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Sequence Tag Site and Host Range Assays Demonstrate that *Radopholus similis* and *R. citrophilus* are not Reproductively Isolated

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Abstract: Males of citrus-parasitic *Radopholus citrophilus* (FL1) were mated with non-citrus-parasitic *R. similis* (FL5) females. Progeny inherited a 2.4-kb sequence tag site (DK#1) and the ability to reproduce in citrus from the paternal parent (FL1); both traits were absent in the maternal line (FL5). The hybrid progeny produced offspring in roots of citrus seedlings over an 8-month period and therefore were considered reproductively viable. Genomic DNA hybridization studies indicated that one or more copies of DK#1 were present in *R. citrophilus* FL1. It is not likely that DK#1 represents a citrus parasitism gene because it was amplified from some burrowing nematode isolates that did not parasitize citrus and because DK#1 contains no open reading frames. Inability to reliably test individual nematodes for their ability to parasitize citrus was a constraint to obtaining F2 data required for definitive genetic characterization of citrus parasitism in burrowing nematodes, and alternate approaches will be required. Although the physical relationship of DK#1 and the citrus parasitism locus remains undefined, results of controlled mating studies using these parameters as genetic markers enabled us to identify hybrid F_1 progeny. Therefore, *R. similis* and *R. citrophilus* are not sibling species since gene flow between the two does not appear to be restricted via geographic isolation (sympatric in Florida) or by genetics.

Key words: banana, citrus, Florida, genetics, hybrid, nematode, PCR, quarantine, *Radopholus citrophilus, Radopholus similis,* RAPD, STS, taxonomy.

Burrowing nematodes (*Radopholus citrophilus* Huettel, Dickson & Kaplan) that attack citrus in Florida are morphologically indistinguishable from *R. similis* (Cobb) Thorne that attack banana worldwide and appear to share a high degree of genome similarity (DuCharme, 1959; DuCharme and Birchfield, 1956; Fallas et al., 1996; Hahn et al., 1994; Kaplan and Opperman, 1997; Kaplan et al., 1996). Although burrowing nematode isolates have been demonstrated to differ in their reproductive fitness and pathogenicity (Fallas et al., 1996; Kaplan and O'Bannon, 1985), there is a complete lack of knowledge of the genetics of burrowing nematode parasitism.

Use of the polymerase chain reaction (PCR) has greatly facilitated genetic analysis, and several assays based on selective amplification of polymorphic DNA have been developed (Persing, 1996). PCR-based genetic analysis is dependent upon identification of polymorphic loci to which sequence-specific primers can be generated (Williams et al., 1990). To this end, a polymorphic DNA fragment (DK#1), first detected in a RAPD analysis that compared citrus-parasitic with non-citrus-parasitic burrowing nematode strains, was converted to a sequence tag site (Kaplan et al., 1996). Subsequent PCR experiments suggested that the sequence tag site DK#1 did not represent a citrus parasitism gene since homologous fragments

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The DNA sequences of the DK#1 homologues amplified from the citrus-parasitic (DK2) and the non-citrus-parasitic (DK3) burrowing nematodes were assigned GenBank accession numbers U54823 and U54824, respectively.

were amplified from several burrowing nematode isolates that did not parasitize citrus (Kaplan and Opperman, 1997). The purpose of this study was to determine the nucleic acid sequence of the DK#1 fragment and to compare DK#1 homologues amplified from multiple burrowing nematode isolates. We also studied the use of DK#1 as a genetic marker and the use of a citrus bioassay to identify hybrid progeny from matings of citrus-parasitic *R. citrophilus* and non-citrus-parasitic *R. similis*.

Materials and Methods

Nematodes: Fourteen burrowing nematode isolates previously characterized for their ability to parasitize sour orange (*Citrus aurantium* L.) and rough lemon (*C. limon* (L.) Burm F.) were cultured on excised carrot disks and extracted from culture by enzymatic maceration (Kaplan and Davis, 1990). Laboratory designations for *Radopholus* isolates collected from Florida were FL1-FL5 and FL7; Hawaii, HI1; Belize, BZ1; Costa Rica, CR1-CR3; Puerto Rico, PR1 and PR2. *Radopholus* isolates FL1-FL4 and FL7 parasitize citrus, whereas *Radopholus* isolates BZ1, FL5, FL6, HI1, CR1-3, and PR1-2 do not parasitize citrus (Kaplan et al., 1996) (Table 1).

DNA cloning and sequencing: Primers and cloned DNA were designated according to the guidelines published for plant-parasitic nematodes (Bird and Riddle, 1994). The primers DK#101 (CAATCGCCGTTAATTGCTGTGTTT) and DK#102 (CAATCGCCGTAGAATGCCATCATC) previously designed to complement the terminal sequences of the 2.4-kb DNA fragment (DK#1) were used to amplify DK#1 (Kaplan et al., 1996). DK#1 amplified from the *R. citrophilus* FL1 and HI1 were individually excised from ethidium bromide-stained agarose gels and recovered with Geneclean (Bio-101, La Jolla, CA) according to manufacturer's protocols. The isolated DNA was ligated into the Eco RV site of pT7Blue (Novagen, Madison, WI) and transformed into *Escherichia coli* strain Nova Blue; recombinants were identified by blue/white selection. The sequence of both strands of the 2.4-kb DNA fragment was determined using the Taq Dye Primer and Taq Dye Deoxy Terminator cycle sequencing protocols (Applied Biosystems, Perkin-Elmer, Foster City, CA). Labeled extension products were analyzed on an Applied Biosystems Model 373a DNA Sequencer (Perkin Elmer). Oligonucleotide primers were synthesized at the DNA Synthesis Core Laboratory at the University of Florida, Gainesville, Florida. Nucleotide sequences were aligned and assembled using programs in the Sequencher software package (Gene Codes, Ann Arbor, MI) and compared to GenBank entries at the nucleic and deduced amino acid levels with the BlastX and BlastN. Similarity was considered significant if the percentage of nucleotide identity was higher than 50% for more than 100 bp.

Restriction analysis: The 2.4-kb DNA fragment was amplified using DNA from the burrowing nematodes BZ1, FL1-FL4, FL7, HI1, and PR2 and cloned as described above. A restriction map of the 2.4-kb fragments from the burrowing nematodes FL1 and HI1 was generated with Cutter Software (http://firstmarket.com/firstmarket/cutter/) revealing several restriction sites for commercially available enzymes. The 6-base endonucleases Pvu I (Boehringer Mannheim, Indianapolis, IN), Hae II, Kpn I, Nru I, Pst I, Sac I, Sph I (New England BioLabs, Beverly, MA), Cla I (Promega, Madison, WI), Eco RI, Stu I (Sigma, St. Louis, MO),

Table 1. Primary designation and acronym for burrowing nematodes and their collection sites.

Nematode population	Origin
FL1	Lake Wales, Florida
FL2	Orlando, Florida
FL3	Clermont, Florida
FL4	Lake Alfred, Florida
FL5	Orlando, Florida
FL7	Avon Park, Florida
HI1	Panaewa, Hawaii
BZ1	Bladen Bridge, Toledo, Belize
CR1	Coyles, Costa Rica
CR2	Guanacoste, Costa Rica
CR3	West Reventazon River, Costa Rica
PR1	Puerto Rico
PR2	Puerto Rico

and Ava II, Bam HI, Hin dIII, Sma I, Spe I, and Xho I (Stratagene, La Jolla, CA) were used according to manufacturer's recommendations to identify restriction fragment-length polymorphisms (RFLP) among the cloned 2.4-kb fragments from each of the eight nematode strains. Three independent clones per strain were analyzed. The endonucleases were chosen for the analysis because they yielded DNA fragments of 0.3–4.0kb that could be observed using 1.0% agarose gels in 1X TAE (40mM Tris acetate, 1mM EDTA, pH 8.0) at 80V for 2 h. Gels were stained with ethidium bromide (5µg/100 ml) and viewed on a UV transilluminator. Molecular weight markers were bacteriophage DNA cut with Pst I and BioMarker EXT (Bio Ventures, Murfreesboro, TN).

Genomic DNA hybridizations: Cesium chloride-purified DNA prepared from *R. citrophilus* FL2 and *R. similis* FL5 was quantified as previously described (Kaplan et al., 1996). Genomic DNA (15 µg) was digested with either Eco RI or Stu I according to manufacturer's recommendations and fractionated in 1.0% agarose with ethidium bromide (5 µg per 100 ml) and viewed on a UV transilluminator. Digoxigenin(DIG)-labeled DNA molecular weight marker III (Boehringer Mannheim Corp., Indianapolis, IN) was used to identify the relative sizes of restriction fragments. DNA blots were made as described previously (Kaplan et al., 1996).

Single nematode amplifications: Individual burrowing nematodes were placed in the bottom of 0.5-ml Eppendorf tubes and crushed with the side of a silanized pipette tip. Then, 5-µl lysis buffer (1X PCR buffer containing 50 g/ml proteinase K) was added to each tube and the tubes were incubated at −80 °C for 20 minutes. A drop of mineral oil was added to each tube, and tubes were incubated at 60 °C for 1 hour and then 95 °C for 15 minutes (Williamson et al., 1997). Twenty microliters of PCR mix containing 0.75 U of Taq DNA polymerase (Perkin Elmer, Norwalk, CT), 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.75 mM $MgCl_2$, 100 M of each dNTP, and 0.2 M primers DK#101 and DK#102 was added to the 5 µl of nematode homogenate. Two types of control treatments were included in the experiment: a negative control consisting of lysis buffer without nematodes, and a positive control consisting of 25 ng of CsCl-purified DNA (DK2 or DK6). PCR was performed in a Perkin Elmer Cetus thermal cycler using a preheated block (94 °C) for 45 cycles (94 °C, 1 minute; 63 °C, 1 minute; 72 °C, 2 minutes). Reacting tubes were subsequently held at 4 °C until retrieval. Electrophoresis conditions were as for the restriction analysis described above.

Mating study: Two or three males of *R. citrophilus* FL1 (citrus parasitic) extracted from carrot disk culture (Kaplan and Davis, 1990) were placed on a thin layer of 0.1% agarose in a 60-mm-diam. petri dish. An active female *R. similis* FL5 (non-citrus-parasitic) was then placed on the agarose surface among the males. Mating was observed at ×100 with a Nikon TMS inverted light microscope. Once copulation was completed, the non-citrus-parasitic female was removed from the agarose surface and placed in a 2.0-µl drop of sterile water on the surface of a carrot disk within a tube. After 25 days, the carrot disks were macerated and progeny were extracted (Kaplan and Davis, 1990). This experiment was repeated three times. Mating procedures were conducted so that only the progeny were tested for citrus parasitism.

A second set of experiments was performed in which 100 female *R. similis* FL5 burrowing nematodes that did not parasitize citrus and 100 citrus-parasitic *R. citrophilus* FL1 male burrowing nematodes were transferred to the surface of a carrot disk in 2.0-µl drops of water. Disks were maintained, macerated, and progeny extracted after 25 days as described above.

Progeny were used individually in PCR reactions under conditions described above (single nematode amplification) to determine if the DK#1 DNA fragment could be amplified. Forty individual F_1 progeny were screened for the presence of DK#1 for the first mating experiments. A total of 150 nematodes were evaluated in the second mating experiments (bulk matings).

The F_1 progeny from both experiments were assayed to determine if they could re-

produce on citrus. Ten seedlings of rough lemon (*Citrus limon*) and tomato (*Lycopersicon esculentum*) were inoculated with 100 F_1 (mixed life-cycle stages) and examined 60 days later in a laboratory host-index assay (Kaplan, 1994a). Nematodes collected from citrus and tomato plants subsequently were used to inoculate new citrus and tomato seedlings, and individuals were assayed for DK#1 as described above throughout an 8-month test period.

Results

The nucleic acid sequence of the 2.4-kb DK#1 homologues amplified from the citrus-parasitic burrowing nematode isolate FL2 and from a non-citrus-parasitic burrowing nematode, isolate HI1, was 99% identical. No open reading frames were identified, and search of the GenBank database revealed no significant match to previously reported DNA sequences. A restriction map was generated that identified several restriction sites (Table 2). The restriction fragment lengths for the DK#1 homologues from burrowing nematode isolates BZ1, FL1-FL4, FL7, HI1, and PR2 were identical and yielded fragments of predicted size for all endonucleases except Cla I. Two restriction sites (244 and 1427) were predicted for Cla I based on the nucleotide sequence. However, only a 5483-bp fragment was observed, indicating that digestion occurred at only one site. Digestion did not occur at the 1427-bp site (Kaplan, unpublished data). Two restriction patterns were observed for most endonucleases with more than one restriction site, since the DK#1 homologues inserted randomly into the cloning vector in either forward or reverse orientation (Fig. 1, Table 2).

DNA hybridization experiments were performed to determine if DK#1 could be detected in *R. citrophilus* FL2 (citrus-parasitic) and *R. similis* FL5 (non-citrus-parasitic). DIG-labeled DK#1 hybridized strongly with two DNA bands (5.0 and 9.2 kb) when 15 or 20 µg of genomic DNA purified from FL2 was digested with Stu I. DIG-labeled DK#1 also strongly hybridized with two DNA bands (3.7 and 4.0 kb) when a similar amount of genomic DNA from FL2 was digested with Eco RI (Fig. 2). The DIG-labeled DK#1 probe did not hybridize with any of the Stu I restriction products from FL5, but weak hybridization with an Eco RI digestion product (3.4 kb) was observed.

Two types of mating experiments were performed to determine if *R. citrophilus* citrus-parasitic males could mate with *R. similis* non-citrus-parasitic females to produce hybrid progeny. In the first experiment, individual *R. similis* FL5 females (non-citrus-parasitic; DK#1 negative) were placed next

Table 2. Nucleotide base pair fragment length patterns and restriction sites for DK#1 PCR-amplified from eight burrowing nematode isolates cloned in T7Blue Vector.

	Fragments		Restriction site	
Endonuclease	Forward	Backward	Vector	DK#1
Eco RI	5172	3150	120	2309
	311	2333		
Pvu II	2873	2873	212	NRS
	2514	2514	2726	
Sac I	4919	3407	118	2054
	564	2076		
Sca I	3331	4054	1177	346
	2152	1427		
Hae II	2941	2683	659	218
	1639	1639	667	
	523	781	2306	
	370	370	2676	
	8*			
Hind III	3318	3881	45	1045
	1094	1069		2114
	1069	531		
Ava II	1779	1990	1294	314
	1723	1512	1516	1371
	1057	1057		1554
	417	417		1654
	222*	222*		2071
	183*	183*		
	100*	100*		
Cla I	5483	5483	NRS	244
				1427
Spe I	5483	5483	81	NRS
Sma I	5483	5483	106	NRS
Kpn I	5483	5483	112	NRS
Pst I	5483	5483	61	NRS
Sph I	5483	5483	55	NRS
Stu I	NRS	NRS	NRS	NRS
Nru I	NRS	NRS	NRS	NRS
Xho I	NRS	NRS	NRS	NRS

*Not visible.
NRS = No restriction site.

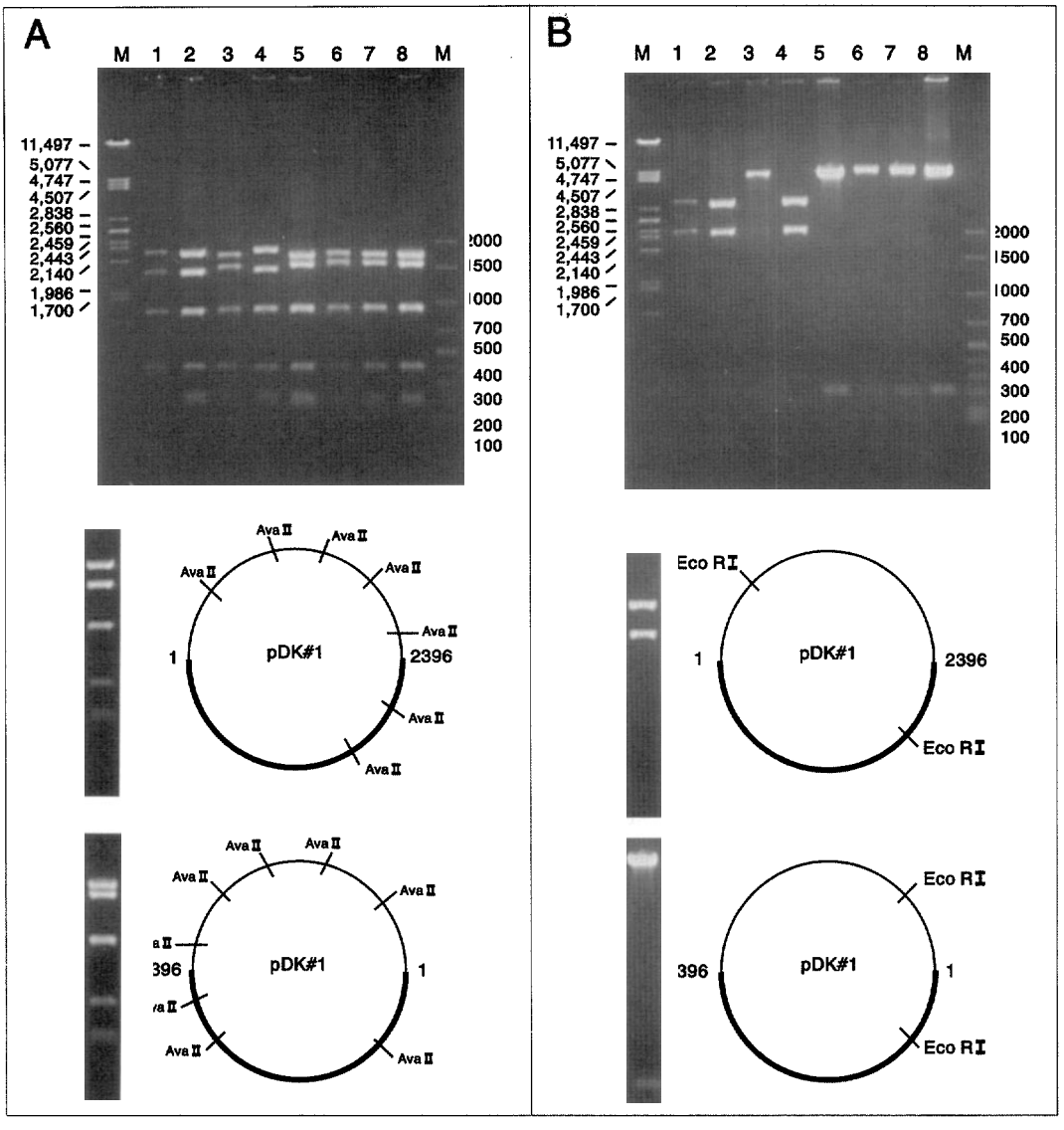

FIG. 1. Restriction patterns of pT7Blue clones of the 2.4-kb, DK#1 fragment from *Radopholus citrophilus* isolates FL1, FL2, HI1, FL3, FL4, FL7, BZ1, and PR2 (lanes 1–8, respectively). Molecular weights are indicated in base pairs. A. Ava II digests. B. Eco RI digests. The DK#1 insert in lanes 1, 2, and 4 is in one orientation, and in the opposite orientation in lanes 3 and 5–8, resulting in apparent differences in restriction patterns as per diagrams. Lanes M are molecular weight markers bacteriophage DNA cut with Pst I or BioMarker EXT (Bio Ventures, Murfreesboro, TN). Table 2 identifies restriction sites and fragment size(s).

to 2–3 *R. citrophilus* FL1 males (citrus-parasitic, DK#1 positive). Typical nematode mating behavior and coitus were observed within 5 to 45 minutes. Females were individually transferred to carrot disk cultures after insemination and maintained for 25 days. Following extraction from carrot disks, the DK#1-specific primers (DK#101 and DK#102) were successfully used to amplify the DK#1 sequence tag site from each of 40 individual progeny. Thus, the progeny were hybrids (Fig. 3). Siblings of these progeny reproduced in roots of citrus seedlings in related host assays. The progeny produced offspring that remained citrus-parasitic and from which DK#1 was readily amplified over an 8-month period.

In the second experiment, nematode mat-

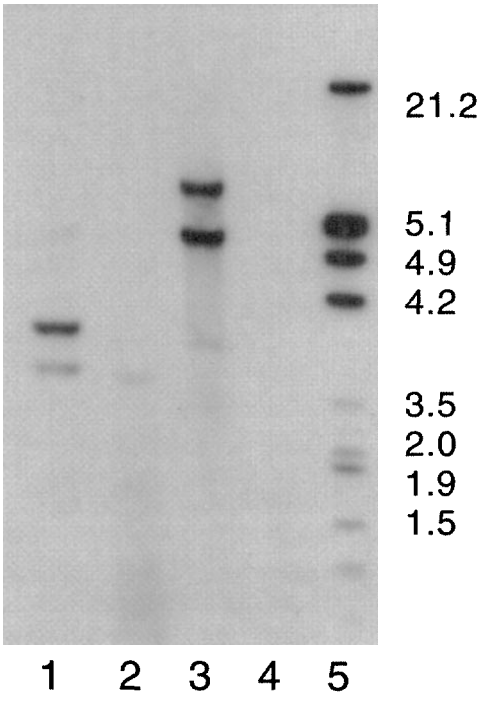

FIG. 2. Genomic digest performed with 15 µg of CsCl-purified genomic DNA from *Radopholus citrophilus* FL2 and *Radopholus similis* FL5. Lane 1, Eco RI-digested DNA from FL2; Lane 2, Eco RI-digested DNA from FL5; Lane 3, Stu I-digested DNA from FL2; Lane 4, Stu I-digested DNA from strain FL5; Lane 5, DIG-labeled DNA molecular weight markers. Hybridization filters were probed with DIG-labeled DK#1. Exposure time was 10 minutes.

ing behavior could not be observed on the carrot disks to which 200 nematodes (100 citrus-parasitic males and 100 non-citrus parasitic females) had been added. However, mating apparently occurred since the DK#1 fragment was amplified from some of the progeny. In contrast to the first experiment, DK#1 was detected in reduced frequency (4 out of 150 progeny).

DISCUSSION

Results of genomic DNA hybridization experiments suggested that, when present, one or more copies of DK#1 exist in the burrowing nematode genome. DK#1 may be present in the *R. citrophilus* FL2 genome in two or more copies, but DK#1 is apparently absent in the *R. similis* FL5 genome (Fig. 2). We believe this to be true because the DIG-labeled DK#1 probe hybridized strongly with two DNA digestion products (9.2 kb and 4.5 kb) when genomic DNA from *R. citrophilus* was digested with Stu I, despite the absence of Stu I cleavage sites in DK#1. Two Eco RI restriction products from *R. citrophilus* genomic DNA (3.0 and 2.7 kb) also hybridized strongly with DIG-labeled DK#1. Although an Eco RI restriction site is present within DK#1, its location at the terminal 85 bp suggests that strong hybridization of the DIG-labeled DK#1 probe represented sequence complementary of DK#1 with the larger 2309-bp restriction fragment. The DIG-labeled DK#1 probe did not hybridize to any of the Stu I restriction products from *R. similis*, and weak hybridization with the Eco RI digestion products of the *R. similis* genomic DNA were probably the result of limited sequence homology between portions of the relatively large 2.4 kb DIG-labeled probe with portions of the *R. similis* genome.

Results of the restriction analysis of DK#1 indicated that the sequence tag site was highly similar among all burrowing nematode isolates in the study. The restriction patterns for all endonucleases, except Cla I, were also as predicted according to the restriction map. However, Cla I was predicted to cut at two sites (244 and 1426), but the single 5483-bp restriction fragment indicated that Cla I cut only at a single site. Combining Cla I and Pst I, we determined that Cla I did not cut at the 1426-bp site (Kaplan, unpublished data). While we cannot rule out methylation of guanine at this site, which would prevent restriction by Cla I, methylated DNA has not been detected in nematodes (Emmons, 1988). It is more likely that this reflects an error in the nucleic acid sequence of DK#1 such that the putative restriction site may not be as predicted.

Inability to amplify DK#1 from some burrowing nematode isolates using DK#1-specific primers suggests they may also lack the sequence tag site (Kaplan and Opperman 1997; Kaplan et al., 1996), but only genomic DNA hybridization studies can verify this lack since differences in primer binding sites could also prevent amplification.

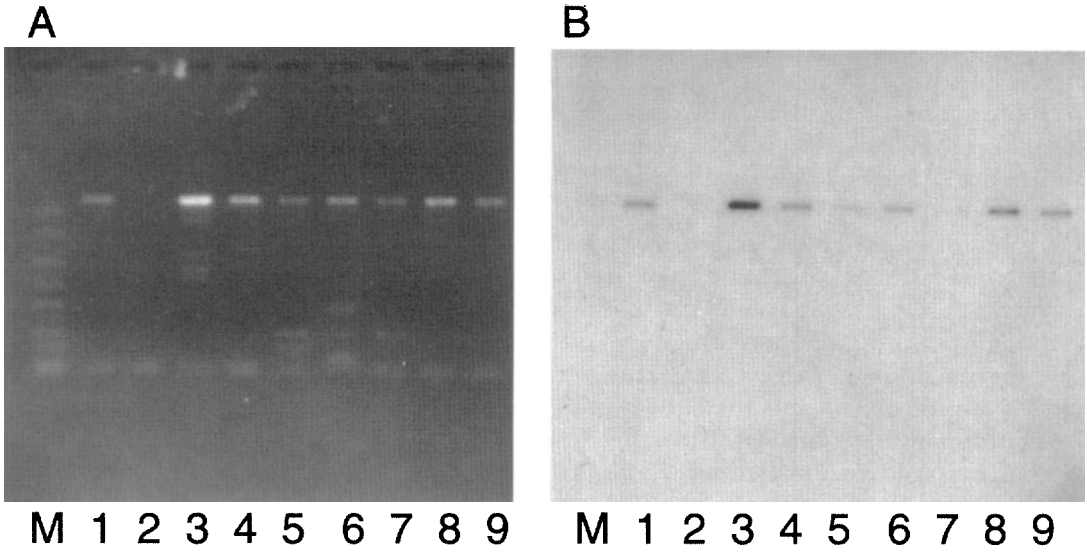

FIG. 3. Inheritance of DK#1 in the F_1 of a cross between FL1 and *Radopholus similis* FL5. Lane M, marker; Lane 1, *R. citrophilus* FL1 (paternal parent); Lane 2, *R. similis* FL5 (maternal parent); Lanes 3–9, PCR amplification of DK#1 from individual F_1 progeny using the DK#1-specific primers (DK#101 and DK#102; Kaplan et al., 1996). A) Ethidium bromide-stained agarose gel. B) DNA blot of Figure 3A probed with DIG-labeled DK#1.

Although both DK#1 and citrus parasitism were inherited by the F_1 progeny, the physical relationship between the citrus parasitism locus and DK#1 remains unknown. Since no open reading frames were detected in DK#1, it is probably not expressed, and since DK#1 was detected in some burrowing nematode isolates that do not parasitize citrus, it is unlikely that DK#1 represents a citrus parasitism gene (Kaplan and Opperman, 1997; Kaplan et al., 1996). To further characterize the genetics of citrus parasitism in the burrowing nematode, segregation analysis must be carried out to the F_2 generation, which requires that individual progeny be evaluated for their ability to parasitize citrus. Inability to attain consistent infection of roots inoculated with individual nematodes poses a technical barrier that, at present, precludes further genetic analysis of citrus parasitism loci in burrowing nematodes.

Demonstration of successful mating of citrus-parasitic *R. citrophilus* with *R. similis* that do not parasitize citrus differs from previous findings where the two were considered to be reproductively isolated (Huettel et al., 1982). Under our experimental conditions, mating and copulatory behavior were consistently observed shortly after the citrus-parasitic and non-citrus-parasitic nematodes were placed together. In the former study, Huettel et al. (1982) incubated the "citrus and banana races" of *R. similis* together in the dark for 2 hours prior to observation. Our experience suggests that mating would likely have occurred during that period, and this may explain why mating was not observed.

Since Huettel et al. (1982) proposed that a pheromone might function as a barrier to mating between the "citrus and banana races" of *R. similis,* experiments were designed so that nematodes had to migrate short distances on agar to locate mates. This raised concern that the hybrid progeny identified herein might be an experimental artifact since the design of our first experiment (placing males and females in immediate contact until they mated) may have circumvented a behavioral barrier to mating. However, we do not believe this to be true since the two mating types were transferred *en masse* to carrot disks without staging matings on an agar surface, and hybrid progeny were later detected. This suggests that pheromones are not barriers to mating between the two burrowing nematodes, as pre-

viously proposed (Huettel et al., 1982, 1984b).

The hybrid progeny were considered to be hemizygous for DK#1 because the maternal line (FL5) lacked DK#1. Furthermore, the hybrid progeny were reproductively competent; offspring of the hybrid progeny increased in roots of both rough lemon and tomato seedlings and in carrot disks throughout a subsequent 8-month period. Although performed under laboratory conditions, our findings suggest that mating of citrus-parasitic burrowing nematodes with non-citrus-parasitic *R. similis* will produce hybrid progeny in vivo. Furthermore, both putative sibling species are present in Florida (DuCharme and Birchfield, 1956; Huettel et al., 1983a; 1983b Kaplan and Opperman, 1997). Gene flow between the two does not appear to be restricted by geographic isolation (sympatric in Florida) or by genetic barriers. Therefore, burrowing nematodes that are similar in appearance but which differ with respect to citrus parasitism should no longer be considered to be reproductively isolated.

Burrowing nematodes that attack citrus in Florida are morphologically indistinguishable from those that attack banana worldwide; thus, they had been considered to be the citrus race of *R. similis* (DuCharme and Birchfield, 1956; Gowen and Queneherve, 1990). However, 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 between single *R. similis* and *R. citrophilus* isolates also were identified (Huettel and Yaegashi, 1988). Siddiqi (1986) later classified the sibling species as two subspecies, *R. similis similis* and *R. similis citrophilus.*

More recently, genomic analyses have raised concern regarding the designation of the former "citrus and banana races" of *R. similis* as sibling species because their genome appeared to be highly conserved (Fallas et al., 1996; Hahn et al., 1994; Kaplan, 1994b; Kaplan et al., 1996, 1997). 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 (C. Valette and J.-L. Sarah, pers. comm.). Furthermore, burrowing nematodes with the karyotype of n = 5 (considered specific to *R. citrophilus* by Huettel and Dickson, 1981) have been detected in non-citrus-parasitic burrowing nematodes collected from Puerto Rico, Sri Lanka, and the Ivory Coast (Hahn et al., 1996; Rivas and Roman, 1985a, 1985b).

In summary, these findings indicate that burrowing nematodes that parasitize citrus are not reproductively isolated from burrowing nematodes that are unable to parasitize citrus. The inheritance of the DK#1 marker and ability to parasitize citrus by reproductively viable progeny derived from matings of select nematode isolates suggests that gene flow is not restricted between the two burrowing nematodes, and therefore they can no longer be considered as distinct species.

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