

Phylogenetic Analysis of Geographically Diverse *Radopholus similis* via rDNA Sequence Reveals a Monomorphic Motif

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Abstract: The nucleic acid sequences of rDNA ITS1 and the rDNA D2/D3 expansion segment were compared for 57 burrowing nematode isolates collected from Australia, Cameroon, Central America, Cuba, Dominican Republic, Florida, Guadeloupe, Hawaii, Nigeria, Honduras, Indonesia, Ivory Coast, Puerto Rico, South Africa, and Uganda. Of the 57 isolates, 55 were morphologically similar to *Radopholus similis* and seven were citrus-parasitic. The nucleic acid sequences for PCR-amplified ITS1 and for the D2/D3 expansion segment of the 28S rDNA gene were each identical for all putative *R. similis*. Sequence divergence for both the ITS1 and the D2/D3 was concordant with morphological differences that distinguish *R. similis* from other burrowing nematode species. This result substantiates previous observations that the *R. similis* genome is highly conserved across geographic regions. Autapomorphies that would delimit phylogenetic lineages of non-citrus-parasitic *R. similis* from those that parasitize citrus were not observed. The data presented herein support the concept that *R. similis* is comprised of two pathotypes—one that parasitizes citrus and one that does not.

Key words: banana, citrus, evolution, genetics, ITS1, 28S, nematode, pathotype, phylogeny, quarantine, race, *Radopholus similis*, rDNA, species concepts, taxonomy.

Sequence comparison for nuclear ribosomal DNA (rDNA) has been evaluated as a means to clarify phylogenetic relationships among populations and species of numer-

ous organisms including nematodes (Adams et al., 1998; Baldwin et al., 1997; Blaxter et al., 1998; Hillis and Dixon, 1991; Powers et al., 1997). The multicopy ribosomal RNA genes of many eukaryotic organisms appear to evolve in a concerted pattern whereby the majority of the repeat copies within a genome of a species are of a single sequence. By contrast, sequence differences are found between species (Brown et al., 1972). Empirical evidence suggests that a concerted pattern is also found in nematode rDNA evolution (Fitch, 1997; Baldwin et al., 1997; Thomas et al., 1997). Nematode rDNA is organized in much the same manner as that of other eukaryotic organisms, being comprised of multiple repeats of three ribosomal genes interspersed between spacer regions of DNA (Files and Hirsh, 1981). For *Caenorhabditis elegans*, the nucleic acid sequence of rDNA is highly conserved between strains, but differences exist between *C. elegans* and the closely related species *C. briggsae* (Files and Hirsch, 1981). Highly conserved sequences found in the rDNA genes are useful for the design of “universal” primers that will amplify the orthologous fragment from all taxa with those priming sites and provide a comparative se-

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quence to aid species identification. We selected two sets of universal rDNA primers to compare 57 burrowing nematode isolates.

The first pair of primers targeted the 18S and the 5.8S genes in the tandem rDNA repeat. Thus, we amplified the first internal transcribed spacer region (ITS1) located between these genes. The ITS1 has provided a reliable means of resolving phylogenetic relationships between closely related nematode taxa (Adams et al., 1998). Interspecific differences in sequence often appear to be more pronounced in spacer regions that lie between genes in the tandem rDNA gene clusters than in the genes themselves (Fedoroff, 1979). Diagnostic assays to identify plant-parasitic nematodes based on rDNA have also been reported (Powers et al., 1997; Uehara et al., 1998).

The second set of primers amplified the most rapidly evolving coding region of the rDNA genes. This region of the large subunit rDNA gene includes the D2 and D3 expansion segments and is flanked by highly conserved sequences. These sequences served as the priming sites for PCR-based amplification and subsequent automated sequencing. The D2/D3 expansion segment of the 28S rDNA subunit has also been demonstrated to vary between species and has been used to distinguish plant-parasitic nematode taxa and to study phylogeny (Baldwin et al., 1997; Thomas et al., 1997).

The genome of putative *R. similis* isolates appears to be highly conserved (Fallas et al., 1996; Hahn et al., 1994; Kaplan, 1994b; Kaplan et al., 1996, 1997; Kaplan and Opperman, 1997). Differences in host range or in relative aggressiveness of different burrowing nematode isolates within this group are well documented, but the genetic basis has not been identified. Fallas et al. (1996) suggested that there are two genetic groups within *R. similis* in Africa based upon RAPD and isozyme analyses; however, these groupings were not correlated with differences in aggressiveness toward banana. Marin et al. (1999) also identified variation within and between burrowing nematode populations, but this was not correlated with differences in host range or aggressiveness but rather

with collection site. Recent genetic studies demonstrated that citrus- and non-citrus-parasitic burrowing nematodes were not reproductively isolated (Kaplan et al., 1997). Staining polar bodies with 4', 6-diamidino-2-phenylindole (DAPI) in *Radopholus* eggs, it was determined that the haploid karyotype ($n=5$) was consistent among all burrowing nematode isolates (Kaplan and Opperman, 2000).

Phylogenetic trees representing the taxonomic relationship of burrowing nematode isolates collected globally have previously been generated on the basis of RAPD analysis (Hahn et al., 1994; Kaplan et al., 1996). However, comparisons of nucleic acid sequences are preferable because they can be compared among a broad range of eukaryotic organisms and nucleic acid sequences can be selected on the basis of their inherent tendency to evolve. Furthermore, nucleic acid sequences serve as a basis for systematic taxonomy that is unambiguous and easily communicated.

To further understand the relationship between citrus- and non-citrus-parasitic burrowing nematodes, we examined the phylogeny of burrowing nematode isolates collected from diverse geographic sites, based upon the extent of sequence identity of nuclear rDNA ITS1 and for 710 bp D2/D3 expansion segment of the 28S rDNA gene. We also determined if 21 burrowing nematode isolates that had not been previously assayed were able to parasitize citrus.

MATERIALS AND METHODS

Nematodes: Fifty-seven burrowing nematode isolates collected in Australia, Belize, Cameroon, Costa Rica, Dominican Republic, Florida, Guadeloupe, Guatemala, Hawaii, Puerto Rico, South Africa, and Uganda were cultured on excised carrot disks and extracted from culture by enzymatic maceration (Kaplan and Davis, 1990). Collection sites and acronyms for each isolate are listed in Table 1. *Tylenchulus semipenetrans* included in the study were extracted from roots of a rough lemon seedling (*Citrus limon*) L. Raf.) and grown as a laboratory cul-

TABLE 1. Collection sites and reproduction on rough lemon (*Citrus limon* L. Raf.) and tomato (*Lycopersicon esculentum* L.) for burrowing nematode isolates.

Nematode isolate	Collection site	Rough lemon ^a	Tomato
AS1	Tully, Queensland, Australia	-	+
AS2	Tully, Queensland, Australia	-	+
AS3	Darwin, Northern Territory, Australia	-	+
AS4	Pimpama, Queensland, Australia	-	+
BZ1	Bladen Bridge, Toledo, Belize	-	+
BZ2	Big Creek, Belize	-	+
BZ3	Stann Creek, Belize	-	+
CM1	Cameroon	-	+
CM2	Island 5, Sanga River, Cameroon	-	+
CR1	Coyles, Costa Rica	-	+
CR2	Guanacoste, Costa Rica	-	+
CR3	West Reventazon River, Costa Rica	-	+
CR4	Pococi, Costa Rica	-	+
CR5	Sixaola, Costa Rica	-	+
CR6	Sixaola, Costa Rica	-	+
CR7	Unknown, Costa Rica	-	+
CR8	Coyoles, Costa Rica	-	+
CU1	Villa Clara, Cuba	-	+
DR1	Hato Viejo, Dominican Republic	-	+
FL1	Lake Wales, Florida	+	+
FL2	Orlando, Florida	+	+
FL3	Clermont, Florida	+	+
FL4	Lake Alfred, Florida	+	+
FL5	Orlando, Florida	-	+
FL7	Avon Park, Florida	+	+
FL8	Frostproof, Florida	+	+
FL9	Frostproof, Florida	+	+
FL10	Frostproof, Florida	+	+
FL11	Frostproof, Florida	+	+
GD1	Neuf Chateau, Guadeloupe	-	+
GN1	Balikoire, Guinea	-	+
GT1	Yuma Farm, Guatemala	-	+
GT2	Creek Farm, Guatemala	-	+
GT3	Lanquin Farm, Guatemala	-	+
HI1	Panaewa, Hawaii	-	+
HI2	Pahoa, Hawaii	-	+
HI3	Panaewa, Hawaii	-	+
HI4	Keeau, Hawaii	-	+
HI5	Pahoa, Hawaii	-	+
HI6	Keeau, Hawaii	-	+
HI7	Waimanalo, Oahu, Hawaii	-	+
HI9	Punaluu, Oahu, Hawaii	-	+
HI10	Hilo, Hawaii	-	+
HI11	Pelekunu Preserve, Molokai, Hawaii	-	+
HI12	Keeau, Hawaii	-	+
HI13	Hilo, Hawaii	-	+
HI14	Watts Panawea (Hawaii)	-	+
HN1	Sula Valley, Honduras	-	+
HN2	Coyoles, Honduras	-	+
IC	Ivory Coast	-	+
NI1	IITA, Onne Station, Nigeria	-	+
PR1	Puerto Rico	-	+
PR2	Puerto Rico	-	+
SA1	Hectorspruit, Mpumalanga, South Africa	-	+
SA2	Hazyview, Mpumalanga, South Africa	-	+
UG1	IITA, Namulonge Station, Uganda	-	+

^a + = Median values of 100 to 1,200 nematodes per plant with burrowing nematodes detected in >95% of test plants; - = Median values of 0 to 30 nematodes per plant with burrowing nematodes detected in fewer than 5% of test plants (Kaplan, 1994a; Kaplan and Opperman, 1997; Kaplan et al., 1997).

ture (Kaplan, 1994a). Primers and cloned DNA were designated according to the guidelines published for plant-parasitic nematodes (Bird and Riddle, 1994).

Identification of citrus-parasitic isolates: Citrus parasitism for each burrowing nematode isolate was estimated using a laboratory host-index system for 21 isolates that had not been previously characterized (Kaplan, 1994a; Kaplan et al., 1997). Each burrowing nematode isolate was evaluated three times for ability to parasitize citrus. Nematode isolates were considered to be citrus-parasitic if mean population densities for each treatment exceeded 100 nematodes/test plant at the end of each experiment. Conversely, burrowing nematode isolates were considered to be non-citrus-parasitic if mean populations on citrus did not exceed 30 nematodes/plant. Tomato was considered to be a host of each burrowing nematode isolate if population densities exceeded 100 nematodes/plant.

Sequence identity in rDNA ITS1 and D2/D3 expansion regions: DNA from each nematode isolate (Table 1) was extracted from approximately 1,000 nematodes, which were ground for 15 seconds in disposable microhomogenizer tubes as described for extraction of plant genomic DNA (Edwards et al., 1991) with 10 μ M dithiothreitol (DTT) added to the extraction buffer. The DNA was subsequently resuspended in 100 μ l of 1 \times TE (10 mM Tris, 1 mM EDTA, pH 8.0). Because significant amounts of RNA were present in the mini-prep DNA (D. T. Kaplan, unpubl.), DNA concentration was not quantified spectrophotometrically. Instead, a dilution series was used to ascertain the amount of DNA solution required to obtain reproducible PCR results for each of the crude DNA samples.

The ITS1 was amplified using the primers TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') synthesized at the DNA Synthesis Core Laboratory, University of Florida, Gainesville, Florida. The rDNA expansion segment was amplified with the primers D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGC-

TACTA-3'). Reaction conditions used for amplification of ITS1 and the D2/D3 expansion segment were identical. Reactions were performed in 25- μ l volumes containing 1.0 U of *AmpliTaq* polymerase (Perkin Elmer, Norwalk, CT), with final concentrations of 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 200 μ M dNTP, 0.2 μ M primer, and 1.0 μ l of nematode DNA. PCR was performed in a PTC-100 MJ Thermal Cycler (MJ Research, Inc., Watertown, MA), where samples were placed in a preheated block (94 °C) for 1 minute followed by 35 cycles (94 °C, 1 minute; 55 °C, 1 minute; and 72 °C, 1 minute). The reaction tubes were subsequently held at 4 °C until retrieved.

The ITS1 for each isolate was amplified in three independent PCR reactions. The ITS1 fragments were cloned as indicated below. A 10- μ l sample of each reaction was electrophoresed on 1.0% agarose stained with ethidium bromide in 1 \times TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.0) at 80 V for 3 hours. Gels were stained with ethidium bromide (5 μ g/100 ml) and viewed on a UV transilluminator. The ITS1 fragments were individually isolated by cutting out the portion of the agarose gel containing each ITS1 DNA band and placing it in 200 μ l of TE in dialysis tubing (molecular weight cutoff 6,000–8,000) (Spectrum Medical Industries, Los Angeles, CA) that had been clamped at one end. The open end of the dialysis tubing was then clamped closed, and the assembly was placed back into the electrophoresis chamber with voltage set at 100V. After the ethidium bromide-stained DNA had eluted from the agarose, 200 μ l of the DNA suspension in TE buffer was transferred to a microfuge tube. Following phenol/chloroform/isopropanol extraction, the DNA was precipitated overnight in ethanol. Pellets were hydrated in 20 μ l of sterile double-distilled water. The DNA in each sample was quantified spectrophotometrically and ligated into the Eco RV site of pT7Blue (Novagen, Madison, WI) and transformed into *Escherichia coli* strain Nova Blue (Novagen, Madison, WI). Recombinants were identified by blue/white selection and by digestion with Spe I and Sph I (New En-

gland Bio Labs, Beverly, MA). The sequence of three independently cloned ITS1 DNA fragments were determined using DNA sequencing on an automated fluorescence-based sequencer at the Molecular Genetics Facility, University of Georgia, Athens, Georgia. The three nucleotide sequences for each isolate were aligned and assembled using programs in the Sequencher software package (Gene Codes, Ann Arbor, MI). The ITS1 nucleotide sequences amplified for all nematode isolates were then aligned and their sequence compared using Clustal W 1.7 (Thompson et al., 1994).

Sequencing the D2/D3 expansion segments was performed following manufacturer's protocol with 400–600 ng template using the dRhodamine Terminator Cycle Sequencing Ready Reaction Mix (ABI/Perkin-Elmer, Foster City, CA) with AmpliTaq DNA polymerase FS (Perkin-Elmer, Foster City, CA), except reactions were performed in one half the suggested volume. Primers for sequencing were D3B, ID3B (5'-TAGWTCRCCATCTTTTCGGG-3'), and D2B (5'-AATCCGTGTTTCAAGACGGG-3'). Sequences were run on an ABI377 automated sequencer (ABI/Perkin-Elmer, Foster City, CA) running ABI Prism DNA sequencing software version 1.1 in the DNA Core Facility, University of Missouri, Kansas City, Missouri. Sequences were aligned by eye using Eyeball Sequence Editor (Cabot and Beckenbach, 1989).

Phylogenetic trees were estimated from the ITS1 and D2/D3 data for each nematode species via maximum likelihood using PUZZLE ver 4.0.2 (Strimmer and von Haeseler, 1996, 1997). The estimation used 1,000 quartet puzzling steps and assumed the HKY model of sequence substitution with a uniform rate heterogeneity (Hasegawa et al., 1985). Transition/transversion parameters and nucleotide frequencies were estimated empirically from the data set (Strimmer and von Haeseler, 1996, 1997).

RESULTS

The sequences of rDNA ITS1 and the D2/D3 expansion segment amplified from the

55 burrowing nematodes that resembled *R. similis* were identical. The ITS1 and D2/D3 sequences for *R. bridgei*, *Radopholus* sp., and *T. semipenetrans* were each distinct. Citrus- and non-citrus-parasitic burrowing nematodes that are morphologically similar to *R. similis* could not be separated as distinct species (Fig. 1). Further, *R. similis* is a sister taxon to *R. bridgei*, and the two share a most recent common ancestor to the exclusion of *Radopholus* spp. Results of citrus bioassays indicated that none of the burrowing nematode isolates collected outside of Florida parasitized citrus (Table 1).

DISCUSSION

Adams (1998) recently proposed a novel method of species delimitation that utilizes the discovery operations of the phylogenetic species concept to recover species as defined by the evolutionary species concept. Selection of appropriate genetic loci for species delimitation is important, but sole reliance on them can result in erroneous conclusions. Further, Doyle (1992) warned that gene trees do not necessarily reflect phylogenetic relationships among species, particularly if species delimitation was based on a single genetic locus that had multiple alleles, could be heterozygous, or was non-recombining, such as mitochondrial DNA (Doyle, 1995). The most accurate inference of phylogenetic relationships is likely to be attained when a range of traits are considered together (Eernisse and Kluge, 1993; Fitch, 1997; Thomas et al., 1997).

The goal of the research reported herein was to characterize two variable regions within the rDNA repeat to define the taxonomic/phylogenetic relationship of burrowing nematodes resembling *R. similis* that parasitize citrus with those that do not. The current findings are compatible with other studies suggesting the taxonomic unity of *R. similis*. Specifically, ability to parasitize citrus was not correlated with differences in karyotype, gametogenesis, or reproductive strategy (Kaplan and Opperman, 2000). Genetic variation, as assessed by RAPD and isozyme analysis, was not correlated with citrus para-

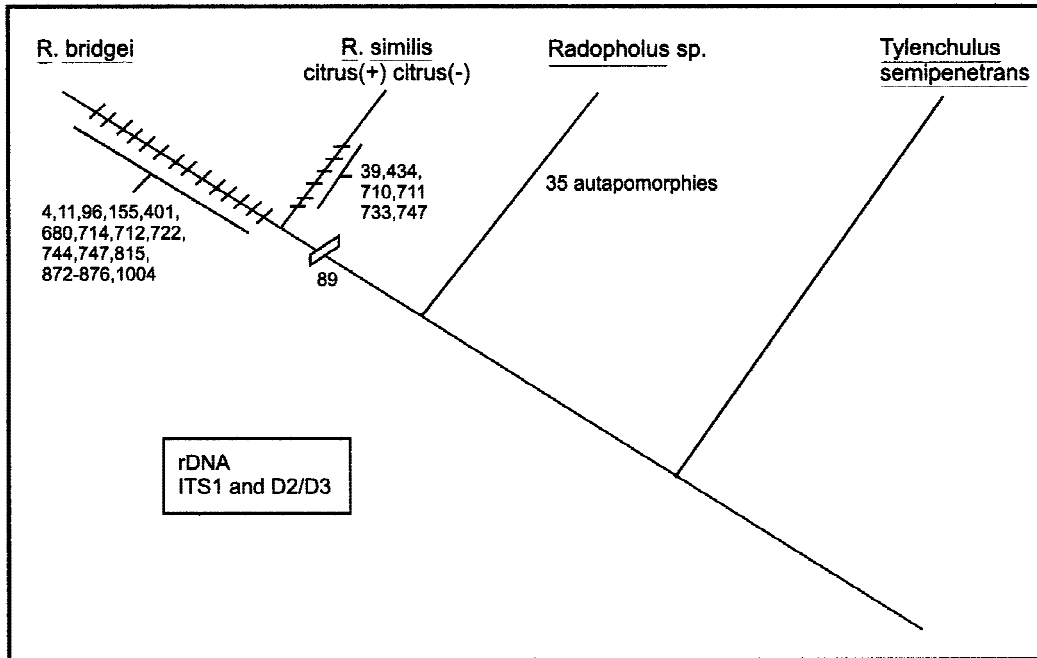


FIG. 1. Phylogenetic relationships of *Radopholus bridgei*, *R. similis*, *Radopholus* sp., and *Tylenchulus semipenetrans*. Autapomorphies (uniquely derived characters) are noted by nucleic acid position in contiguous sequence of ITS1 and D2/D3 expansion segment. Note: The genetic sequence of the ITS1 and the D2/D3 expansion segment of the large rDNA gene were identical for citrus- and non-citrus-parasitic burrowing nematodes; hence, there are no autapomorphic nucleotide character states among the two parasitic forms.

sitism (Kaplan et al., 1996; Kaplan and Opperman, 1997). Furthermore, morphological differences (Elbardri et al., 1998, 1999; Valette et al., 1998) and reproductive barriers between citrus- and non-citrus-parasitic burrowing nematodes could not be demonstrated (Kaplan et al., 1997).

The nucleic acid sequence of rDNA ITS1 for the burrowing nematode isolates was compared because it is considered to be a non-coding, homozygous, nuclear DNA, useful for understanding phylogeny (Adams, 1998; Baldwin et al., 1995). The ITS1 has been used to estimate phylogeny and to identify species for several nematode species including animal, plant, and insect parasites (Campbell et al., 1995; Chilton et al., 1995; Ferris et al., 1993, 1994, 1995; Hoste et al., 1995; Thiery and Mugniery, 1996; Vrain et al., 1992). However, in some instances, nematodes have been demonstrated to have intra-individual ITS1 regions that can complicate analyses and possibly lead to erroneous conclusions. To date, only *Belonolaimus*

(Cherry et al., 1997), *Meloidogyne* (Xie et al., 1994; Zijlstra et al., 1995, 1997), and *Heterodera zea* isolates from India (Szalanski et al., 1997) have been demonstrated to have variable ITS1 regions. In general, these studies indicated that nematode species that could be recognized on the basis of their morphology could also be distinguished by restriction digestion patterns of the rDNA ITS regions.

The nucleic acid sequence for the rapidly evolving D2/D3 expansion segment of the 28S rDNA gene can distinguish taxa at the species level (Thomas et al., 1997). Recently, a dendrogram based upon the D2/D3 sequence was used to describe the phylogeny of 22 *Pratylenchus* spp. (Duncan et al., 1999). Sequence homology of this single gene locus and a principal component analysis based upon select morphological features also were used to estimate the relationship among the lesion nematode isolates. Results of the D2/D3 analysis for lesion nematodes were concordant with results of the princi-

pal component analysis. Lesion nematode isolates also were assayed for ITS1 amplification using PCR primers that were designed to selectively amplify rDNA from *P. coffeae* or *P. loosi* (Uehara et al., 1998).

Nadler (1992) inferred the phylogeny of ascaridoid nematodes by comparison of 18S and 28S rRNA sequences because there was a lack of available morphological characteristics upon which phylogeny could be estimated. Therefore, heterologous nucleic acids in rDNA sequences were treated as autapomorphic characters upon which ascaridoid phylogeny was estimated. Results were in agreement with a prior classification based on evolutionary trends, but differed from the more widely accepted classification based upon a set of variable ascaridoid physical traits. For burrowing nematodes that were morphologically similar, we determined that the nucleic acid sequence for the ITS1 and D2/D3 of rDNA were identical, respectively, regardless of collection site or ability to parasitize citrus. While two species could have the same sequence for a particular genetic locus, the likelihood that the nucleic acid sequence of the ITS1 and the D2/D3 as well as other traits each being identical among different species is exceedingly small.

Therefore, burrowing nematodes that resemble *R. similis* but parasitize citrus should not be considered a sibling species (Huettel et al., 1984) or a subspecies (Siddiqi, 1985). The citrus-parasitic form has the autapomorphic trait of being able to parasitize citrus and therefore can be distinguished from *R. similis* that cannot parasitize citrus. However, the non-citrus-parasitic burrowing nematodes that resemble *R. similis* can only be defined as "not being citrus-parasitic." They lack any discernible autapomorphy, and therefore the two types of burrowing nematodes cannot be recognized as distinct species.

This suggests that burrowing nematodes that are morphologically similar to *R. similis* but differ with respect to their ability to parasitize citrus are pathotypes of *R. similis*. DuCharme and Birchfield (1956) identified differences in host range between *R. similis*

isolates and designated them as races. However, use of the term "race" infers that the genetic basis of citrus parasitism is known. To date, the genetic basis of citrus parasitism in *R. similis* remains elusive.

We concur with Valette et al. (1998) and Elbardri et al. (1998, 1999), who hypothesized that *R. citrophilus* should be considered a junior synonym of *R. similis*. Morphological characters previously reported to differentiate *R. citrophilus* from *R. similis* by Huettel and Yaegashi (1988) were present among non-citrus-parasitic *R. similis* collected in Africa and Europe (Valette et al., 1998). Our molecular, biological, morphological, and cytological findings involving a global collection of citrus and non-citrus-parasitic burrowing nematodes bring us to the same conclusion (Kaplan and Opperman, 1997, 2000; Kaplan et al., 1996, 1997). *Radopholus citrophilus* is an invalid species designation. Citrus-parasitic burrowing nematodes are correctly synonymized with *R. similis* and should be considered as a pathotype.

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