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Discrimination of Six *Pratylenchus* Species Using PCR and Species-Specific Primers[1]

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Abstract: A PCR-based assay for identification of six species of *Pratylenchus* common in California is described. In this assay, five forward species-specific primers were designed from the internal variable portion of the D3 expansion region of the 26S rDNA and were each used with a single, common reverse primer. The optimized species-specific primers produced unique amplicons from their respective target and did not amplify DNA from other *Pratylenchus* species. With this assay we were able to identify single females to species level. This method obviates the need for subsequent RFLP or sequence analysis of the PCR product and can be used as a rapid diagnostic tool in epidemiological and management studies.

Key words: D3 26S rDNA, diagnostic, *Pratylenchus*, PCR, species-specific primers.

Root-lesion nematodes, *Pratylenchus* spp., are among the most economically damaging plant-parasitic nematodes and are found in a wide variety of crops. In California, 12 *Pratylenchus* species have been reported (Siddiqui et al., 1973). Although *Pratylenchus* species are polyphagous, there are clear differences in host preference among the species. For example, whereas *P. vulnus* and *P. penetrans* are commonly found on a range of perennial fruit crops in California, *P. brachyurus* is mainly associated with cotton (McKenry and Roberts, 1985). Also, whereas some species are considered damaging (e.g., *P. vulnus* on fruit trees and roses and *P. penetrans* on fruit trees and bulbous ornamentals), damage by other species is rarely observed (e.g., *P. brachyurus* on cotton, and *P. thornei* and *P. neglectus* on sorghum and wheat) (McKenry and Roberts, 1985). Thus, to predict risks of crop damage based on pre-plant nematode levels, proper identification of *Pratylenchus* populations to species is essential.

Until recently, identification of *Pratylenchus* relied on morphological differences among species. However, the general morphology of all species is similar. In addition, there is strong intra-specific variation with few species-specific diagnostic characteristics (Loof, 1978; Orui, 1996). As a result, morphological identification of *Pratylenchus* species requires examination of several adult female specimens by an experienced taxonomist. A simple, quick, reliable, and relatively inexpensive diagnostic method for *Pratylenchus* species would allow growers to anticipate potential problems caused by these species and provide for nematode management. DNA-based techniques have been used previously to discriminate among species of plant-parasitic nematodes (Orui, 1996; Powers and Harris, 1993; Uehara et al., 1999; Waeyenberge et al., 2000). Orui (1996) and Waeyenberge et al. (2000) identified *Pratylenchus* species using PCR-RFLP techniques, but this required the digestion of PCR products with several restriction enzymes. Recently, Uehara et al. (1998) were able to distinguish between *P. loosi* and *P. coffeae* using DNA amplification with species-specific primer sets. In this paper, we describe the development of species-specific primer sets to distinguish six *Pratylenchus* species.

Materials and Methods

Populations: Six *Pratylenchus* species were used in this study: *P. brachyurus, P. neglectus, P. scribneri, P. penetrans, P. thornei,* and *P. vulnus*. Two populations of each species were obtained from different geographical locations and hosts (Table 1).

Nematodes were extracted either from soil using a sieving-and-decanting method or from roots placed in a misting chamber (Niblack and Hussey, 1985). Mixed stages of *Pratylenchus* were hand-picked from the extracted suspensions under 40-fold magnification and were either killed and fixed in hot buffered formalin (Humason, 1972) or used to establish pure cultures. Cultures were started by inoculation of a single gravid female around roots of plants grown in a greenhouse (*P. neglectus*), onto root explants (*P. brachyurus, P. scribeneri, P. penetrans, P. thornei, P. vulnus*) (Huettel and Rebois, 1985), or onto carrot discs (*P. brachyurus, P. scribeneri, P. penetrans, P. thornei, P. vulnus*) (O'Bannon and Taylor, 1968). Nematodes from these cultures were used either as a source of total DNA or to obtain adult females. Individual females were placed in 0.2-ml tubes containing 10 µl sterile water and stored at −80 °C until used.

Morphological characteristics: Permanent mounts (10 females/population, collected from the cultures) were made from nematodes fixed in buffered formalin (Seinhorst, 1959). Quantitative and qualitative morphological characteristics were determined using a compound light microscope. Nematodes were identified to the species level using both original descriptions

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TABLE 1. Sources of *Pratylenchus* populations used in this study.

Pratylenchus species	Code	Locality (USA)	Host	Source
P. brachyurus (Godfrey, 1929)	Pbra1	Kern Co., CA	*Gossypium*	A. Ploeg
	Pbra2	FL	*Aster*	E. Caswell-Chen
P. neglectus (Rensch, 1924)	Pneg1	Santa Barbara Co., CA	*Lactuca*	A. Ploeg
	Pneg2	Monterey Co., CA	*Pyrethrum*	B. Westerdahl
P. penetrans (Cobb, 1917)	Ppen1	MD	*Medicago*	D. Chitwood
	Ppen2	Curry Co., OR	*Lilium*	B. Westerdahl
P. scribneri Steiner, 1943	Pscr1	Riverside Co., CA	Mixed	A. Ploeg
	Pscr2	Kern Co., CA	*Vitis*	B. Westerdahl
P. thornei Sher and Allen, 1953	Ptho1	Santa Barbara Co., CA	*Pisum*	A. Ploeg
	Ptho2	Yolo Co., CA	*Triticum*	H. Ferris
P. vulnus Allen and Jensen, 1951	Pvul1	Fresno Co., CA	*Prunus amygda*	M. McKenry
	Pvul2	Yolo Co., CA	*Juglans*	B. Westerdahl

and diagnostic keys (Café Filho and Huang, 1989; Handoo and Golden, 1989).

Primer selection: DNA sequences for the 26S rDNA D3 expansion region from 10 *Pratylenchus* species, including all species from the present study, were obtained from Genbank (Al-Banna et al., 1997). An alignment of these DNA sequences was used to guide the design of six species-specific forward primers, each 20 nucleotides in length (Table 2). Forward primer sequences were selected for compatibility with the annealing temperature of the common reverse primer D3B (5′-TCGGAAGGAACCAGCTACTA-3′).

DNA extraction: Total DNA was extracted by incubating approximately 100 individual nematodes of mixed stages (stored at –80 °C for at least 1 hour) in 0.3 ml of extraction buffer (0.1 mM Tris-HCl 0.05 mM EDTA, 0.2 mM NaCL, 1% SDS, and 0.5 mg proteinase K/ml extraction buffer) at 65 °C for 1 hour. The lysate was then extracted with phenol/chloroform, and the DNA was precipitated with ethanol and subsequently dissolved in TE (10mM Tris-HCl, and 1mM EDTA, pH 8.0).

Amplification of the D3 region: The D3 region was amplified either from single females or from total DNA from each of the 12 populations using the primers D3A (5′-GACCCGTCTTGAAACACGGA-3′) and D3B (Al-Banna et al., 1997).

Amplification using species-specific primers: Preliminary experiments were conducted to optimize the PCR conditions by testing different annealing temperatures for each primer set using total DNA as the template. After identifying the optimum annealing temperatures, the diagnostic assay was performed on both the D3 amplification products and on single females previously frozen in 0.2-ml tubes containing 10 µl water. The amplified D3 region was used as a template in the species-specific primer assay instead of total DNA from bulked nematodes because the number of nematodes available from some populations was limited. The amplification was performed in a 25-µl reaction mix containing the DNA template, 2 units of Taq polymerase (Promega, Madison, WI), 200 µM dNTPs, 0.8 µM of each primer, 2.5 mM $MgCl_2$, and the buffer provided by the manufacturer. The thermal cycling was performed in a Peltier Thermal cycler (PT-200, MJ Research, Watertown, MA) as follows: a hot start at 95 °C for 3 minutes; 35 amplification cycles at 95 °C for 1 minute; 62, 63, or 68 °C, depending on the species-specific primer (Table 2) for 1 minute; 72 °C for 1 minute; and a final extension step for 7 minutes at 72 °C. Aliquots of 10 µl of the PCR products were analyzed by electrophoresis in 1.7% agarose gels, stained with ethidium bromide, and visualized under UV light. This experiment was performed a total of three times.

Amplification using both species-specific and conserved D3 primers on the same individual female: A single female from each of three *Pratylenchus* species (*P. neglectus, P. penetrans,* and *P. vulnus*) was digested individually in 5 µl extraction buffer with proteinase K for 1 hr at 65 °C, frozen overnight at –80 °C, and heated at 95 °C for 1 minute and at 99 °C for 3 minutes. The total extract of each individual (5 µl) was divided into two equal aliquots. The first one was subjected to the amplification of the conserved D3 region and the second aliquot was amplified using species-specific primers (either PPEN

TABLE 2. Species-specific primers designed from the DNA sequence of the D3 expansion region of the 26S rDNA of *Pratylenchus* species.

Pratylenchus spp.	Primer code	Primer sequence	Annealing temperature (°C)	PCR product size (bp)
P. neglectus	PNEG	5′-ATGAAAGTGAACATGTCCTC-3′	63	290
P. penetrans	PPEN	5′-TAAAGAATCCGCAAGGATAC-3′	62	278
P. scribneri	PSCR	5′-AAAGTGAACGTTTCCATTTC-3′	63	286
P. thornei	PTHO	5′-GAAAGTGAAGGTATCCCTCG-3′	68	288
P. vulnus	PVUL	5′-GAAAGTGAACGCATCCGCAA-3′	68	287

or PVUL). Amplification conditions were followed as described earlier. The two amplification reactions using the same template DNA were combined before analysis by gel electrophoresis. This experiment was performed three times.

Results

Characterization of populations: All the qualitative characters including number of lip annuli, spermatheca (presence and shape), and tail shape of the six species of *Pratylenchus* agreed with the original descriptions. Most of the quantitative characters of the populations also agreed with the original descriptions. However, a few characters fell outside the range of the original descriptions. For example, individuals of both populations of *P. penetrans* had greater body lengths (0.51 to 0.79 mm and 0.60 to 0.70 mm) than those reported in the orignial description (0.43 to 0.65 mm).

The D3 region was amplified from all populations studied. DNA sequences from these fragments were identical to the previously reported sequences from Genbank, thus supporting the morphological identification.

Amplification using species-specific primers: Each species-specific primer was constructed to amplify DNA from the target species but to preclude the amplification of non-target *Pratylenchus* species. The optimum annealing temperatures for these primers are listed in Table 2. Due to limited sequence variation, we were not able to design a forward primer that was unique to *P. brachyurus*. Each of the optimized primers amplified a unique PCR product from its respective target and did not produce an amplicon from other *Pratylenchus* species (Fig. 1). Amplicon sizes were in agreement with the fragment sizes predicted from the known nucleotide sequences. Two populations for each species were used to ascertain the specificity of the primer set (Fig. 1). Furthermore, the presence and the size of the amplification product obtained from individual female nematodes were similar to those obtained from the D3 amplification products (Fig. 1).

Diagnostic application: A common need in molecular diagnostics of lesion nematodes is to distinguish *P. vulnus* from *P. penetrans* in field samples. A test assay was performed using single females of *P. penetrans*, *P. vulnus,* and *P. neglectus*. Extracts of single females were divided into two aliquots and amplified in two reactions

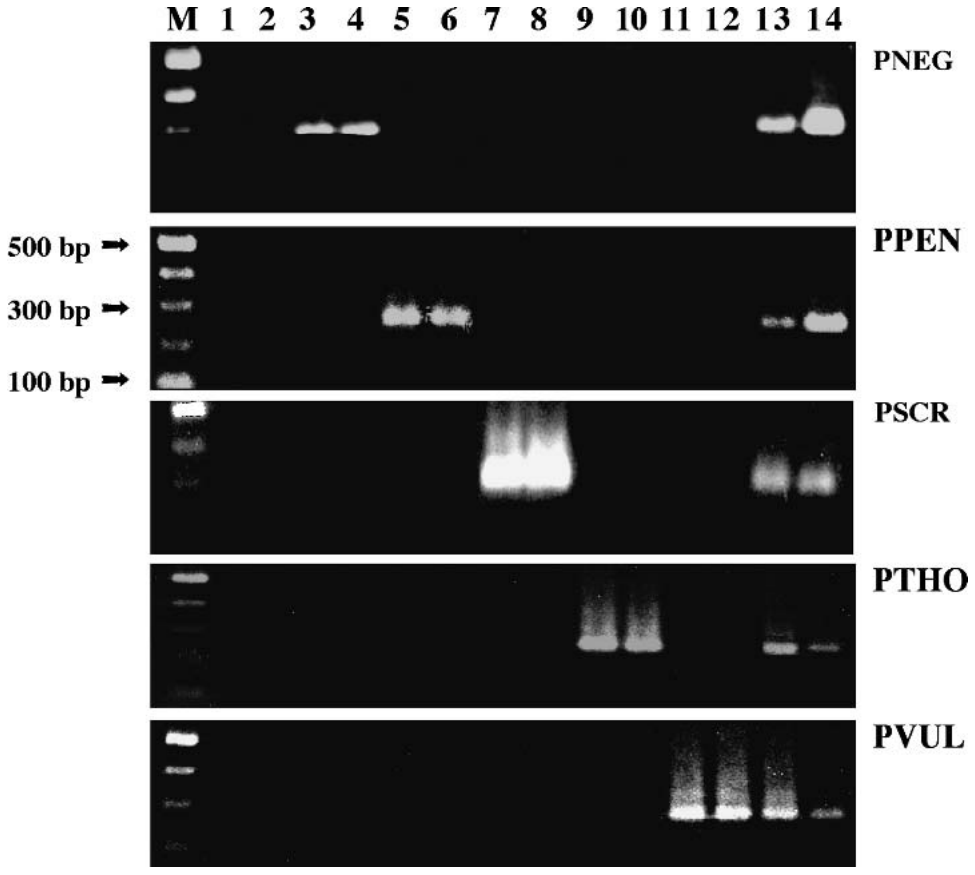

Fig. 1. Agarose gels of PCR products using species-specific primers of *Pratylenchus*. A common D3B primer and the following species-specific primers were used: PNEG; PPEN; PSCR; PTHO; PVUL. In lanes 1 to 12, the amplified D3 region was used as a template. Nematode codes are as abbreviated in Table 1. Lane 1, Pbra1; lane 2, Pbra2; lane 3, Pneg1; lane 4, Pneg2; lane 5, Ppen1; lane 6, Ppen2; lane 7, Pscr1; lane 8, Pscr2; lane 9, Ptho1; lane 10, Ptho2; lane 11, Pvul1; lane 12, Pvul2. In lanes 1 to 14, single females of the species for which the primers were designed were used as template. Lane M, DNA size markers.

using conserved and species-specific primers. This assay showed that conserved D3 primers amplified DNA from all individuals of the three species, thus confirming the overall reliability of the PCR for a given specimen tested. In contrast, the PPEN primers amplified DNA from *P. penetrans* but not from *P. neglectus* or *P. vulnus*. Similarly, PVUL primers amplified only DNA from *P. vulnus* but not from *P. neglectus* or *P. penetrans* (Fig. 2). Together these results confirm that the absence of amplification of the non-target species was due to the species-specificity of the primers and not due to experimental artifact.

Discussion

Certain morphological characters, such as the number of lip annules, the shape of the spermatheca, and the structure of the lateral field, are generally reliable for identification of *Pratylenchus* species (Loof, 1978). However, recognition of these characters requires substantial and specialized training and, even then, multiple adult female specimens are necessary for reliable species diagnosis. The qualitative morphological characteristics of the *Pratylenchus* populations used in this study followed the original species descriptions, but some morphometric discrepencies were found. Such morphometric variations in *Pratylenchus* species have been previously reported (Doucet et al., 1998, 2001; Townshend, 1991). Townshend (1991) reported that morphometric variations existed between populations of *P. penetrans* associated with strawberry and those associated with celery in Ontario, Canada. Furthermore, variations in size were also found between populations recovered from the same host (strawberry) but collected from different geographical areas (Townshend, 1991). Similarly, Doucet et al. (2001) reported that temperature significantly influenced the morphometrics of individuals derived from a single isolate of *P. vulnus*. Together these findings indicate that morphometric characters are not always reliable as primary characters for *Pratylenchus* species identification.

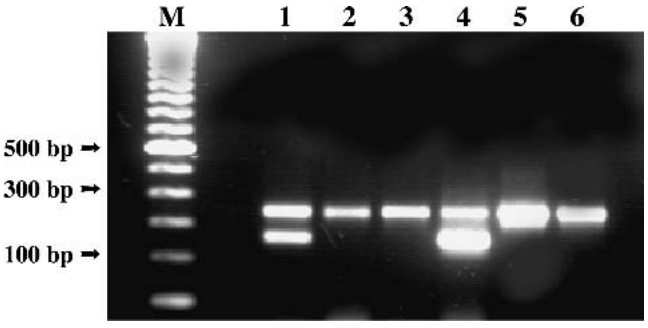

Fig. 2. Agarose gel of combined PCR products obtained using conserved and species-specific primers on aliquots of single digested females. The D3 region of an aliquot of a single proteinase K digested female of the respective species was amplified using primers D3A and D3B (top band in lanes 1 to 6). Amplification reactions with the PPEN-specific primer pair were mixed with the common amplification product in lanes 1, 3, and 5; reactions with the PVUL-specific primer pair were mixed with the common amplification product in lanes 2, 4, and 6. Nematode females, codes as abbreviated in Table 1, are: Lanes 1 & 2, Ppen2; lanes 3 & 4, Pvul2; lanes 5 & 6, Pneg1; Lane M, DNA size markers. The bottom bands of lane 1 and lane 4 represent the amplified template using PPEN, and PVUL species-specific primers, respectively.

Unlike sequencing and PCR-RFLP methods, the PCR products produced using species-specific primers generate a 'yes or no' result without need for subsequent analysis of the amplicon. Species-specific primers have been developed for distinguishing parasitic nematodes (Roos and Grant, 1993; Uehara et al., 1998; Williamson et al., 1997). In our study, species-specific amplifications of several *Pratylenchus* species were achieved through the use of primers designed to the limited variable region within the D3 expansion region of the 26S rDNA. Since the D3 is found in a repeat and does not show intra-specific variation in *Pratylenchus* spp. (Al-Banna et al., 1997), it is a suitable region to use for this purpose and improves the specificity of the PCR and the sensitivity of detection.

We designed and tested five primer sets to discriminate six species of root lesion nematodes that are known to be dominant on the Pacific coast of the United States. Based on the original descriptions, *P. penetrans* females can be distinguished from *P. vulnus* by the presence of a shorter postuterine branch, round spermatheca, and a rounder tail. However, it has been reported that many geographical isolates identified as *P. vulnus* exhibit characters similar to *P. penetrans* (Doucet et al., 1996, 1998; Gao et al., 1999). Such variation within a species may lead to misidentification of these two species, which are serious pests of fruit trees and ornamentals in California. Accurate identification of these agronomically important nematodes is particularly relevant to ongoing work on the development of resistant cultivars and rootstocks (Baldridge et al., 1998; Hafez et al., 2000; Schneider et al., 1995). As an example of an application of our assay, we have shown that *P. vulnus* and *P. penetrans* can be differentiated from each other and from *P. neglectus*. These species-specific primers should be applicable to diagnostics of both pure as well as mixed *Pratylenchus* populations, since single females can be tested. However, until the PCR identification has been validated on a wide collection of field isolates, parallel identification with qualitative morphological techniques is recommended for key samples.

We did perform multiplex PCR on single nematodes using the conserved D3A and species-specific forward primers with the common D3B reverse primer. However, results were inconsistent, probably due to the competition between the two forward primers as well as the small and variable amount of DNA released from the single nematode (not shown).

In conclusion, five species-specific primers were designed to provide a simple and rapid means of identi-

fying root lesion nematodes. Since only a single band is amplified with the species-specific primer set, this assay should be adaptable to rapid, real-time PCR assays (Oliveira et al., 2002; Schaad et al., 1999) for the quantitation of *Pratylenchus* species in root and soil samples.

Literature Cited

Al-Banna, L., V. Williamson, and S. L. Gardner. 1997. Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26 S rDNA. Molecular Phylogenetics and Evolution 7:94–102.

Baldridge, G. D., N. R. O′Neill, and D. A. Samac. 1998. Alfalfa (*Medicago sativa* L.) resistance to the root-lesion nematode, *Pratylenchus penetrans:* Defense-response gene mRNA and isoflavonoid phytoalexin levels in roots. Plant Molecular Biology 38:999–1010.

Café Filho, A. C., and C. S. Huang. 1989. Description of *Pratylenchus pseudofallax* n. sp. with a key to species of the genus *Pratylenchus filipjev*, 1936 (Nematoda: Pratylenchidae). Revue de Nématologie 12:7–16.

Doucet, M., J. Pinochet, and J. A. Di Rienzo. 1996. Comparative analysis of morphological and morphometrical characters in six isolates of *Pratylenchus vulnus* Allen & Jensen, 1951 (Nemata: Tylenchida). Fundamental and Applied Nematology 19:79–84.

Doucet, M. E., P. Lax, and J. Pinochet. 1998. Variability of some external characters in *Pratylenchus vulnus* Allen & Jensen, 1951 (Nematoda; Tylenchida). Fundamental and Applied Nematology 21: 205–206.

Doucet, M., P. Lax, J. A. Di Rienzo, J. Pinochet, and P. Baujard. 2001. Temperature-induced morphometrical variability in an isolate of *Pratylenchus vulnus* Allen & Jensen, 1951 (Nematoda; Tylenchida). Nematology 3:1–8.

Gao, X., H. Cheng, and C. Fang. 1999. New diagnostic characters for *Pratylenchus vulnus*. Nematologia Mediterranea 27:9–13.

Hafez, S. L., D. Miller, and P. Sundararaj. 2000. Screening of alfalfa cultivars to the lesion nematode *Pratylenchus penetrans* for commercial release. Nematologia Mediterranea 28:157–161.

Handoo, Z. A., and A. M. Golden. 1989. A key and diagnostic compendium to the species of the genus *Pratylenchus filipjev*, 1936 (lesion nematodes). Journal of Nematology 21:202–218.

Huettel, R. N., and R. V. Rebois. 1985. Culturing plant-parasitic nematodes using root explants. Pp. 155–158 *in* B. M. Zuckermann, W. F. May, and M. B. Harrison, eds. Plant nematology laboratory manual. Amherst, MA: Agricultural Experiment Station, University of Massachusetts.

Humason, G. L. 1972. Animal tissue techniques 3rd edition. San Francisco, CA: W. H. Freeman.

Loof, P. A. A. 1978. The genus *Pratylenchus filipjev*, 1936 (Nematoda: Pratylenchidae): A review of its anatomy, morphology, distribution, systematics, and identification. Research Information Center. Swedish University of Agricultural Sciences. Uppsala, Sweden.

McKenry, M. V., and P. A. Roberts. 1985. Phytonematology study guide. Publication No. 4045. Cooperative Extension, University of California. Division of Agricultural and Natural Resources. Oakland.

Niblack, T. L., and R. S. Hussey. 1985. Extracting nematodes from soil and plant tissue. Pp. 201–206 *in* B. M. Zuckermann, W. F. May, and M. B. Harrison, eds. Plant Nematology Laboratory Manual. Amherst, MA: Agricultural Experiment Station, University of Massachusetts.

O′Bannon, J. H., and L. Taylor. 1968. Migratory endoparasitic nematodes reared on carrot discs. Phytopathology 58:385.

Oliveira, O. C., M. A. Vallim, C. P. Semighini, W. L.. Araujo, G. H. Goldman, and M. A. Machado. 2002. Quantification of *Xylella fastidiosa* from citrus trees by real-time polymerase chain reaction assay. Phytopathology 92:1048–1054.

Orui, Y. 1996. Discrimination of the main *Pratylenchus* species (Nematoda; Pratylenchidae) in Japan by PCR-RFLP analysis. Applied Entomology and Zoology 31:505–514.

Powers, T. O., and T. S. Harris. 1993. A polymerase chain reaction method for identification of five major *Meloidogyne* species. Journal of Nematology 25:1–6.

Roos, M. H., and W. N. Grant. 1993. Species-specific PCR for the parasitic nematodes *Haemonchus contorus* and *Trichostrongylus colubriformus*. International Journal of Parasitology 23:419–421.

Schaad, N. W., Y. Berthier-Schaad, A. Sechler, and D. Knorr. 1999. Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. Plant Disease 83:1095–1100.

Schneider, J. H. M., J. J. S'Jacob, and P. A. van de Pol. 1995. *Rosa multiflora* 'Ludiek', a rootstock with resistant features to the root lesion nematode *Pratylenchus vulnus*. Scientia Horticulturae 63:37–45.

Seinhorst, J. W. 1959. A rapid method for the transfer of nematodes from fixation to anhydrous glycerin. Nematologica 4:67–69.

Siddiqui, I. A., S. A. Sher, and A. M. French. 1973. Distribution of plant-parasitic nematodes in California. Sacramento, CA: State of California Department of Food and Agriculture.

Townshend, J. L. 1991. Morphological observations of *Pratylenchus penetrans* from celery and strawberry in southern Ontario. Journal of Nematology 23:205–209.

Uehara, T., T. Mizukubo, A. Kushida, and Y. Momota. 1998. Identification of *Pratylenchus coffeae* and *P. loosi* using specific primers for PCR amplification of ribosomal DNA. Nematologica 44:357–368.

Uehara, T., A. Kushida, and Y. Momota. 1999. Rapid and sensitive identification of *Pratylenchus* spp. using reverse dot blot hybridization. Nematology 1:549–555.

Waeyenberge, L., A. Ryss, M. Moens, J. Pinochet, and T. C. Vrain. 2000. Molecular characterization of 18 *Pratylenchus* species using rDNA restriction fragment length polymorphism. Nematology 2:135–142.

Williamson, V. M., E. P. Caswell-Chen, B. B. Westerdahl, F. F. Wu, and G. Caryl. 1997. A PCR assay to identify and distinguish single juveniles of *Meloidogyne hapla* and *M. chitwoodi*. Journal of Nematology 29:9–15.