

Genome Similarity Implies that Citrus-Parasitic Burrowing Nematodes do not Represent a Unique Species

D. T. KAPLAN¹ AND C. H. OPPERMAN²

Abstract: Burrowing nematodes from Central America, Dominican Republic, Florida, Guadeloupe, Hawaii, and Puerto Rico were characterized for their ability to parasitize citrus, but citrus parasites were found only in Florida. Sequence tag sites originally amplified from a citrus-parasitic burrowing nematode were polymorphic among 37 burrowing nematode isolates and were not correlated with citrus parasitism, nematode isolate collection site, or amplification of a 2.4-kb sequence tag site (DK#1). Results of a RAPD analysis and characterization of the isozymes phosphoglucose isomerase, lactate dehydrogenase, and malate dehydrogenase indicated that the burrowing nematode isolates were highly similar. Citrus parasitism in Florida appears to be associated with limited changes in the burrowing nematode genome. Findings did not substantiate a previous report that *R. citrophilus* was present in Hawaii. Overall, these data do not support assignment of sibling species status to burrowing nematodes that differ with respect to citrus parasitism.

Key words: anthurium, banana, citrus, evolution, genetics, isozymes, molecular biology, nematode, phylogeny, quarantine, *Radopholus*, RAPD, STS, taxonomy.

Burrowing nematodes, *Radopholus* spp., are migratory, endophytoparasitic nematodes that are prevalent in many tropical and subtropical regions throughout the world. They damage a wide range of plants by extensively wounding cortical tissues as they feed in roots (Blake, 1961; DuCharme, 1959). *Radopholus* spp. are considered to be among the 10 most damaging plant-parasitic nematodes worldwide (Sasser and Freckman, 1987). Their effect on citrus and banana is well documented (Duncan and Cohn, 1990; Esser et al., 1988; Gowen and Queneherve, 1990; Holdeman, 1986). *Radopholus similis* has been detected in numerous countries throughout the tropics and subtropics (Gowen and Queneherve, 1990), causing black head toppling disease and toppling disease of banana (Loos and Loos, 1960).

Although citrus is present in most countries where burrowing nematodes are commonly associated with damage to banana, burrowing nematodes cause spreading decline of citrus only in Florida (Holdeman, 1986; Suit and DuCharme, 1953). Classic spreading decline symptoms are strongly dependent upon edaphic conditions (Duncan and Cohn, 1990). Burrowing nematodes that attack citrus in Florida are morphologically indistinguishable from those that attack banana worldwide and thus had been considered to be the citrus race of *R. similis* (DuCharme and Birchfield, 1956). The citrus race was elevated to species status as *R. citrophilus* (Huettel et al., 1984b) on the basis of putative biochemical, physiological, and karyotypic differences that distinguished the citrus and banana races of *R. similis* (Huettel and Dickson, 1981; Huettel et al., 1982, 1983a, 1983b, 1984a). Minor morphological differences in the female head and vulva regions and in male cloacal ornamentation also were reported to be present in a comparison of an *R. similis* isolate with an *R. citrophilus* isolate (Huettel and Yaegashi, 1988).

In 1986, *R. citrophilus* was reported to be present in Hawaii on the basis of putative similarity in karyotype, isozyme, and protein content of a single burrowing nematode isolate collected from *Anthurium andraeanum*, with that of citrus-parasitic burrowing nematodes from Florida. However, this isolate did

Received for publication 16 April 1997.

¹ USDA-ARS, U.S. Horticultural Research Laboratory, 2120 Camden Rd., Orlando, FL 32803.

² Departments of Plant Pathology and Genetics, North Carolina State University, Raleigh, NC 27612.

E-mail: dkaplan@asrr.arsusda.gov

Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

The authors thank Carrie Vanderspool, Diana Johnson, and Sarah Hayne for technical assistance; Mark E. Hilf, Mark A. Conkling, David McK. Bird, and James G. Baldwin for technical advice; and Douglas Marin, Lyle Wong, Mark Enriques, Brent Sipes, Tim R. Gottwald, Stephen M. Garnsey, Jean Michel Risede, Mark E. Tudor, Peter Hunt, John Bridge, Julio C. Borbon, and Van Kashiwamura for assisting in collection of burrowing nematode isolates. Gratitude is expressed to Mike Conway for historical information related to the anthurium industry in Hawaii.

not parasitize citrus (Huettel et al., 1986). Results of subsequent RAPD analyses involving the same burrowing nematode isolate collected in Hawaii suggested that burrowing nematodes attacking anthurium in Hawaii were closely related to the citrus-parasitic burrowing nematodes from Florida (Kaplan et al., 1996). It was unclear if these findings were representative of other burrowing nematode isolates associated with anthuriums in Hawaii or for burrowing nematode isolates from other countries.

Acceptance of taxonomic revisions of *R. similis* by federal and state regulatory agencies has varied, and many have maintained the taxonomic status of these nematodes as races of *R. similis sensu lato* (Holdeman, 1986). Siddiqi (1986) classified the sibling species as subspecies *R. similis similis* and *R. similis citrophilus*. However, assigning sibling or subspecies status to the citrus- and non-citrus-parasitic burrowing nematodes may not be warranted because the burrowing nematode genome appears to be highly conserved (Fallas et al., 1996; Hahn et al., 1994; Kaplan, 1994b; Kaplan et al., 1996). In addition, morphological structures reported to be specific to *R. citrophilus* (Huettel and Yaegashi, 1988) have been observed in African isolates where citrus-parasitic burrowing nematodes have not been detected (Valette, pers. comm.).

Molecular characterization of the burrowing nematode genome should help clarify the taxonomic status of burrowing nematodes and likely will result in the identification of genetic loci controlling host range. We previously cloned a DNA fragment (DK#1) from *R. citrophilus*, sequenced its termini, and designed primers to selectively amplify DK#1 (Kaplan et al., 1996). The DK#1 fragment appears to be specific to the genus *Radopholus* and was amplified from all citrus-parasitic burrowing nematodes and from a few non-citrus-parasitic isolates (Kaplan et al., 1997). DK#1 subsequently was used as a genetic marker, and citrus parasitism was used as a phenotypic trait to demonstrate that citrus- and non-citrus-parasitic burrowing nematodes were not reproductively isolated (Kaplan et al., 1997).

This suggests that the morphologically identical citrus and non-citrus-parasitic burrowing nematodes should not be considered as independent species.

In this study, 14 burrowing nematode isolates collected from Hawaii, 10 isolates from Florida, and 13 isolates collected throughout Central America, Puerto Rico, Dominican Republic, and Guadeloupe were compared with molecular and biochemical techniques. Each isolate also was characterized for ability to parasitize citrus, and a field survey was conducted in Hawaii in which citrus and anthurium roots were analyzed for burrowing nematodes.

MATERIALS AND METHODS

Nematodes: Fourteen burrowing nematode isolates collected in Hawaii, 10 from Florida, and 13 from Central America, Dominican Republic, Guadeloupe, and Puerto Rico were cultured on excised carrot disks and extracted from culture by enzymatic maceration (Kaplan and Davis, 1990). The collection site and acronym for each isolate are listed in Table 1. Designations for primers and cloned DNA were made according to the guidelines published for plant-parasitic nematodes (Bird and Riddle, 1994).

Identification of citrus-parasitic isolates: Citrus parasitism was estimated with a laboratory host-index system (Kaplan, 1994a). Each burrowing nematode isolate was evaluated three times for its ability to parasitize citrus. Nematode isolates were considered to be citrus-parasitic if mean population densities for each treatment exceeded 100 nematodes per test plant at termination of each experiment. Conversely, burrowing nematode isolates were considered to be non-citrus-parasitic if mean populations on citrus did not exceed 10 nematodes per plant.

Field survey in Hawaii: Citrus roots were collected from 11 sites in Hawaii in 1995 and 1996 and analyzed for burrowing nematodes. Samples of citrus roots (ca. 6.0 g fresh weight) grown in association with anthuriums for the past 50 to 70 years were collected, rinsed free of soil, and placed in mist chambers or in jars (Young, 1954). Anthur-

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 ^a |
|------------------|------------------------------------|--------------------------|---------------------|
| FL1 | Lake Wales, Florida | + | + |
| FL2 | Orlando, Florida | + | + |
| FL3 | Clermont, Florida | + | + |
| FL4 | Lake Alfred, Florida | + | + |
| FL5 | Orlando, Florida | - | + |
| FL6 | Apopka, Florida | - | + |
| FL7 | Avon Park, Florida | + | + |
| FL8 | Frostproof, Florida | + | + |
| FL9 | Frostproof, Florida | + | + |
| FL10 | Frostproof, Florida | + | + |
| BZ1 | Bladen Bridge, Toledo, Belize | - | + |
| BZ2 | Big Creek, Belize | - | + |
| CR1 | Coyles, Costa Rica | - | + |
| CR2 | Guanacoste, Costa Rica | - | + |
| CR3 | West Reventazon River, Costa Rica | - | + |
| DR1 | Hato Viejo, Dominican Republic | - | + |
| GD1 | Neuf Chateau, Guadeloupe | - | + |
| GT1 | Yuma, Guatemala | - | + |
| GT2 | Creek, Guatemala | - | + |
| HI1 | Panaewa, Hawaii | - | + |
| HI2 | Pahoa, Hawaii | - | + |
| HI3 | Panaewa, Hawaii | - | + |
| HI4 | Keeau, Hawaii | - | + |
| HI5 | Pahoa, Hawaii | - | + |
| HI6 | Keeau, Hawaii | - | + |
| HI7 | Waimanalo, Oahu, Hawaii | - | + |
| HI8 | Kona, 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 | - | + |
| PR1 | Puerto Rico | - | + |
| PR2 | Puerto Rico | - | + |

^a + = Median values from 100 to 1,200 nematodes per plant with burrowing nematodes detected in >95% of test plants; - = Median values from 0 to 30 nematodes per plant with burrowing nematodes detected in less than 5% of the test plants.

ium roots (ca. 4.0 g fresh weight) from each site also were collected and processed in a similar manner. After 5 days, nematodes were extracted and each sample was examined for the presence of burrowing nematodes with a light microscope. Data were reported as presence or absence of burrowing nematodes in each sample. Ability to parasitize citrus was determined for each burrowing nematode isolate collected from *Anthurium* with the citrus host assay system described previously.

Genomic survey for sequence tag sites DK#1,

DK#3, DK#4, and DK#5: DNA from each nematode isolate (Table 1) was extracted from approximately 1,000 nematodes, which were ground for 15 seconds in disposable micro-homogenizer tubes as described for extraction of plant genomic DNA (Edwards et al., 1991) with 10 µM dithiothreitol (DTT) added to the extraction buffer. The DNA subsequently was resuspended in 100 µl of 1X TE (10mM Tris, 1mM EDTA, pH 8.0). Because significant amounts of RNA are present in the mini-prep DNA (Kaplan, unpubl.), DNA concentration was not quan-

tified 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.

Amplification reactions for the DK#1-specific primers (DK#101 and DK#102), DK#3 specific primers (DK#103 and DK#104), DK#4-specific primers (DK#107 and DK#108), and DK#5 specific primers (DK#105 and DK#106) were performed as previously described (Kaplan et al., 1996). DNA blots were made as described by Maniatis et al. (1982). The blots were probed with digoxigenin (DIG)-labeled DK#1, DK#3, DK#4, or DK#5 and developed using the Genius non-radioactive Labeling Kit III (Boehringer Mannheim, Indianapolis, IN). The Flash Membrane (Stratagene, La Jolla, CA) was hybridized with probes at both high and low stringency (68 °C or 42 °C) and processed as previously described (Kaplan et al., 1996).

RAPD analysis using bulk DNA: DNA was extracted from nematodes as described above for the genomic survey; however, in this experiment, nematodes were sorted into five groups. Group 1 was comprised of burrowing nematodes collected in Florida that were citrus-parasitic and from which DK#1 could be amplified (Table 2). Groups 2A and 2B were not citrus-parasitic and were collected in Hawaii; isolates in Group 2A were DK#1-positive, whereas DK#1 could not be amplified from the DNA of isolates included in Group 2B. Burrowing nematode isolates included in Groups 3A and 3B were not citrus-parasitic and were collected in Central America, the Caribbean, and Florida. Group 3A was DK#1 positive, but Group 3B was not (Table 2). Five μ l of DNA solution at concentrations that gave comparable PCR amplification from each burrowing nematode isolate within each group were combined and used in the following protocol.

Five sets of random decamer primers (OPB01-B20, OPC01-OPC20, OPG01-G20, OPM01-M20, and OPR01-R20; Operon Technologies, Alameda, CA) were used for RAPD analyses (Williams et al., 1990) as de-

TABLE 2. Burrowing nematode isolates combined into groups on the basis of parasitism, amplification of DK#1, and collection site. Crude DNA pooled for each group was used in a RAPD analysis involving 100 primers.

| 1 ^a | 2A | 2B | 3A | 3B |
|----------------|------|-----|-----|-----|
| FL1 | HI1 | HI2 | PR1 | FL5 |
| FL2 | HI4 | HI6 | CR3 | FL6 |
| FL3 | HI5 | HI8 | GT1 | CR1 |
| FL4 | HI7 | HI9 | GT2 | CR2 |
| FL7 | HI10 | | HN1 | DR1 |
| | HI11 | | HN2 | |
| | HI12 | | | |
| | HI13 | | | |
| | HI14 | | | |

^a Group 1: Citrus-parasitic, positive for DK#1, collected in Florida; Group 2: Non-citrus-parasitic and collected in Hawaii; Group 3: Non-citrus-parasitic and collected in Florida, Central America, Guadeloupe and Puerto Rico. Groups 2 and 3 were further divided into two groups based on presence of DK#1. Groups 2A and 3A were DK#1-positive. DK#1 could not be amplified from isolates in Groups 2B and 3B.

scribed previously (Kaplan et al., 1996). At least three independent reactions were performed for each nematode-primer combination. A 10- μ l sample of each reaction was electrophoresed on 1.0% agarose. Molecular weight markers were lambda-DNA cut with Nsi I or BioMarker EXT (Bio Ventures, Murfreesboro, TN). Cluster and phenogram analysis of the RAPD data was performed by the unweighted pair group method (UPGMA) (Sneath and Sokal, 1973) using the PC version of Numerical Taxonomy and Multivariate Analysis System (Exeter Software, Setauket, NY).

Isoelectric focusing (IEF) isozyme analysis: Sample preparation and comparison of phosphoglucose isomerase isoforms were performed with isoelectric focusing (IEF) as described by Fallas et al. (1996), with the exception that the nematodes analyzed represented all life-cycle stages since they were collected by tissue maceration (Kaplan and Davis, 1990).

Cellulose acetate electrophoresis isozyme analysis: Samples were prepared as for IEF (above) in order to characterize malate dehydrogenase, lactate dehydrogenase, and phosphoglucose isomerase for seven citrus and non-citrus-parasitic burrowing nematode isolates (FL1-4, FL6-7, HI1) using cel-

lulase acetate as previously described (Herbert and Beaton, 1993). Staining for enzyme activity was done as described (Allendorf et al., 1977).

RESULTS

Three independent host range assays were performed to determine which of the 37 burrowing nematode isolates were citrus parasites. Only burrowing nematode isolates from Florida (FL1-FL4 and FL7-FL10) were citrus-parasitic. Population densities of all burrowing nematode isolates increased in roots of susceptible tomato controls (Table 1). Furthermore, burrowing nematodes were not detected in roots collected from citrus trees sampled in Hawaii (Table 3), where burrowing nematode-infected anthuriums had been propagated under citrus tree canopies for as long as 70 years (M. Conway, pers. comm.).

A total of 452 bands were amplified when 100 RAPD primers representing the OPB, OPC, OPG, OPM, and OPR series were used on the 37 burrowing nematode isolates. These products sorted into five groups (Table 2). Ninety of the primers amplified reproducible banding patterns that could be scored. Seventy-three bands were polymorphic; however, only six were amplified solely from the citrus-parasitic group of burrowing nematode isolates. The hierarchical cluster analysis (SAHN) and UPGMA genomic similarity coefficients (>93%) derived from these data indicated that the five groups of burrowing nematode isolates were highly similar (Fig. 1).

Comparison of phosphoglucose isomerase (PGI) isoforms for the 37 burrowing nematode isolates confirmed the high degree of similarity among the nematode isolates. All nematode isolates had the same PGI profiles (negatively charged dimer) as determined by IEF (Fig. 2). Lack of variation in isoform for PGI also was observed with cellulose acetate electrophoresis. Variation in malate dehydrogenase isoforms (monomeric) was not observed; however, burrowing nematode isolates FL1, FL2, FL4, FL6, and FL8 were dimorphic for lactate dehy-

TABLE 3. Hawaiian field survey of *Anthurium andraeanum* and associated citrus trees for presence of burrowing nematodes, *Radopholus* spp.

| Location | Plant | Burrowing nematode |
|--------------------|-----------|--------------------|
| 1996 | | |
| Kurtistown, Site 1 | Anthurium | + |
| | Citrus | - |
| | Citrus | - |
| | Citrus | - |
| Hilo, Site 2 | Anthurium | + |
| | Anthurium | + |
| | Citrus | - |
| | Citrus | - |
| Hilo, Site 3 | Anthurium | + |
| | Citrus | - |
| | Citrus | - |
| Kurtistown, Site 4 | Anthurium | + |
| | Citrus | - |
| | Citrus | - |
| Mt. View, Site 5 | Anthurium | + |
| | Citrus | - |
| | Citrus | - |
| | Citrus | - |
| Mt. View, Site 6 | Anthurium | + |
| | Citrus | - |
| | Citrus | - |
| | Citrus | - |
| | Citrus | - |
| 1995 | | |
| Kurtistown, Site A | Citrus | - |
| | Citrus | - |
| | Citrus | - |
| Pahoa, Site B | Anthurium | + |
| | Anthurium | - |
| | Citrus | - |
| Pahoa, Site C | Anthurium | + |
| | Citrus | - |
| | Citrus | - |
| | Citrus | - |
| Pahoa, Site D | Anthurium | + |
| | Anthurium | + |
| | Citrus | - |
| Panaewa, Site E | Anthurium | + |
| | Anthurium | + |
| | Anthurium | + |
| | Citrus | - |

drogenase (LDH), whereas HI3 and FL4 were monomorphic. Differences in LDH did not appear to be consistent with ability to parasitize citrus, with presence of DK#1, or with collection site for a subset of eight burrowing nematode isolates.

The genomes of the 37 burrowing nematode isolates were compared for the presence of the four sequence tag sites DK#1,

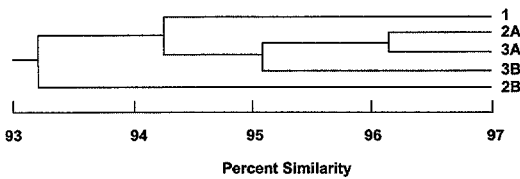


FIG. 1. Phenogram based on RAPD analysis showing the relationships of 37 burrowing nematode isolates sorted into five groups on the basis of geographic collection site, ability to parasitize citrus, and amplification of the sequence tag site DK#1.

DK#3, DK#4, and DK#5 that were originally amplified from the citrus-parasitic burrowing nematode, FL2. The DK#1-specific primers amplified the anticipated 2.4-kb fragment from a wide range of burrowing nematode isolates collected in Hawaii, Central America, and Florida. However, the 2.4-kb fragment was not amplified from two citrus-parasitic burrowing nematode isolates (FL8, FL10) or from 10 non-citrus-parasitic burrowing nematodes (CR2, DR1, FL5, FL6, GD1, HI2, HI6, HI8, HI9, and HI14). DNA hybridization of Southern blots with DIG-labeled DK#1 verified that amplification

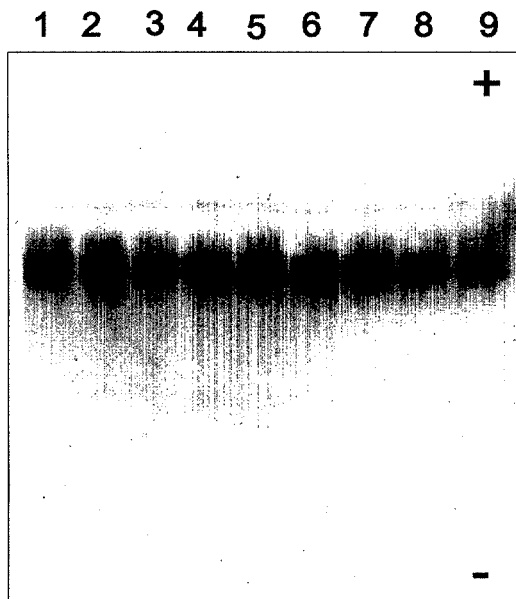


FIG. 2. Isoelectric focusing gel stained for phosphoglucose isomerase (PGI) for nine burrowing nematode isolates. PGI isoforms were identical for all 37 burrowing nematode isolates. Lane 1, FL1; 2, FL2; 3, CR1; 4, HI1; 5, FL3; 6, FL4; 7, FL5; 8, FL6; 9, FL7. Acronyms for nematode isolates are listed in Table 1.

products observed in ethidium bromide were homologous to DK#1 (Fig. 3).

The sequence tag site DK#3 (1.2 kb) was amplified from all burrowing nematode isolates except those collected from Guatemala (GT1, GT2), Honduras (HN1, HN2), and one isolate from Hawaii (HI14) (Fig. 4). Amplification and hybridization were weak for FL5, FL6, and HI1. Three additional fragments also were amplified (0.4, 0.6, and 1.4 kb), but the 0.4-kb fragment did not hybridize with DIG-labelled DK#3.

Overall, the DK#4-specific primers amplified more polymorphic DNA among the 37 burrowing nematode isolates than any of the other sequence-tag-site primers (Fig. 5). Two DNA fragments (1.4 and 0.9 kb) were strongly amplified from five Hawaiian isolates (HI6-HI10). These fragments also were amplified from isolates CR1, CR2, CR3 and PR1; however, the 1.4-kb fragment did not hybridize with the DIG-DK#4 probe for isolates CR3 and PR1. The 0.9-kb fragment also was amplified from isolates FL3, FL5, FL7, BZ1-2, CR3, PR1, GT1-2, HN1-2, GD1, HI2-5, HI12, and a 0.7-kb fragment became apparent after DNA hybridization. Fragments amplified from isolates FL6 and HI11 (0.5-kb fragment) and HI8 and HI13 (1.3 kb and 0.95 kb) were polymorphic. DNA fragments were not amplified from isolates FL1, FL4, FL8-10, PR2, and HI14.

Two DNA fragments (0.9 and 0.75 kb) were amplified from 26 of the 37 burrowing nematode isolates using the DK#5-specific primers (Fig. 6). Isolates BZ2 and FL2 were polymorphic (0.9-kb and 0.8-kb amplified fragments). Finally, the DK#5-specific primers did not amplify any DNA fragments from six of the ten citrus-parasitic burrowing nematodes (FL3, FL4, FL7, FL8, FL9, FL10).

DISCUSSION

Burrowing nematodes that parasitize citrus in Florida do not appear to be a distinct species from morphologically similar burrowing nematodes that cannot parasitize citrus, since isolates collected from a wide range of geographically distinct sites were greater than 93% similar. The high extent of

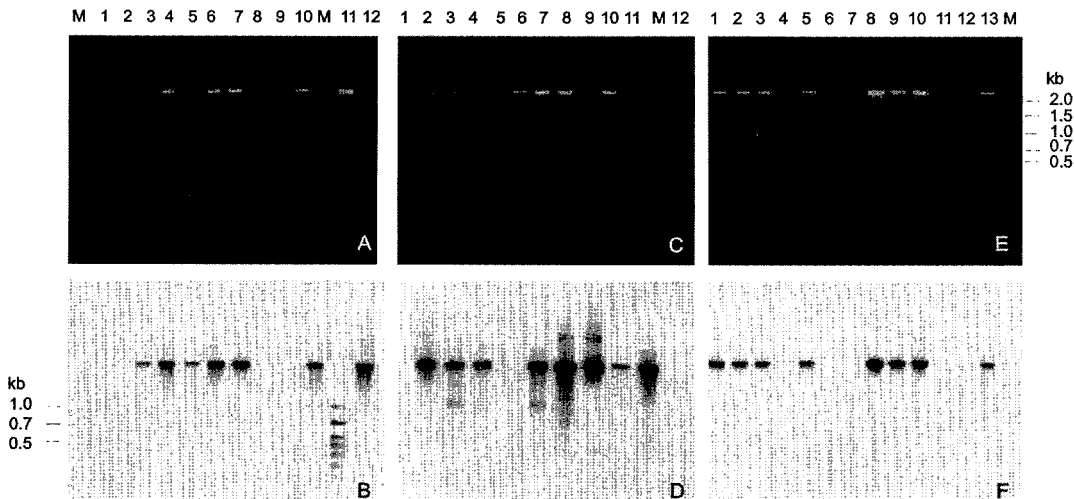


FIG. 3. Amplification of DK#1 from crude DNA from 37 burrowing nematode isolates using DK#1-specific primers DK#101 and DK#102. A,C,E) Ethidium bromide-stained agarose gels. B,D,F) DNA blots of A, C, and E, respectively, probed with digoxigenin-labeled DK#1. Lane designations for A,B) 1, FL8; 2, FL10; 3, FL9; 4, FL1; 5, FL2; 6, FL3; 7, FL4; 8, FL5; 9, FL6; 10, FL7; M = Molecular Weight Marker III; 11, BZ1; 12, CR1. C,D) 1, CR2; 2, CR3; 3, PR1; 4, PR2; 5, DR1; 6, GT1; 7, GT2; 8, HN1; 9, HN2; 10, BZ2; 11, GD1; M = BioMarker EXT; 12, HI2. E,F) 1, HI3; 2, HI4; 3, HI5; 4, HI6; 5, HI7; 6, HI8; 7, HI9; 8, HI10; 9, HI11; 10, HI12; 11, HI13; 12, HI14; 13, HI1; M = BioMarker EXT. Acronyms for nematode isolates are listed in Table 1.

genome similarity is in accordance with previous studies (Hahn et al., 1994; Kaplan, 1994b; Kaplan et al., 1996; Kaplan et al., 1997). In contrast, Fallas et al. (1996) proposed that two distinct gene pools were present among banana-parasitic burrowing nematodes collected in Africa, Australia,

and Guadeloupe on the basis of RAPD and PGI analyses. Diversity among burrowing nematodes may be greater in Africa than in Hawaii, Florida, Central America, Puerto Rico, and Guadeloupe. Previously we determined that DK#1 could not be amplified from African burrowing nematode isolates,

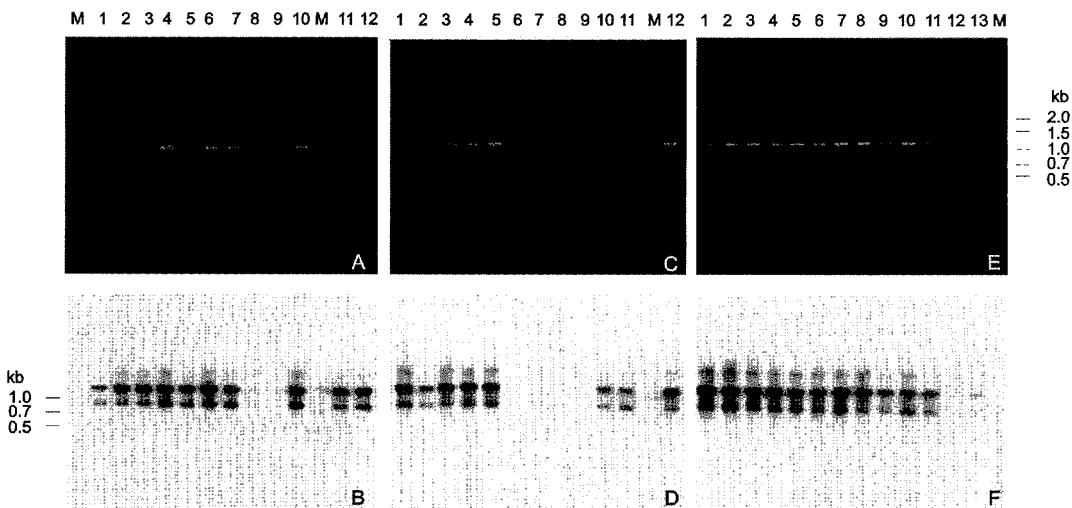


FIG. 4. Amplification of DK#3 from crude DNA from 37 burrowing nematode isolates using DK#3-specific primers DK#103 and DK#104. A,C,E) Ethidium bromide-stained agarose gels. B,D,F) DNA blots of A, C, and E, respectively, probed with digoxigenin-labeled DK#3. Lane designations as for Fig. 3. Acronyms for nematode isolates are listed in Table 1.

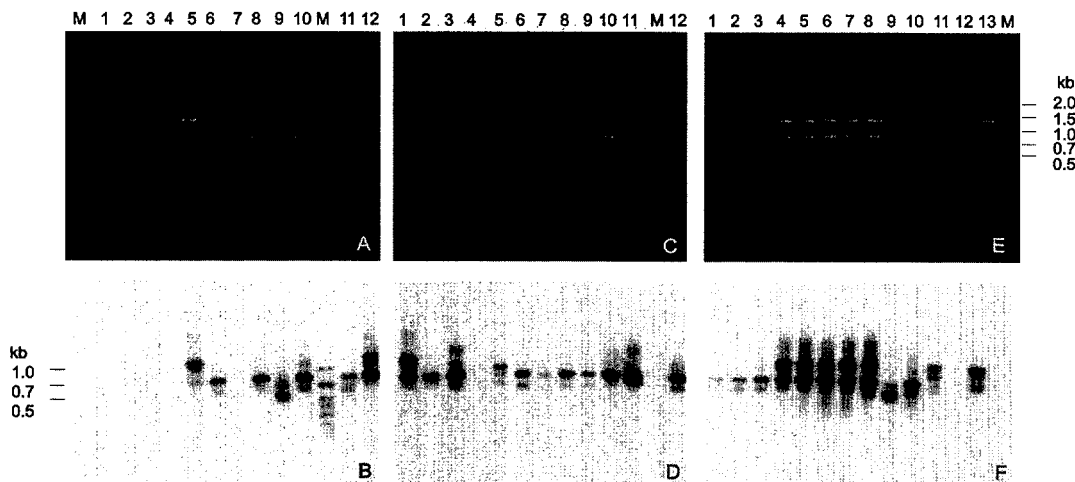


FIG. 5. Amplification of DK#4 from crude DNA from 37 burrowing nematode isolates using DK#4-specific primers DK#107 and DK#108. A,C,E) Ethidium bromide-stained agarose gels. B,D,F) DNA blots of A, C, and E, respectively, probed with digoxigenin-labeled DK#4. Lane designations as for Fig. 3. Acronyms for nematode isolates are listed in Table 1.

whereas DK#1 was readily amplified from burrowing nematodes collected in the Western Hemisphere (Kaplan, unpubl. data).

The high degree of genome similarity among burrowing nematodes suggests that they share a common ancestry and have been dispersed relatively recently. Burrowing nematode-infested plants have been transported throughout the world and may have been transported with a crop from its

origin or become infested from populations encountered in transit or if planted in infested soils. However, *Radopholus* may contain a genetic mechanism through which the genome remains relatively constant (Kaplan et al., 1996).

Although it is unknown how and when burrowing nematodes were first introduced to each country where they currently persist, it can be assumed that they were transported

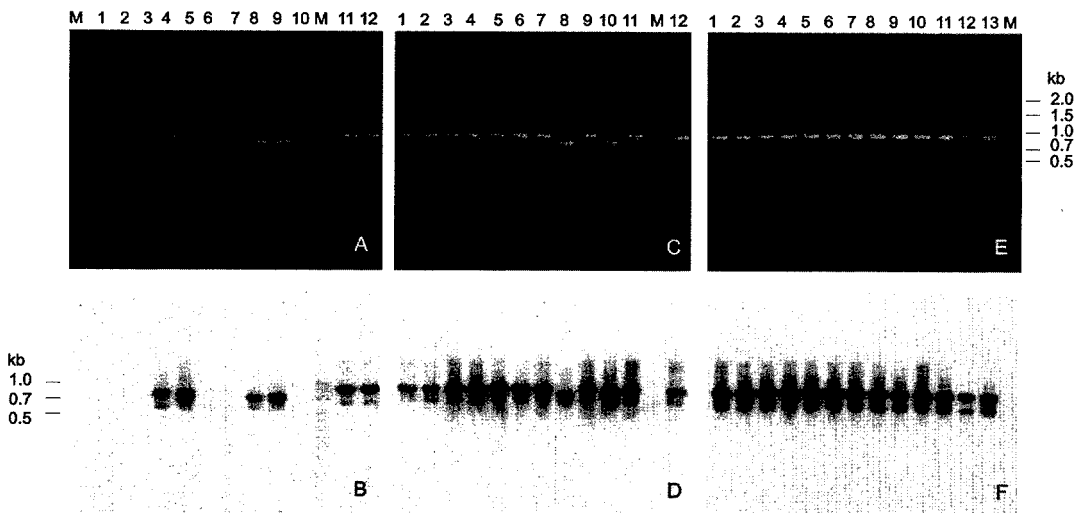


FIG. 6. Amplification of DK#5 from crude DNA from 37 burrowing nematode isolates using DK#5-specific primers DK#105 and DK#106. A,C,E) Ethidium bromide-stained agarose gels. B,D,F) DNA blots of A, C, and E, respectively, probed with digoxigenin-labeled DK#5. Lane designations as for Fig. 3. Acronyms for nematode isolates are listed in Table 1.

in the roots of crops carried from infested to uninfested sites. For instance, anthuriums are native to Colombia but grow throughout Central America where *Radopholus* is prevalent. The first anthuriums were brought to Hawaii in 1889 by Samuel M. Damon, who obtained them from the Royal Botanic Gardens, Kew, England. It is unknown if these plants were infested with burrowing nematodes; however, in 1985, burrowing nematodes were detected in roots of *A. andraeanum* growing in a glasshouse at the Royal Botanic Gardens at Kew (Kaplan, unpubl. data). Thus, Damon could have transported burrowing nematodes to Hawaii in anthurium roots in the late 1800s via Kew as proposed by Huettel et al. (1986). Sher documented the first association of *R. similis* with *A. andraeanum* in roots of plants collected in Hilo and Honomu, Hawaii, in 1954 (Sher, 1954).

Burrowing nematodes probably were introduced to Hawaii from Australasia or Indonesia by settlers who transported infested plants prior to the importation of anthuriums. Burrowing nematodes also have been transported in recent times in corms used to propagate bananas (Gowen and Queneherve, 1990). Thus, it is likely that burrowing nematodes were transported to and from Hawaii, Central America, and Caribbean countries on numerous occasions. In contrast, Florida does not appear to be the origin of plants recognized for use as ornamentals or food. Although a significant ornamentals industry is present in Florida, the Florida Department of Consumer Services, Division of Plant Industry nursery certification program instituted nearly 40 years ago has ensured that citrus-parasitic burrowing nematode-infested crops are not exported.

Root samples collected from citrus trees in Pahoia, Hawaii, where anthuriums have been grown since the 1930s (Mike Conway, pers. comm.), did not contain burrowing nematodes. However, burrowing nematodes were detected in roots of anthuriums grown under citrus tree canopies. This suggests that the genetic potential for citrus parasitism is absent among burrowing nematodes infecting anthuriums in Hawaii. Previously,

Sher (1954) sampled citrus trees in Pahoia but did not detect burrowing nematodes. Clark (1983) conducted greenhouse studies and demonstrated that burrowing nematodes isolated from bananas and anthurium in Hawaii did not reproduce in citrus roots. In 1995, citrus trees were sampled in three independent sites in Hawaii, but burrowing nematodes were not detected in their roots (M. Enriques, pers. comm.).

The prior designation of a non-citrus-parasitic burrowing nematode isolated from anthurium in Hawaii as *R. citrophilus* (Huettel et al., 1984b) appears to be unwarranted. Burrowing nematodes associated with *A. andraeanum* were reported to be *R. citrophilus* based upon karyotype, isozymes, proteins, and morphological traits (female head, annules interrupted by vulva, cloacal ornamentation), which were considered to be specific to *R. citrophilus* (Huettel and Dickson, 1981; Huettel et al., 1982, 1983a, 1983b, 1984a). However, burrowing nematodes in Africa, where citrus-parasitic burrowing nematodes have never been detected, have morphological traits and isozyme profiles similar to those reported for *R. citrophilus* (Fallas et al., 1996; Valette et al., pers. comm.). Furthermore, non-citrus-parasitic burrowing nematodes with the karyotype ($n = 5$) considered specific to *R. citrophilus* by Huettel et al. (1986) have been detected in Puerto Rico, Sri Lanka, and the Ivory Coast (Hahn et al., 1996; Rivas and Roman, 1985a, 1985b).

Another citrus-parasitic burrowing nematode (*R. citri*) collected in East Java has been described recently, but its morphology is distinct from *R. similis*, with RAPD profiles confirming that *R. citri* is a distinct species (Hahn et al., 1994; D. Kaplan, unpubl. data; Machon and Bridge, 1996). Hahn et al. (1996) reported that an apparent *R. similis* collected in Sri Lanka also was citrus-parasitic, but this finding has not been adequately documented. In the study by Hahn et al. (1996) burrowing nematodes apparently were detected in citrus seedling roots 60 days after inoculation in a single test, and limited decline in nematode population densities in roots of test seedlings suggested that the burrowing nematodes might actu-

ally be reproducing in citrus. In a second test, however, burrowing nematodes failed to persist in roots of citrus seedlings. Accurate interpretation of such assays are dependent upon understanding how burrowing nematodes interact with citrus. Non-citrus-parasitic burrowing nematodes can persist for long periods of time in citrus roots, and even juveniles can be detected. However, the numbers of nematodes eventually decline to non-detectable levels. This is similar to the dynamics associated with the decline of citrus-parasitic burrowing nematode populations in nematode-resistant citrus rootstocks. Furthermore, citrus declines attributed to burrowing nematodes have not been reported from Sri Lanka. In other host-range studies, citrus was assigned non-host status for burrowing nematodes collected from a variety of crops in Honduras, Panama, Natal, Fiji, Australia, and Rhodesia (O'Bannon, 1977).

Identification of polymorphic DNA can lead to development of genetic markers that can facilitate mapping of the burrowing nematode genome to improve our understanding of parasitism in burrowing nematodes, clarify relationships among burrowing nematode populations throughout the world, and contribute to the development of novel methods for nematode control. For example, the sequence tag site DK#1 and citrus parasitism were co-inherited by progeny produced in controlled matings of citrus-parasitic, DK#1-positive males with non-citrus-parasitic, DK#1-negative females. The resultant F₁ progeny from this cross were citrus-parasitic, DK#1-positive, and reproductively viable (Kaplan et al., 1997). These findings indicated that citrus and non-citrus-parasitic burrowing nematodes were not reproductively isolated (Kaplan et al., 1997) as previously reported (Huettel et al., 1982). However, DK#1 and citrus parasitism loci are not sufficiently linked so as to enable DK#1 to be used to identify citrus-parasitic burrowing nematodes or to make broad inferences regarding the extent of genome variation among burrowing nematodes. The four sequence-tag sites (DK#1, DK#3, DK#4, DK#5) appear to represent variable regions

in the burrowing nematode genome and should prove useful in mapping experiments involving controlled matings where parental lines differ with respect to these genetic sequences.

Additional polymorphic fragments identified in this study will be converted to sequence-tag sites for study of burrowing nematode genetics and mapping of the burrowing nematode genome.

Our data indicate that the geographic distribution of citrus-parasitic burrowing nematodes morphologically identical to *R. similis* remains restricted to Florida. How burrowing nematodes came to parasitize citrus in Florida is unknown, but thus far our findings suggest that citrus parasitism appears to be associated with limited changes in the burrowing nematode genome.

LITERATURE CITED

- Allendorf, F. W., N. Mitchell, N. Ryman, and G. Stahl. 1977. Isoenzyme loci in brown trout. *Salmo trutta* L.: Detection and interpretation from population data. *Hereditas* 86:179-190.
- Bird, D. McK., and D. L. Riddle. 1994. A genetic nomenclature for parasitic nematodes. *Journal of Nematology* 26:138-143.
- Blake, C. D. 1961. Root rot of bananas caused by *Radopholus similis* (Cobb) and its control in New South Wales. *Nematologica* 6:295-310.
- Clark, D. A. C. 1983. Identification of physiological races of *Radopholus similis* in Hawaii. M.S. thesis, University of Hawaii, Manoa, HI.
- DuCharme, E. P. 1959. Morphogenesis and histopathology of lesions induced on citrus roots by *Radopholus similis*. *Phytopathology* 49:388-395.
- DuCharme, E. P., and W. Birchfield. 1956. Physiological races of the burrowing nematode. *Phytopathology* 46:615-616.
- Duncan, L. W., and E. Cohn. 1990. Nematode parasites of citrus. Pp. 321-346 in M. Luc, R. A. Sikora, and J. Bridge, eds. *Plant parasitic nematodes in subtropical and tropical agriculture*. St. Albans, UK: CAB International.
- Edwards, K., C. Johnstone, and C. Thompson. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* 19:1349.
- Esser, R. P., J. H. O'Bannon, and C. C. Riherd. 1988. The citrus nursery site approval program for burrowing nematode and its beneficial effect on the citrus industry of Florida. *EPP0 Bulletin* 18:579-586.
- Fallas, G. A., M. L. Hahn, M. Fargette, P. R. Burrows, and J. L. Sarah. 1996. Molecular and biochemical diversity among isolates of *Radopholus* spp. from different areas of the world. *Journal of Nematology* 28:422-431.
- Gowen, S., and P. Queneherve. 1990. Nematode

parasites of bananas, plantains, and abaca. Pp. 431–460 in M. Luc, R. A. Sikora, and J. Bridge, eds. Plant parasitic nematodes in subtropical and tropical agriculture. St. Albans, UK: CAB International.

Hahn, M. L., P. R. Burrows, N. C. Gnanaoragasam, J. Bridge, N. Vines, and D. J. Wright. 1994. Molecular diversity amongst *Radopholus similis* populations from Sri Lanka detected by RAPD analysis. *Fundamental and Applied Nematology* 17:275–281.

Hahn, M. L., D. J. Wright, and P. R. Burrows. 1996. The chromosome number in *Radopholus similis*—a diagnostic feature? *Nematologica* 42:382–386.

Hebert, P. D. N., and M. J. Beaton. 1993. Methodologies for allozyme analysis using cellulose acetate electrophoresis. Beaumont, TX: Helena Laboratories.

Holdeman, Q. L. 1986. The burrowing nematode *Radopholus similis*, *sensu lato*. Sacramento, CA: California Department of Food and Agriculture.

Huettel, R. N., and D. W. Dickson. 1981. Karyotype and oogenesis of *Radopholus similis* (Cobb) Thorne. *Journal of Nematology* 13:16–20.

Huettel, R. N., and T. Yaegashi. 1988. Morphological differences between *Radopholus citrophilus* and *R. similis*. *Journal of Nematology* 20:150–157.

Huettel, R. N., D. W. Dickson, and D. T. Kaplan. 1982. Sex attraction and behavior in the two races of *Radopholus similis*. *Nematologica* 28:360–369.

Huettel, R. N., D. W. Dickson, and D. T. Kaplan. 1983a. Biochemical identification of two races of *Radopholus similis* by starch gel electrophoresis. *Journal of Nematology* 15:338–344.

Huettel, R. N., D. W. Dickson, and D. T. Kaplan. 1983b. Biochemical identification of two races of *Radopholus similis* by polyacrylamide gel electrophoresis. *Journal of Nematology* 15:345–348.

Huettel, R. N., D. W. Dickson, and D. T. Kaplan. 1984a. Chromosome number of populations of *Radopholus similis* from North, Central and South America, Hawaii, and Indonesia. *Revue de Nématologie* 7:113–116.

Huettel, R. N., D. W. Dickson, and D. T. Kaplan. 1984b. *Radopholus citrophilus* sp. n. (Nematoda), a sibling species of *Radopholus similis*. *Proceedings of the Helminthological Society of Washington* 51:32–35.

Huettel, R. N., D. W. Dickson, and D. T. Kaplan. 1986. Characterization of a new burrowing nematode population, *Radopholus citrophilus*, from Hawaii. *Journal of Nematology* 18:50–54.

Kaplan, D. T. 1994a. Measuring burrowing nematode virulence to select rootstocks for Florida citrus groves. *Proceedings of the Florida State Horticultural Society* 107:84–89.

Kaplan, D. T. 1994b. Molecular characterization of the burrowing nematode sibling species, *Radopholus citrophilus* and *R. similis*. Pp. 77–83 in F. Lamberti, C. De Georgi, and D. McK. Bird, eds. *Advances in molecular plant nematology*. New York: Plenum Press.

Kaplan, D. T., and E. L. Davis. 1990. Improved nematode extraction from carrot disk culture. *Journal of Nematology* 22:399–406.

Kaplan, D. T., M. C. Vanderspool, C. Garrett, S. Chang, and C. H. Opperman. 1996. Molecular polymorphisms associated with host range in the highly conserved genomes of burrowing nematodes, *Radopholus* spp. *Molecular Plant-Microbe Interactions* 9:32–38.

Kaplan, D. T., M. C. Vanderspool, and C. H. Opperman. 1997. Sequence tag site and host range assays demonstrate that *Radopholus similis* and *R. citrophilus* are not reproductively isolated. *Journal of Nematology* 29: (In press.)

Loos, C. A., and S. B. Loos. 1960. The blackhead disease of bananas. *Proceedings of the Helminthological Society of Washington* 27:189–193.

Machon, J. E., and J. Bridge. 1996. *Radopholus citri* n. sp. (Tylenchida: Pratylenchidae) and its pathogenicity on citrus. *Fundamental and Applied Nematology* 19:127–133.

Maniatis, T. A., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: A laboratory handbook*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

O'Bannon, J. H. 1977. Worldwide dissemination of *Radopholus similis* and its importance in crop production. *Journal of Nematology* 9:16–25.

Rivas, X., and J. Roman. 1985a. Oogenesis y reproducción de una población de *Radopholus similis* de Puerto Rico. *Nematropica* 15:19–25.

Rivas, X., and J. Roman. 1985b. Estudio sobre la gama de hospederos de una población de *Radopholus similis* de Puerto Rico. *Nematropica* 15:165–170.

Sasser, J. N., and D. W. Freckman. 1987. A world perspective on nematology: The role of the society. Pp. 7–14 in J. A. Veech and D. W. Dickson, eds. *Vistas on nematology*. Hyattsville, MD: Society of Nematologists.

Sher, S. A. 1954. Observation on plant-parasitic nematodes in Hawaii. *Plant Disease Reporter* 38:687–689.

Siddiqi, M. R. 1986. *Tylenchida: Parasites of plants and insects*. St. Albans, UK: CAB International.

Sneath, P. H. A. and R. R. Sokal. 1973. *Numerical taxonomy*. San Francisco, CA: Freeman and Company.

Suit, R. F., and E. P. DuCharme. 1953. The burrowing nematode and other parasitic nematodes in relation to spreading decline. *Citrus Leaves* 33:8–33.

Williams, J. G., A. R. E. Kubelik, K. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research* 18:6531–6535.

Young, T. W. 1954. An incubation method for collecting migratory endoparasitic nematodes. *Plant Disease Reporter* 38:794–795.