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Randomly Amplified Polymorphic DNA Differs with Burrowing Nematode Collection Site, but not with Host Range[1]

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Abstract: The genetic variability of 12 burrowing nematode (*Radopholus* sp.) isolates from Central America, the Caribbean, and Florida, and one isolate from Ivory Coast were compared with RAPD analysis. A high degree of genetic similarity (>0.82) was determined for isolates from the Western Hemisphere. Genome similarity was greatest among isolates collected within a country. Among isolates collected in Central America and the Caribbean, burrowing nematodes from Belize and Guatemala were genetically more distant. However, the genome of the isolate from Ivory Coast was most dissimilar (>0.30). These results suggest that African and American burrowing-nematode isolates may have had different origins or that they have been geographically isolated for a sufficient amount of time to have accumulated genetic changes detectable by RAPD analysis. No relationship was found between the genomic similarity and extent of reproduction or damage to banana or citrus roots. Morphometric analysis involving eight of the isolates indicated that they were morphologically identical and values for morphometric parameters were well within the range previously published for banana and citrus-parasitic burrowing nematodes.

Key words: Banana, biogeography, citrus, genetic diversity, molecular biology, *Musa,* nematode, *Radopholus,* RAPD.

Burrowing nematodes, *Radopholus similis* (Thorne) Cobb, are widely distributed among banana plantations in Central America and the Caribbean (Bridge, 1993; Gowen and Queneherve, 1990; Marin et al., 1998), where infestations are often associated with significant economic losses. However, the extent of crop damage often is influenced by banana cultivar, edaphic factors, and differences in aggressiveness (plant damage and rate of reproduction) of nematode populations (Gowen, 1979; Gowen and Queneherve, 1990; Marin et al., 1989; Pinochet, 1988; Sarah et al., 1993; Stover and Simmonds, 1987). To date, differences in aggressiveness among burrowing nematode populations have not been associated with variation in burrowing nematode morphology or genomic traits (Hahn et al., 1995; Kaplan et al., 1996; Valette et al., 1998), although morphological variation has been associated with differences in host preference and reproductive rates (Tarte et al., 1981). Considerable genome similarity among *R. similis* isolates collected throughout the world has been demonstrated repeatedly in RAPD analyses (Hahn et al., 1996a, 1996b; Kaplan, 1994b; Kaplan et al., 1996; Kaplan and Opperman, 1997; Kaplan et al., 1997; Sarah and Fallas, 1996). This similarity contrasts with that in most plant-parasitic nematode genera, where species and races that differ in host range, geographic distribution, reproduction, or damage can be readily distinguished by RAPD or restriction analysis (Bakker and Gommers, 1994; Blok et al., 1997; Burrows et al., 1996; Caswell-Chen et al., 1992; Ferris et al., 1993; Lopez Brana et al., 1996).

We are interested in gaining an understanding of the genetic basis of parasitism in burrowing nematodes in order to improve crop protection strategies. Understanding the genetic basis of banana parasitism should contribute to development of novel nematode management strategies and to im-

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proved methods for classification and identification of burrowing nematodes. A limited number of burrowing nematode isolates from Central America and the Caribbean have been included in prior genome characterization studies (Kaplan et al., 1996), but findings were not correlated with the relative aggressiveness and reproduction of burrowing nematode isolates on banana.

The objective of this study was to correlate *Radopholus* isolate genomes with aggressiveness and reproduction of the isolates on banana and citrus. Inherent differences in aggressiveness (increase in nematode population size, density, and root damage on banana) were estimated under controlled conditions and subsequently compared using an RAPD analysis.

Materials and Methods

Nematodes: Thirteen burrowing nematode isolates were collected from roots in banana-growing regions in Belize, Guatemala, Honduras, Costa Rica, Dominican Republic, Florida, Puerto Rico, and Ivory Coast. Burrowing nematodes historically have been associated with crop damage in these regions (Pinochet 1988, 1992, 1996). All isolates were cultured on excised carrot disks (O'Bannon and Taylor, 1968) and extracted from culture by enzymatic maceration (Kaplan and Davis, 1990). The collection site, relative rating of burrowing nematode crop damage associated with each site, and acronym for each isolate are listed in Table 1. Isolates were maintained in culture for the duration of the project (2 years). A citrus-parasitic burrowing nematode isolate included in the study was obtained from a stock carrot disk culture that had been maintained for several years.

Morphometrics: Morphometrics for eight of the burrowing nematode isolates were determined with a light microscope (Table 2). Ten females from each nematode isolate were fixed in formalin-acetic acid (FAA) for a minimum of 48 hours. The specimens were then placed on previously ringed microscope slides and measured with a micrometer fitted on a compound light microscope. Measurements of body and stylet length, vulva to tail end, anus to tail end, and greatest body width were determined. Mean values and standard deviations were calculated for each trait, and values were compared with published descriptions for burrowing nematodes (Huettel et al., 1984; Sher, 1968).

Table 1. Collection sites, level of damage associated with nematodes at each collection site, and host from which nematodes were isolated.

Isolate	Collection site	Damage[a]	Host
FL6	Orlando, Florida	NA	Banana
FL7	Avon Park, Florida	++	Citrus
BZ3	Stann Creek, Belize	–	Banana
GT1	Yuma Farm, Guatemala	–	Banana
GT2	Creek Farm, Guatemala	+	Banana
GT3	Lanquin Farm, Guatemala	++	Banana
HN1	Santa Rosa Farm, Honduras	–	Banana
HN2	Coyoles, Honduras	++	Banana
CR1	Balatana Farm, Costa Rica	++	Banana
CR3	PAIS Farm, Costa Rica	++	Banana
PR1	Puerto Rico	++[b]	Banana
DR1	Dominican Republic	NA	Anthurium
IC1	Anguededou, Ivory Coast	++	Banana

[a] Relative rating of damage historically associated with burrowing-nematode infestation: (–) low damage, control measures are rarely needed; (+) intermediate damage, control measures are usually recommended; (++) high damage, uprooting losses are important if control measures are not taken, nematicides are always used; (NA) information not available.

[b] *R. similis* in Puerto Rico more damaging to plantain than to banana.

RAPD analysis: DNA was extracted from approximately 1,000 nematodes of each isolate (Table 1). Nematodes 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 was subsequently resuspended in 100 µl of 1X TE (10mM Tris, 1mM EDTA, pH 8.0). 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.

Seventy random decamer primers

TABLE 2. Simple matching coefficients (SMC)[a] generated by comparison of RAPD profiles from burrowing-nematode isolates from Central America and the Caribbean, Florida, and Ivory Coast.

	Nematode isolate												
	FL6	FL7	BZ3	GT1	GT2	GT3	HN1	HN2	CR1	CR3	PR1	DR1	IC1
L6	1.00												
L7	0.87	1.00											
Z3	0.82	0.80	1.00										
T1	0.82	0.82	0.78	1.00									
T2	0.78	0.76	0.73	0.87	1.00								
T3	0.78	0.75	0.73	0.82	0.82	1.00							
N1	0.84	0.80	0.79	0.82	0.79	0.77	1.00						
N2	0.84	0.82	0.81	0.84	0.82	0.80	0.86	1.00					
R1	0.82	0.80	0.79	0.82	0.80	0.77	0.82	0.86	1.00				
R3	0.78	0.78	0.74	0.80	0.76	0.76	0.78	0.82	0.86	1.00			
R1	0.82	0.78	0.78	0.79	0.75	0.73	0.83	0.85	0.87	0.87	1.00		
R1	0.79	0.80	0.76	0.77	0.74	0.74	0.78	0.80	0.84	0.84	0.87	1.00	
C1	0.65	0.65	0.71	0.68	0.66	0.63	0.70	0.70	0.76	0.75	0.76	0.78	1.00

[a] SMCs among populations were calculated from 298 scorable DNA bands generated with 70 Operon random primers, except for population CR3, where 223 DNA bands were used from 60 Operon random primers.

(OPA01–OPA20, OPB01–OPB02, OPB05–OPB20, OPM01–OPM20, and OPT01–OPT12) (Operon Technologies, Alameda, CA) were used for RAPD analyses (Welsh and McClelland, 1990; Williams et al., 1990). Amplification reactions were performed using 25µl of PCR mix containing 1.0U of Taq DNA polymerase (Promega, Madison, WI, or Boehringer Mannheim, Indianapolis, IN), 10 mM Tris-Cl, pH 8.3, 50mM KCl, 2.0mM $MgCl_2$, 150µM of each dNTP, 32 ng primer, 52.0 µg bovine serum albumin, and 1.0 µl of nematode DNA solution. DNA amplification by PCR was performed in a MJ Thermal Cycler (MJ Research, Watertown, MA); using a preheated block (95 °C) for 45 cycles (95 °C, 1 minute; 35 °C, 1 minute; and 72 °C, 2 minutes). Reaction tubes were subsequently held at 4 °C until retrieval. A 10-µl sample of each reaction was electrophoresed on 1.2% agarose gel in 1X tris-borate buffer (TBE) (pH 8.0) at 90V for 4 hours. Molecular weight markers were a 1-kb lambda DNA ladder (GIBCO BRL, Life Technologies, Gaithersburg, MD). Amplification products were detected with ethidium bromide (1 µg/ml) on a UV transilluminator and photographed with a video system (UVP Image Store 7500, UVP, Upland, CA). Three independent reactions were performed for each nematode isolate-primer combination in each amplification. Two positive controls, using purified *Radopholus* DNA previously extracted by phenol-chloroform, and two negative reactions (no DNA template) were included for each primer tested.

The presence and absence of DNA fragments were scored and used for grouping the nematode isolates. Simple matching coefficients were calculated as described by Digby and Kempton (1987) and used in hierarchical cluster analysis. Cluster analysis, by the unweighted pair-method using arithmetic averages (UPGMA), was performed with the SAHN procedure of NTSYS-PC (version 1.80, Exeter Software, Setauket, NY).

Host range assays: Reproduction for each of the burrowing nematode isolates (Table 1) except for isolate GT1 were determined on banana and rough lemon, *Citrus limon* (L.) Raf., using host-index assay systems described previously by Marin et al., (1999) for banana and by Kaplan (1994a) for citrus. Total number of burrowing nematodes per plant and nematode density per gram of root were determined 8 weeks after inoculation. The relative aggressiveness of each nematode isolate was further estimated by comparing root necrosis on *Musa* AAA cv. Grande Naine.

To determine the relationship of the molecular data with the damage and reproduc-

tion data for banana, a nested analysis of variance was performed with clusters and isolates within clusters. Clusters were defined based on molecular data. Nematode reproduction data and root necrosis were transformed with $\log_{10}$ (x + 1) and square root of (x), respectively, prior to analyses of variance. Statistical analyses were performed with SAS (SAS Institute, Cary, NC).

Results

A total of 298 bands were consistently amplified for 57 of the 70 primers included in the RAPD analysis. A high degree of similarity among the burrowing nematode isolates from Central America, Caribbean, Florida, and Ivory Coast was apparent when RAPD profiles were compared visually and when simple matching coefficients were calculated from the data set (Table 2). The simple-matching-coefficient matrix used to generate a molecular similarity dendrogram was based on a hierarchical cluster analysis (Fig. 1). The greatest similarity was observed among isolates collected from the same geographic areas. The primary clusters observed were FL6 and FL7, HN1 and HN2, CR1 and CR3, PR and DR, and GT1 and GT2, with all isolates from the Western Hemisphere being ≥0.78 similar. The isolate from Ivory Coast was less similar to all other isolates but was still highly similar to them (0.70) (Table 2, Fig. 1). Morphometrics for eight of the isolates included in this study indicated that their appearance was identical (Table 3).

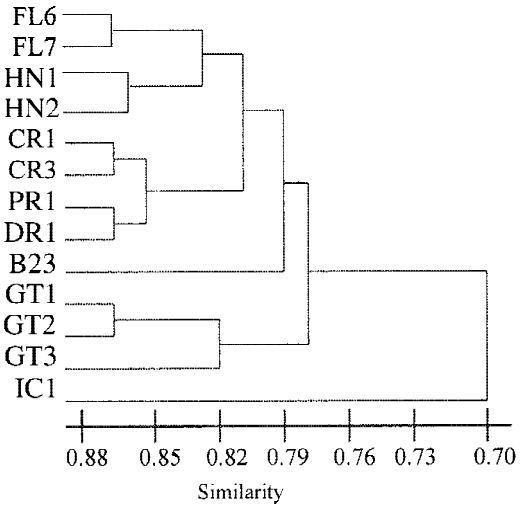

Fig. 1. Molecular similarity tree of different burrowing nematode isolates determined by hierarchical cluster analysis based on the unweighted pair-group method using arithmetic averages.

Total size of nematode populations in roots of each test plant, population densities, and root damage varied between burrowing nematode isolates (Table 4). However, the only burrowing nematode isolate that reproduced in citrus was FL7, which was originally isolated from citrus in Florida (Table 4).

Nested ANOVA was performed with three separate clusters or groups of isolates that were delimited on the basis of the dendrogram generated from the RAPD analysis (Fig. 1). Group 1 included the populations FL6, FL7, HN1, and HN2; cluster 2 contained CR1, CR3, PR1, and DR1; and cluster 3 included the three isolates from Guatemala (GT1, GT2, GT3). Isolates from Belize (BZ) and Ivory Coast (IC) were not included in the analysis since they were relatively more distant. The sources of variation evaluated were ''among clusters'' and ''isolates within a cluster'' for the total number of nematodes per plant, nematode densities in root tissue, and root necrosis on banana (Table 5). There were no differences among clusters when they were compared for reproduction on banana ($P = 0.67$), population densities between isolates ($P = 0.70$), and root necrosis ($P = 0.85$), whereas, isolates within clusters were significant for all variables ($P < 0.001$).

Discussion

Burrowing nematodes isolated from North and Central America and from Caribbean islands appear to share a high level of genome conservation (similarity indexes were >0.80 among nematode isolates). In contrast, the burrowing nematode isolate from Ivory Coast was more distant (30% dissimilar to other isolates). These levels of genome similarity are in agreement with previously reported RAPD analyses involving

TABLE 3. Morphological measurements (mean ± standard deviation) of burrowing nematode females from Florida, Central America, and the Caribbean.[a]

Isolate	Body length (µm)	Vulva-tail distance (µm)	Anus-tail distance (µm)	Greatest body width (µm)	Stylet length (µm)	Vulva position (%)
CR1	619.2 ± 32.9	257.2 ± 17.9	67.0 ± 4.7	27.2 ± 1.0	20.0 ± 0.9	58.4 ± 2.4
CR3	611.2 ± 20.2	270.5 ± 21.4	67.0 ± 3.9	23.4 ± 1.3	20.2 ± 1.5	55.7 ± 3.4
DR1	660.0 ± 38.0	275.2 ± 30.5	68.6 ± 4.4	22.2 ± 1.8	19.8 ± 1.1	58.4 ± 2.8
FL7	694.1 ± 25.5	296.6 ± 20.7	72.6 ± 7.2	23.6 ± 1.6	20.8 ± 1.0	57.3 ± 1.6
FL8	680.7 ± 25.7	289.2 ± 15.4	70.7 ± 4.9	22.0 ± 1.6	19.4 ± 1.3	57.5 ± 1.6
PR1	651.3 ± 30.1	274.5 ± 21.7	72.6 ± 7.2	23.6 ± 1.6	20.6 ± 1.0	57.9 ± 2.2
HN1	590.5 ± 63.5	262.5 ± 45.4	71.3 ± 4.8	23.0 ± 1.9	19.4 ± 1.0	55.8 ± 3.8
HN2	642.6 ± 44.6	285.2 ± 30.9	67.4 ± 3.9	25.4 ± 2.7	19.0 ± 1.1	55.7 ± 2.5
R. similis[b]	520–880	–	–	–	17–20	55–61
R. citrophilus[c]	600–764.1	242.2–409.4	62.4–80.8	21.6–30.4	18–20	46–58

[a] Values are means of 10 females per isolate.
[b] Data from Sher (1968)
[c] Data from Huettel et al. (1984).

burrowing nematodes collected from different areas of the world (Fallas et al., 1996; Hahn et al., 1994, 1996a; Kaplan, 1994b; Kaplan et al., 1996; Kaplan and Opperman, 1997). Further evidence of a high level of genome conservation among burrowing nematode isolates was obtained through comparison of rDNA sequence, isozyme patterns, and restriction analysis of sequence tag sites (Fallas et al., 1996; Kaplan, 1994b; Kaplan and Opperman, 1997; Kaplan et al., 1997).

The basis for the apparently highly conserved nature of the *Radopholus* genome has not been elucidated (Kaplan, 1994b) although Kaplan et al. (1996) suggested that it might be related to reproductive mode, dissemination through planting material, wide host range, and migratory parasitic habit of burrowing nematodes. Hahn et al. (1996a) also inferred that genome conservation is probably due to dissemination of nematodes by vegetative propagation of crops but suggested that genetically divergent populations might also occur in the same geographical area (Hahn et al., 1994). However, burrowing nematodes morphologically similar to *R. similis* but with distinct genomes (detectable by RAPD analysis) have not been widely reported. In contrast, morphologically distinct burrowing nematodes (other *Radopholus* spp.) have RAPD profiles that are much different from that of *R. similis* (Hahn et al., 1994; D. Kaplan, unpubl. data). Valette et al. (1998) concluded on the basis of an ultrastructural study that morphological features reported to be specific to citrus-parasitic burrowing nematodes were present among banana-parasitic burrowing nematodes in Africa, and proposed that *R. citrophilus* be synonymized with *R. similis*.

Results of our RAPD analysis suggest that variation in the burrowing nematode genome was more restricted within geographic regions. However, isolates from Belize and Guatemala were more distant than the other populations from Central America and

TABLE 4. Reproduction of 13 burrowing-nematode isolates in rough lemon and banana.

Nematode isolate	Reproduction on rough lemon[a]	Population levels on bananas[b]
GT3	– (0)	3,835
CR1	– (4)	3,672
FL6	– (3)	3,326
HN1	– (3)	2,914
GT2	– (5)	2,709
HN2	– (1)	2,067
IC1	– (2)	1,567
CR3	– (4)	1,051
PR1	– (3)	503
BZ3	– (1)	447
GT1	NT[c]	286
FL7	+ (10)	75
DR1	– (1)	24

[a] + = $P_f > 30$ nematodes/plant; – = $P_f < 30$ nematodes/plant (Kaplan, 1994a). Numbers in parentheses represent the number of plants in which burrowing nematodes were detected.
[b] Nematodes per plant 8 weeks after inoculation.
[c] NT: not tested.

TABLE 5. F-values and probabilities (P) for nested analyses of variance[a] conducted with reproductive fitness, nematode density, and total-root necrosis caused by burrowing-nematode isolates on bananas (*Musa* AAA, cv. Grande Naine).

Source of variation	Reproduction		Nematode density		Total-root necrosis	
	F value	P > F	F value	P > F	F value	P > F
Cluster	0.42	0.668	0.37	0.703	0.17	0.847
Isolate (cluster)[b]	59.00	<0.001	72.44	<0.001	37.63	<0.001

[a] Three clusters were considered for analyses based on grouping obtained with hierarchical cluster analysis of molecular data. Cluster 1 included populations FL6, FL7, HN1, and HN2; cluster 2 included CR1, CR3, PR1, and DR1; and cluster 3 included GT1, GT2, and GT3. Populations BZ3 and IC1 were not used.

[b] Isolate (cluster) = nematode isolates within a cluster.

Florida. The reason for this separation is unclear, but these subtle differences may reflect independent introductions of burrowing nematodes into these areas. Burrowing nematodes probably have been present in Central America and Florida for 200–300 years; therefore, significant shifts in the genome due to geographic isolation of these nematode populations appear unlikely (Marin et al., 1998). Although geographic distribution has been associated with genome variation among burrowing nematodes (Hahn et al., 1996a) high levels of genome similarity also have been reported (Fallas et al., 1996; Hahn et al., 1996a; Kaplan, 1994b; Kaplan et al., 1996; Kaplan and Opperman, 1997; Kaplan et al., 1997; Sarah and Fallas, 1996). The relationship between apparent molecular similarity and geographic origin may be related to concurrent dissemination of bananas and burrowing nematodes via long-distance dispersal of infested corms for establishing commercial banana nurseries (Fallas et al., 1996; Marin et al., 1995; Sarah and Fallas, 1996).

Results from a prior RAPD analysis and comparison of phospho-glucose isomerase isoforms for a limited number of burrowing nematode isolates collected in Africa, Australia, and Guadeloupe (Fallas et al., 1996) suggested that *R. similis* populations may be placed in two different genetic groups. Fallas et al. (1996) and Sarah and Fallas (1996) found that the cluster including the Ivory Coast population had a similarity index of 0.70 to a dendrogram cluster of OTUs that included a Costa Rican population. Kaplan and Opperman (1997) suggested that a sequence tag site (DK#1) could not be amplified from African isolates. We estimated that the genome similarity of the same Ivory Coast isolate studied by Sarah and Fallas (1996) with several Central American and Caribbean isolates was 0.70, whereas the Central American, Caribbean, and Florida isolates were more similar to one another ($\geq$0.80). These similarities suggest that the isolates from the Western Hemisphere are genomically similar to isolates from Australia, Nigeria, and Cameroon (Fallas et al., 1996).

Sarah and Fallas (1996) concluded that genomic differences between two *R. similis* isolates collected from Martinique and Guadeloupe reflected two independent introductions of burrowing nematodes. Nematode populations from Guadeloupe were probably introduced from West Africa (Bridge, 1993), which would explain their close relatedness to the Ivory Coast isolate. The introduction of *R. similis* to Martinique was probably associated with introduction of the banana cultivar Gros Michel (Marin et al., 1998). Gros Michel was introduced to Martinique in the early 19th century and then was taken to Jamaica in 1835 (Reynolds, 1927; Simmonds, 1959). Thus, it is likely that burrowing nematodes were introduced at two independent times (Sarah and Fallas, 1996), which may explain why the isolate from Guadeloupe is more closely related to the Ivory Coast isolate than to the isolate from Martinique.

Results of the host range assays substantiated previous reports that only burrowing nematode isolates collected in Florida from

infested citrus groves parasitized citrus (Kaplan, 1994b; Kaplan and Opperman, 1997; Kaplan et al., 1997). These results also agree with the findings of DuCharme and Birchfield (1956), who determined that populations from the ''banana race'' of *R. similis* did not attack citrus.

The high level of molecular and morphological similarity among burrowing nematodes suggests that banana- and citrus-parasitic forms shared a common origin. Citrus parasitism probably evolved under very specific conditions in Florida, and citrus-parasitic burrowing nematodes were probably a recent radiation of banana-parasitic *R. similis* (Kaplan et al., 1996).

In this study, differences in nematode reproduction on *Musa* AAA cv. Grande Naine and citrus were detected but were not associated with differences in genome organization as estimated by RAPD analysis or nested ANOVA, or with morphometric differences as previously reported (Tarte et al., 1981). Clusters were comprised of isolates that had both high and low levels of reproduction on Grande Naine. Similar results were obtained by Fallas et al. (1996). Likewise, Kaplan and Opperman (1997) and Kaplan et al. (1997) could not discriminate between borrowing nematodes that differed with respect to citrus parasitism or with differences in nematode aggressiveness toward citrus.

Variation in reproduction and host range represent economically important gains of function, the genetic and physiological basis of which requires elucidation. However, repeated failure to detect and correlate polymorphic DNA with these traits suggests that a very small portion of the nematode genome confers them. Hypothetically, polymorphic DNA should be associated with parasitism or specific morphological traits (Hahn et al., 1994; Kaplan et al., 1996), but thus far none have been identified (Kaplan and Opperman, 1997; Kaplan et al., 1997). If shifts in host-range or increased aggressiveness can be correlated with polymorphisms, the high degree on genome conservation observed in *Radopholus* may actually facilitate the characterization of genetic loci that confer these traits (Kaplan et al., 1996).

Since genetic markers associated with host range and aggressiveness remain elusive, current selection of nematode isolates used in cultivar development must rely strongly on knowledge of biological variation with respect to nematode potential to damage banana. The global nature of the crop and parasite warrants use of a standardized collection of isolates that represent the range of aggressiveness by programs devoted to improvement of bananas.

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