing and mounting procedures). Reference specimens should be fixed in 4% formalin. This material can be kept in mass collection vials, but a few specimens should be made into slides. A color digital camera with color film can be used to record the presence or absence of cyst tanning typical of *G. rostochiensis*, but not of *G. pallida*. Characters that must be captured if available for each species include female vulva, stylet of any stage, and male spicule. Archive for specimens of critical regulatory importance should include the head and tail of a given specimen on the same slide. Specimen archive for DNA procedures can be made in 95% ethanol, 1 M NaCl, or frozen extraction buffer.

References for molecular identification. Confirmatory molecular methods beyond morphological characters are used for *Meloidogyne* and *Globodera* species identification. Among many possible choices in DNA extraction protocols and purification, selection depends on the desired diagnostic tests. Molecular methods for detecting *M. chitwoodi*, *M. fallax* Karssen, and *M. hapla* have been recently reviewed (Castagnone-Sereno, 2000; OEPP/EPPO, 2004a). Molecular methods for *Globodera rostochiensis* and *G. pallida* have also been reviewed (Fleming and Powers, 1998; OEPP/EPPO, 2004b). General principles of nematode molecular diagnostics are found in Powers (2004) and Blok (2003). The following molecular methods may also be used for identification, depending on the genus and species, where ambiguities between closely related taxon pairs or a suspected new species may be present. For *Meloidogyne* spp. females, esterase and malate dehydrogenase isozyme patterns may be confirmatory with morphology, but *M. chitwoodi* has an esterase pattern indistinguishable from at least one population of *M. incognita*, and *M. hapla* has a pattern difficult to distinguish from *M. incognita* (Esbenshade and Triantaphyllou, 1985). After examination of vermiform specimens by light microscopy and recording of digital images, temporary slides may be

dismantled for subsequent DNA extraction and purification. Three acceptable extraction and PCR protocols for *Meloidogyne*, *Globodera*, and one for *Ditylenchus* are given in Appendices 4-6.

Positive and negative PCR controls for each species. In general, a positive control should be obtained from designated geographic regions where studies have shown documented specimens typical of the species where host range has been tested to determine race, and pure cultures should be carefully maintained in a greenhouse. For *Globodera rostochiensis*, a negative control should include *G. tabacum* (Lownsbery and Lownsbery) Skarbilovich (Cherry *et al*., 1997) and possibly *G. pallida* present in Europe and Canada. For *Meloidogyne chitwoodi*, a negative control should include *M. hapla*, *M. incognita*, and *M. naasi* or *M. graminicola* Golden and Birchfield if present in the region; *M. fallax* and *M. minor* Karssen, Bolk, van Aelst, van den Beld, Kox, Korthals, Molandijk, Zijlstra, van Hoof and Cook would be used if specimens came from outside North America (OEPP/EPPO, 2004a). *Meloidogyne chitwoodi* has molecular sequence similarities with both *M. naasi* (Zijlstra *et al*., 2004) and *M. graminicola* (Castillo *et al*., 2003). *Meloidogyne graminicola* can parasitize potato and other solanaceous plants (MacGowan and Langdon, 1989). Morphologically similar but non-pathogenic *Ditylenchus myceliophagus* Goodey can be used as a negative control for *D. destructor* or *D. dipsaci* (Wendt *et al*., 1994).

Ideal primer sets for species combinations. For *Globodera rostochiensis* and *G. pallida*, specific primer pairs as detailed in Bulman and Marshall (1997) or Fullaondo *et al*. (1999) can be multiplexed for mixed populations as low as 5%, and have shown the most consistently reproducible results within species (Radivojevic *et al*., 2001). The primer pairs of Shields *et al*. (1996) are adequate if *G. pallida* is not present. If ambiguous results with this species do occur, another method of confirmation should be used. RFLPs for species of *Globodera* may be made with ITS 1 and 2 using primers rDNA1 and rDNA2, followed by up to nine restriction enzymes (Subbotin *et al*., 2000; Vrain *et al*., 1992). For *Meloidogyne* spp., species-specific primer pairs capable of being multiplexed for mixed populations, such as those of Petersen *et al*. (1997), Zijlstra (1997), or Williamson *et al*. (1997) are appropriate to distinguish *M. chitwoodi*, *M. fallax* (regulated), and morphologically variable *M. hapla* and *M. incognita*. Universal primers followed by restriction enzymes may be used as described above. Wherever non-specific universal primers are used, test material must be relatively clean, since false-positives for species of *Heterodera*, *Meloidogyne*, and *Pratylenchus* have occurred when diverse environmental organisms were present (Volossiouk *et al*., 2003). While sequencing of specimens is expensive and time-consuming relative to restriction digests or tests with species-specific primers, this may be necessary in case of any uncertainties in gel profiles of target specimens. Multiple nematode sequences are available in GenBank for final verification.

Interpretation of results. A variety of procedures are available to diagnose potato nematodes of quarantine importance. Methods for surveys involve somewhat less rigor than those involving border interceptions. The procedures selected depend on what constitutes a valid sample for reliable diagnosis. Material is often limited, yet accuracy and low laboratory cost are of paramount importance for growers and regulators. It is essential to provide morphological voucher specimens with specific location and molecular information for important trade decisions whenever possible. Because some diagnostically important structures such as stylet knobs may fade with time, it is highly recommended before fixation to photograph stylet, lip, and tail. This would be necessary if only a single juvenile specimen were available. Other important features include spicules and head regions for single males, and perineal and head regions for single females. Although morphological analysis can be sufficient for a diagnosis without protein pattern or PCR procedures, material should be available frozen, in alcohol, in salt (*e.g*. 1 M NaCl), or as amplified genomic DNA (Skantar and Carta, 2005) for subsequent molecular analysis if the morphological identification is challenged. While females are essential for reliable morphological identification, it is difficult to quantify how many are sufficient for morphological ID alone. This depends on the condition of specimens and if diagnostic features are within the typical range. Can a single juvenile or other stage tested by PCR alone be considered acceptable without unambiguous morphology?. If the sample is contaminated with other pathogens, or the likelihood of mixed species in the sample is high (more common with root-knot than cyst nematodes), species-specific primers are important. However, unless the primer pairs are clearly validated, even this could be uncertain. For instance, a single juvenile may have up to a 20% chance of showing a false negative result with one set of species-specific primer pairs for *M. chitwoodi* (Williamson *et al*., 1997). Therefore, molecular procedures should be run two times if possible; confirmation by a second laboratory may be desirable. For diagnostic markers such as ITS that show substantial intraspecific sequence heterogeneity, a second marker may be warranted (Powers, 2004). The report should contain comments on the degree of certainty of the identification. The best molecular diagnostics for phytosanitary labs. involve a single PCR step to allow high throughput, are sensitive to low numbers of individuals, and have been reliably validated against related organisms in the same geographic location (Hübschen *et al*., 2004). *Meloidogyne chitwoodi* protocols will especially benefit from further validation with frequently coexisting *M. hapla*, *M. naasi*, and potentially coexisting (MacGowan and Langdon, 1989) molecular relatives *M. graminicola* and *M. trifoliophila* Bernard and Eisenback (Castillo *et al*., 2003). Validation has been published for some potato cyst nematode primers for *G. rostochiensis* and *G. pallida* (Radivojevic *et al*., 2001); however *G. tabacum* (OEPP/EPPO, 2004b), *G. solanacearum*

(Miller and Gray) Behrens and *G. virginiae* (Miller and Gray) Stone (not parasitic on potato per Miller and Gray, 1968) from North America have not been sufficiently tested and reported. Rapid real-time PCR techniques are also available for *Globodera rostochiensis* and *G. pallida* (Bates *et al*., 2002; Madani *et al*., 2005), although these are currently more expensive than gel-based tests. Also, real-time assays may not save much time for use on an occasional basis unless the PCR equipment is regularly used and calibrated. Diagnostic decisions will improve as this refinement process continues, and comparison with a number of alternative primer sets on multiple individuals. These guidelines may be revised through a NAPPO web-based Appendix every five years or as needed (NAPPO, 2005), as molecular methodologies will undoubtedly continue to improve.

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APPENDICES

Further details on the extraction, killing, fixing and mounting procedures of specimens listed in Appendices 1-3 are given by several authors (Ayoub, 1977; Golden, 1990; Hooper, 1986; Oostenbrink, 1960; Seinhorst, 1959; Taylor, 1971; Thorne, 1961).

Appendix 1. Extraction of nematodes from soil and plant material. A). Direct Sieving (After Ayoub, 1977 and Hooper, 1986). Materials: 10 L bucket, 20-mesh sieve, 60-mesh sieve, and 100-mesh sieve for suspected cyst nematode females, and/or 325-mesh sieve for other stages or genera, 250 mL beakers, watch glasses, squirt bottles or pressurized hose, and stand to hold sieves. Check any available roots separately after gentle rinsing in sample bucket to find embedded females. Procedure: Stack 3 sieves on stand, one on top of the other, 20-, 60- and 100- or 325-mesh. Wet the sieves so water runs freely. For soil, place an approximately 250 mL soil sample into bucket. Stir with hand and break up clumps of soil while adding water to 3/4 the bucket volume. When solution is uniform, wait 15 seconds for some of the heavy sediment to settle. For tubers, wash individually especially around the eyes with brush and running water in a bucket. Lift bucket gently and pour water through the stacked sieves, leaving the settled sediment in the bottom. Discard the contents of the 20-mesh sieve. Collect cysts and large vermiform stages from the 60-mesh sieve by backwashing into a beaker, and other vermiform stages by backwashing the 200- or 325-mesh sieve into a separate beaker. For cysts in 60- and 100- mesh sieves, wash onto labeled filter paper for drying. For other samples, observe liquid in watch glass or continue further clean-up by Baermann funnel or sugar flotation if necessary. Types of nematodes to collect from various sieves: 60-mesh for cysts, white females, some large males and other vermiform stages, 100 mesh for some females, cysts and males, 325-mesh for juveniles and males (200-mesh may also be used to catch some juveniles and males if samples are particularly dirty). B). Baermann Funnel (after Hooper, 1986). Materials: Funnel in support (or petri dish or flat tray), tygon tubing, clamp, wire basket to fit funnel or dish, porous paper, plastic cover. Procedure: Put tube partially over stem of funnel. Place clamp securely below glass stem so water can't leak through. Fill funnel with tap water to a few centimeters below the rim. Open the clamp to release a small amount of water and any bubbles. Nest the paper in the wire basket within the filled funnel. Place 50-60 cc soil in the basket. If necessary, gently add enough water to the soil so it appears half saturated. Place the plastic cover over the basket of soil to reduce evaporation and set up a thermal gradient. Various live nematodes will wiggle through the holes in the paper and collect in the funnel stem or base of tray. Reasonable accumulations occur between 24 and 48 h, but oxygen is limiting in funnels and sensitive species may die after a day. Release nematodes into a sample vial and decant surface column of water after approximately half an hour so that nematodes are in a few centimeters of water. Optimum storage temperature for live material over a few days is 15-20°C. C). Sugar flotation for nematodes (adapted from Carta and Carta, 2000). The specific gravity (SG) of most nematodes is in the range (1.06, 1.10). In water (SG = 1.00) they will sink; in a 1 M sugar solution they will float. Materials: Centrifuge, 50 mL test tubes, wash bottle or hose and fog-nozzle, 65- and 500-mesh sieves, 250 or 500 mL beakers, sugar solution. Mix 2 parts granulated table sugar in 5 parts water by volume, or 227 g sugar/500 mL water for SG > 1.10; (between 1.105 and 1.13 SG checked by pyncnometer or refractometer = 24 BRIX or 16.5 degrees Baume). Procedure: Label pairs of centrifuge tubes, fill with 10 mL soil and water to 50 mL volume. Cap tubes and shake mixture well. Spin at about 1,000 g for 5 min so heavy soil and nematodes concentrate in a pellet, and light organic material floats. Discard water and floating organic material, without disturbing the pellet. Refill the tubes with a 1.11 specific gravity sugar solution. Shake tubes vigorously to resuspend the compacted pellet in the sugar solution. Centrifuge again at 1,000 g for 5 min. The soil will again concentrate in a pellet, but all particles and

nematodes with specific gravity less than 1.11 will float. Nematodes can be recovered from the sugar solution immediately by pouring the solution through a 325-, 400-, or 500-mesh sieve (stack a 65-mesh sieve on top to catch any large particles for discard if necessary). Nematodes retained by the sieves can be collected by backwashing into a collection beaker. Work quickly to avoid osmotic distortion. Nematodes can be concentrated by pouring the beaker contents into a narrow cylinder or wide mouth jar and letting the nematodes fall (about 1 cm/5 min) to the bottom. Pipette water off surface leaving at least one cm of water with nematodes. Allow two hours for nematodes to recover full turgidity before fixation (Caveness and Jensen, 1955). D). Crushing-Sieving-Centrifugation-Flotation (after Sosa-Moss, 1997; Coolen, 1979). Materials: 100-300 cc soil and/or plant material, blender, 20-mesh (840 µm opening), 60-mesh (240 µm), 100-mesh (149 µm), 200-mesh (74 µm), 325-mesh (44 µm) sieves, kaolin powder, 50 mL centrifuge tubes, centrifuge. Procedure: Crush plant material such as potato tuber slices or roots with a slow-speed blender (12,000 rpm) for three runs of 1 second. For soil, mix sample in 2 L water, settle for about 5 seconds, and pour water through sieves. Backwash material from sieves of 100-mesh and higher. Concentrate suspension and place in 50 mL centrifuge tube, then add 1 g kaolin and rapidly mix in a circular motion. Balance tubes by weight and centrifuge 5 min at 2,800 rpm. Kaolin will bind to nematodes present in pellet. Dissolve this precipate in 1 M sucrose (227 g/500 mL water), and centrifuge at 2,800 rpm for 3 min. Sieve floating nematodes in sucrose solution through 325- or 500-mesh sieve quickly, reducing volume to 30 mL for observation.

Appendix 2. Mounting methods for cysts, perineal pattens, and vermiform stages. Cyst mounts (*Globodera* spp.). Cysts are identified by size, colour and morphological characteristics. Moisten cysts in water 24 h before cutting the fenestral area (Sosa Moss, 1997) in 10 µL of sterile distilled water. Cut the posterior part of the cyst in half (or a third part) with a knife. Try to keep the fenestral area at the centre of the cut section under the stereoscope microscope. Cut until obtaining a small piece of cuticle, so that the vulva region remains at the centre. Clean the body tissues adhered to the cuticle thoroughly, without damaging the structures related to the vulva. Cut cyst at or around the lower third area in 3% formaldehyde, or in 45% lactic acid. (Note: press the cyst to release eggs and mount the eggs/released juveniles from the eggs on a temporary slide mount in formaldehyde or lactic acid after tapping the cover slip from the top with a small needle). Clean with a needle and trim cyst portion/pattern with an eye knife near the lower third area. Transfer the cut to other slides and mount the cut in a drop of dehydrated glycerine, and seal it with a wax ring, heating a hot plate to seal and avoid air bubbles. Alternatively, transfer portion of trimmed posterior part and anterior part of cyst into a tiny drop of lactophenol on a slide. Put a round 18 mm cover slip on top of the lactophenol drop, press down gently and ring the edges of the cover slip with Glyceel/Zut. Root-knot nematodes *Meloidogyne* spp., and *Ditylenchus* spp. Various stages: Second-stage juveniles (J2) and males are recovered from infected roots, egg masses or sieved or floated extract and kept in Petri dishes with a small amount of water. Females are dissected from infected roots or tubers. Nematodes should be gently heated and fixed overnight in 3% formaldehyde. Nematodes may initially be killed in an 80°C water bath, followed by addition of the same volume each of cold 4% formaldehyde and hot 4:1 (10 mL 40% formaldehyde + 1 mL glacial acetic acid + 89 mL water) for three days, followed by substitution with 4% formaldehyde before dehydration. Alternative procedures for measuring and preparing specimens are given in Hooper (1986) and Golden (1990); for instance, fixed female cyst cones can be cut and mounted in euparal (Golden and Birchfield, 1972) glycerol, lactoglycerol or lactophenol solutions. Photomicrographs of perineal patterns, J2, and males can be made with 35-mm or digital camera attached to a compound microscope equipped with differential interference contrast optics. Roots with galls can be photographed under a dissecting microscope and light microscopic photographs of fixed nematodes can be taken on a compound microscope. Measurements are made with an ocular micrometer on a compound microscope. Root-knot nematode (*Meloidogyne* spp.) perineal patterns. Tease out females from roots or tubers. On a slide in a drop of formaldehyde, clean then nick and cut off at lower third. Then, mount in a drop of small lactophenol or 45% lactic acid with outer cuticle of posterior end facing upwards and the anterior portion of the same female adjacent to its posterior end. Mount about five anterior and posterior ends on the same slide in a similar manner as described above.

Appendix 3. Specimen processing for slide preparation. A modified Seinhorst (1959) method for nematodes to glycerin is as follows (Golden, 1990): Make up two solutions: Solution I, 1 part glycerol, 20 parts 96% ethanol, 79 parts distilled water; Solution II, 5 parts glycerol, 95 parts 96% ethanol. Nematodes should be hand picked with clean tools in a small deep dish (holding about 10 mL) containing Solution I (about 75% of the volume of the dish). The dish and the solution must also be extremely clean as any small particles will adhere to the specimens. Add sufficient saturated picric acid solution (usually 2 drops) to stain the nematodes. (**Warning**: In the dry state picric acid is an explosive. Keep stock material moist with water. From this stock, add a small amount of picric acid to a small dropping bottle containing pure water, adding just enough to make a saturated aqueous solution.) Place the open small dish into a larger closed glass vessel, *e.g.* a desiccator, dehydrator or staining dish, containing about 1/10 of its volume of 96% ethanol. Leave the dish in this saturated atmosphere for at least 12 h in an oven at 35-40°C. This removes almost all the water and leaves the nematodes in a mixture of glycerol and ethanol. Decrease the volume in the small dish by about half and add Solution II to the dish after the 12 h. Several hours later repeat the removal and replacement of Solution II (less collapsing of

certain nematodes occurs using this modification). Place the small dish in a covered petri dish and replace it in the oven. Keep in covered petri dish in oven and add Solution II every day for a week. Then leave in the oven until the alcohol evaporates and the nematodes in the dish are in pure glycerol and can be mounted. An alternative schedule for glycerin infiltration without picric acid: Nematodes in 4% formaldehyde are held in 40°C incubator for 12 h, then upper solution pipetted off and replaced with Solution I for 3 h, two times, followed by Solution II for 3 h, two times. Pure dehydrated glycerin replaced remaining Solution II for 3 h, followed by maintenance in a dessicator with $CaCl_2$ until mounting.

Appendix 4. Acceptable alternative PCR Techniques for *Meloidogyne*. A protocol used in Canada originated by Vrain *et al*. (1992) with 18S and 26S primers produces an ITS1 and ITS2 PCR fragment, which is then subjected to restriction digestions. ITS-specific markers include CF-ITS, H-18S, I-ITS and HCF-28S (Ziljstra *et al*., 1995; Ziljstra *et al*., 1997). Species-specific forward primers combined in a multiplex PCR with a common reverse primer produce PCR fragments of species-specific size. There is an option of a restriction digestion using *Rsa*I and *Hinf*I ITS-RFLP markers to help distinguish *M. chitwoodi* from *M. fallax*. (Ziljstra *et al*., 1997) and differentiating *M. chitwoodi*, *M. fallax*, *M. incognita* from *M. hapla* (18S: 5'-TTGATTACGTCCCTGCCCTTT-3'; 26S: 5'-TTTCACTCGCCGTTACTAAGG-3'; CF-ITS: 5'-GAATTATACGCACAATT-3'; H-18S: 5'-CTTGGAGACTGTTGATC-3'; I-ITS: 5'-TGTAGGACTCTTTAATG-3'; HCF1-28S: 5'-TCCTCCGCTTACTGATATG-3'). DNA Extraction as modified from Orui (1996): Incubate single juveniles in PCR tubes for 1 hour at 55ºC. Include a tube of lysis buffer as the DNA negative control. Inactivate proteinase K at 95ºC for 10 min. Store at -20ºC. Use 5 µL per 15 µL PCR reaction, using 0.3 µL of 0.4 µM ITS specific primers, and 0.9 µL of 0.6 µM 18 and 26S RFLP markers, 0.075 µL of 5U/µL *Taq*. Cycling conditions for specific markers: Cycling conditions for RFLP markers: initial denaturation 94ºC 1 min, then 35 cycles of secondary denaturing at 94°C 15 sec, anneal at 55°C for 30 sec, extend at 72°C for 11/2 min, final extension at 72ºC for 3 min, hold at 4°C. A protocol is used in Mexico from Petersen *et al*. (1997) from the Ribosomal Intergenic Spacer Regions (IGS) of *M. chitwoodi* using primers 1839 and C64 (1839: 5'-AGCCAAAACAGCGACCGTCTAC-3'; C64: 5'-GATCTATGGCAGATGGTATGGA-3') that produce an amplified segment of 990 bp. DNA Extraction modified from Curran *et al*. (1986): Pelletize the females and eggs centrifuging at 10,000 rpm for 10 min. Add 50 µL of Extraction Buffer (Tris-HCl 1M pH 8.0, EDTA 0.5 M pH 8.0, NaCl 5 M, 20% of SDS) macerate with a clean pestle, and finally add another 50 µL of buffer to complete a total volume of 100 µl. Add 0.05 µL of proteinase K and mix rapidly in a circular motion. Incubate at 65°C for 20 min. Add a volume of phenol-chloroform-isoamyl alcohol (lower part). Mix the phenol with the sample by vortexing rapidly in a circular motion. Centrifuge at 12,000 rpm for 15 min. Remove the supernatant to a clean microcentrifuge tube and add a volume of phenol-chloroform-isoamyl alcohol. Centrifuge at 12,000 rpm for 15 min. Remove the supernatant and place it in another clean tube. Add 2.5 vol of cold absolute alcohol to precipitate the DNA and mix by turning 3 to 4 times. Centrifuge for 10 min at 12,000 rpm. Decant the supernatant and wash the pellet with 200 µL of 75% ethanol. Crumble the pellet with the vortex and centrifuge at 12,000 for 5 min. Decant and let the pellet dry at room temperature. Dissolve the pellet in 50 µL of sterile double distilled H_20, free of DNAses and RNAses. Observe in an agarose gel at 0.8% in UV light. PCR Components per tube: 5.0 µL of 10X PCR Buffer, 5.0 µL of 2.5 mM $MgCl_2$, 1.0 µL of 10 mM dNTP, 2.5 µL of 20 pmol Primer 1(C64), 2.5 µL of Primer 2 (1839) 20 pmol, 0.5 µL of 2.5 U/µL *Taq* Polymerase, 2.0 µL of 50 ng DNA, 31.50 µL sterile distilled H_20, for a final volume of 50.0 µL. Add 10 µL of mineral oil to avoid evaporation. Thermocycler conditions: Initial denaturing at 95°C for 5 min, secondary denaturing at 95°C for 30 sec, annealing at 60°C for 45 sec, extending at 72°C for 1.30 min, final extension at 72ºC for 10 min, hold at 4°C. The DNA extraction and PCR methods of Powers and Harris (1993) and Cherry *et al*. (1997) are used in the United States. Nematodes are individually placed in a 15 µL drop of sterile water on a cover slip, and smashed with a micropipette tip. The solution is placed in individual PCR reaction tubes. A 2-3 µL portion of the solution serves as DNA template for PCR. The first amplification is made with primer set C2F3/1108 (5'-GGTCAATGTTCAGAAATTTGTGG-3' and 5'-TACCTTTGACCAATCACGCT-3') located in the COII and 16S ribosomal genes respectively. PCR reaction master mix typically consists of 1.5 units of a hot-start polymerase such as JumpStart *Taq* (Sigma, St. Louis, MO, USA) final concentration with its 10x buffer at 1x final concentration, total Mg^{+2} at 3.0 mM final concentration, dNTPs each at 200 ìM final concentration, each primer at 0.36 ìM final concentration. From the master mix, 27.0 µL is aliquoted to a PCR tube containing 2-3 µL nematode template and mixed thoroughly. Amplification conditions generally include a modified hot-start, initial denaturation at 94ºC for 3 min, followed by 45 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec and extension at 72ºC for 1 min. Final extension is for 2 min at 72ºC. Time, temperatures and ramping rates vary according to thermal cyclers and primer sets. Initial CF3/1108 PCR products (5.0 µL of each mixed with 1.0 µL loading buffer (gel dye)) are separated on a 1.0% agarose gel (made with Agarose Low EEO Electrophoresis Grade and 0.5XTBE buffer). Size standards are 1 Kb or 100 bp ladders (used at about 185 ng DNA/gel lane). Electrophoresis is conducted in 0.5X TBE running buffer for about 101 volt-hours. Bands are stained with ethidium bromide (EtBr) during the run, and results are recorded on Polaroid 667 film. The sizes of individual products determine

subsequent assays: 1. If the PCR product is 0.52kb it is subjected to a *Dra*I digestion: > 4-25 iL of each PCR product is digested with *Dra*I enzyme (from New England Biolabs) along with NE Buffer4 in 12.2 µL total reaction volumes overnight in a 37°C incubator). Digestion results: 243 bp, 200 bp, 53 bp, and 36 bp (*Meloidogyne hapla*) or 260 bp, 120 bp, 85 bp, 40 bp (*M. chitwoodi*). 2. If digestion suggests *M. chitwoodi* is present, a second amplification is conducted on the original DNA. The 18S primer set (18s1.2/18sr2b: 5'-GGCGATCAGATACCGCCCTAGTT-3'; 5'-TACAAAGGGCAGGGACGTAAT-3') produces an approximately 640 bp. product. Digestion with *Alu*I results in a 350, 115, 85, 50 bp set of products, unique for *M. chitwoodi*. 3. Alternatively, species-specific PCR primer pairs based on species-diagnostic RAPD markers can be used (Williamson *et al*., 1997). The primer set for *M. hapla* (MH0F: 5'-CAGGCCCTTCCAGCTAAAGA-3'; MH1R: 5'-CTTCGTTGGGGAACTGAAGA-3') produces a 960 bp product. The primer set for *M. chitwoodi* (MC3F: 5'-CCAATGATAGAGATAGGCAC-3'; MC1R: 5'-CTGGCTTCCTCTTGTCCAAA-3') produces a 400 bp product. While this technique is more rapid and less expensive than the restriction digestion above, 80-90% of individual J2s were successfully amplified. The primary strong band typical of *M. chitwoodi* did not occur in some, requiring the use of multiple individuals to detect this species (Williamson *et al*., 1997).

Appendix 5. Acceptable alternative PCR Techniques for *Globodera*. In Canada, the method of Fullaondo *et al*. (1999) employing species-specific RAPD primers for multiplex PCR is used after the extraction protocol described by Cherry *et al*. (1997) with primers (*G. rostochiensis*: 5'-GCAAGCCCAGCGTCAGCAAC-3' and 5'-GAACATCAACCTCCTATCGG-3'; and *G. pallida*: 5'-TGTCCATTCCTCTCCACCAG-3' and 5'-CCGCTTCCCCATTGCTTTCG-3') producing 315 bp and 798 bp fragments, respectively. Reaction volume of 25.0 µL includes 50 ng primers, 0.5U *Taq*, and 25 ng DNA, with cycling conditions having initial denaturation of 94°C, 4 min, and 40 cycles of 94°C, 1 min, 60ºC 1 min, 72ºC 2 min, ending with 72ºC for 10 min, hold at 4°C. In Canada, DNA extraction from cysts may be done in two ways:

Method 1. For a positive sample, include at least one of the biological stages for the identification of the nematode (cyst, eggs, juveniles, females and males), separated from other nematodes. A modification of the procedure of Caswell-Chen *et al*. (1992) for cyst extraction is used: Place 1.2 mg of cysts (50 cysts) in a microcentrifuge tube. Soak the cysts in the same tube with a carpel, adding 50 µL of extraction buffer, later on add another 50 µL of the same buffer (100 mM EDTA, 0.5 % SDS, 50 µg/mL proteinase K, pH = 8) with a total final volume of 100 µL. Add 0.05 µL of proteinase K, mixing rapidly in a circular motion two to three times to break the protein structure. Incubate in a water bath at 50°C for 2 h, mixing rapidly in a circular motion at intervals of 10 min. Immediately after the incubation, place the microcentrifuge tube for approximately one minute in ice (to stop the reaction). Centrifuge at 1,000 rpm for one minute at 6°C. Then remove the remaining with a micropipette and place it in another microcentrifuge tube, add a volume of phenol-chloroform: isoamyl alcohol (25:24:1). Mix rapidly in a circular motion two to three times. Centrifuge at 14,000 rpm for 15 min at 6°C. Remove the watery phase with a micropipette and place it in another tube, then add 0.1 volume of NaOAc 3M and 3 volumes of 100% ethanol. Invert two to three times. Incubate at -20°C for 2 h. Then centrifuge at 14,000 rpm for 15 min at 4°C to get a pellet. Discard the supernatant, add 3 volumes of 70% ethanol to the pellet. Detach the pellet with vortex. Centrifuge at 14,000 rpm for 15 min at 4°C. Discard the remaining supernatant in the tube and let the pellet dry at room temperature. Dissolve the pellet in 50 µL of sterile distilled water. Run in 0.8% agarose gel, and observe through a UV transluminator at 254-366 nm.

Method 2. Direct PCR from a single cyst: After morphometric observation the remaining eggs, juveniles and/or cuticle are placed in a 500 µL microcentrifuge tube with 10 µL of sterile distilled water and immediately placed on ice. PCR components detailed above are added directly to the microcentrifuge tube with the rest of the cyst. Anneal temperature of the PCR should be 50°C in a thermocycler with the steps indicated above. The PCR product is run in 1.4% agarose gel and it is observed through the transilluminator. In Mexico, the protocol and primers of Shields *et al*. (1996) modified to differentiate *Globodera rostochiensis* and *G. pallida*, using the primers 5SG and SLG specific for *G. rostochiensis* and *G. pallida* are used. (5SG: 5'-CGCGGATCCTTACGACCATACCACG-3'; SLG: 5'-CTCAAACTTGGGTAATTAAACC-3') The size of the amplified segment for the reaction of PCR is a 914 bp band for *G. rostochiensis* and two bands for *G. pallida*: 853 bp and 914 bp using the primers 5SG and SLG that detects a preserved region of the genome located between the genes 5S rDNA and SL DNA. Oligonucleotides or primers lyophilized are resuspended in Buffer TE, pH 7.2 with a final concentration of 0.5 µg/µL. Before usage, primers are diluted at a concentration of 50 ng/µL or 10 pmol (5SG and SLG). Procedure: 500 µL microcentrifuge tubes for final volume of 50.0 µL, containing 2.0 µL 20 pmol of each primer and 0.06 µL of 0.3 U/iL *Taq* Polymerase and 1.5 µL of 1.5 mM $MgCl_2$, 1 µL dNTP's 10 mM, with 2 µL of DNA overlain with mineral oil. Mix all ingredients in a mixer. Place the tubes in the thermocycler with the following program: Thermocycler conditions: initial denaturing at 94°C 5 min, 30 cycles of secondary denaturing at 94°C 30 sec, anneal at 60°C for 1 min, extend at 72°C for 2 min, final extension at 72ºC for 10 min, hold at 4°C. The PCR product will be run in 1.4% agarose gel and observed through the transluminator. In the United States the extraction protocol of Cherry *et al*. (1997), as above is followed by the ITS rDNA protocol of Bulman and Marshall (1997) using multiplexed species-specific primers. This

protocol results in a 315 bp fragment for *G. rostochiensis* and 798 bp for *G. pallida*. (ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3' White *et al*. (1990); ITS26 (AB28R): 5'-GCGGATCCATATGCTTAAGTTCAGCGGGT-3' Howlett *et al*. (1980); PITSr3: 5'-AGCGCAGACATGCCGCAA-3'; PITSp4: 5'-ACAACAGCAATCGTCGAG-3' Bulman and Marshall (1997). Reaction volume 25.0 µL, 250 µM primers, 0.6U *Taq* polymerase, 1.0 µL DNA. Thermocycler conditions: initial denaturing at 94°C, 2 min, 35 cycles of secondary denaturing at 94°C, 30 sec, anneal at 60°C for 30 sec, extend at 72°C for 30 sec, hold at 4°C. Confirmation of *G. rostochiensis* over a similar ITS profile of *G. tabacum* (Cherry *et al*., 1997) was made with restriction enzymes *Alu*I, *Mae*III, and *Dde* I (Thiéry and Mugniéry, 1996).

Appendix 6. PCR Techniques for *Ditylenchus*. *Ditylenchus dipsaci* and *D. destructor* can be identified with ITS rDNA-related diagnostics (Leal-Bertioli *et al*., 2000; Wendt *et al*., 1994). Ribosomal DNA of the ITS region using 18S and 26S primer pairs (18S: 5'-TTGATTACGTCCCTGCCCTTT-3'; 26S: 5'-TTTCACTCGCCGTTACTAAGG-3') can be employed as RFLPs to distinguish *D. destructor* (1.2-kb band) from the 0.9-kb band of both *D. dipsaci* and *D. myceliophagus*; *D. dipsaci* can then be separated by other restriction enzymes such as BAmH I to produce 180, 220 and 340 bands (Wendt *et al*., 1994). Alternatively, a rapid ITS-based diagnostic test is available for *D. dipsaci* as detailed in Subbotin *et al*. (2005).

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