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Reproductive Strategies and Karyotype of the Burrowing Nematode, *Radopholus similis*

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Abstract: Karyotype, gametogenesis, and gonad morphology were characterized for 56 *Radopholus* spp. isolates collected from Africa, Australia, Central America, Cuba, Dominican Republic, Guadeloupe, Puerto Rico, North America (Florida), and Hawaii. Seven of the isolates, all collected from Florida, were citrus-parasitic. The haploid karyotype for all isolates was $n = 5$, and gonad organization was similar for each. Furthermore, reproduction did not involve parthenogenesis. Initially, spermatids were produced in young adult females and accumulated in the spermatheca prior to differentiation to sperm. At the cessation of spermatogenesis, oogenesis began and continued for the remainder of the nematode's life. Oocytes first entered a mitotic phase, then a transition zone, and remained in pachytene until they reached the proximal end of the ovary. Thus, *Radopholus* can reproduce as a hermaphrodite when amphigony does not occur. The gonad is actually an ovatestis.

Key words: banana, *Caenorhabditis elegans,* citrus, egg, evolution, genetics, hermaphrodite, karyotype, nematode, oogenesis, ovary, ovatestis, parthenogenesis, polar body, quarantine, *Radopholus,* sperm.

Citrus-parasitic burrowing nematodes were reported to have a haploid karyotype of $n = 5$, whereas non-citrus-parasitic burrowing nematodes had a haploid karyotype of $n = 4$ (Huettel and Dickson, 1981). However, non-citrus-parasitic burrowing nematode isolates collected from Hawaii, Puerto Rico, Ivory Coast, and Sri Lanka were more recently also reported to have the haploid karyotype, $n = 5$ (Hahn et al., 1996; Huettel et al., 1984; Rivas and Roman, 1985).

Previously, we determined that reproductively viable hybrid progeny were produced when non-citrus-parasitic burrowing nematode females were mated with citrus-parasitic burrowing nematode males; parents and progeny were morphologically similar to *Radopholus similis* (Kaplan et al., 1997). Progeny were demonstrated to be hybrids because they inherited both the ability to parasitize citrus and the DK#1 marker from the paternal line. We wished to determine the karyotype of the reproductively viable hybrid progeny and the parent lines.

Burrowing nematode karyotype and gametogenesis were estimated previously using propionic orcein to stain chromosomes in isolated nematode ovaries, eggs, and polar bodies (Hahn et al., 1996; Rivas and Roman, 1985). However, we found that these experimental methods yielded results that were difficult to interpret. Staining polar bodies with fluorescent nucleic acid-specific stains such as DAPI and Hoechst 33258 has been used routinely to determine karyotype in a wide array of organisms including nematodes (Rouppe van der Voort et al., 1996). We used fluorescent nucleic acid stains to determine the karyotype of the burrowing nematode parental lines and progeny. We also studied gametogenesis in situ, using nematodes present in egg suspensions. Herein, we report the results of our observations on karyotype and gametogenesis.

Materials and Methods

The karyotypes of parental lines, hybrid progeny, and 53 other burrowing nematode isolates (Table 1) were determined according to the following protocol. Nematodes and eggs were extracted by enzymatic maceration from carrot disk cultures (Kaplan and Davis, 1990) and centrifuged at 13,000*g*

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TABLE 1. Collection sites, reproduction on rough lemon (*Citrus limon* (L.) Raf.), and karyotype for *Radopholus similis* isolates.

Nematode isolate	Collection site	Rough lemon[a]	Karyotype[b]
AS1	Tully, North Queensland, Australia	–	5
AS2	Tully, Queensland, Australia	–	5
AS4	Pimpama, Queensland, Australia	–	5
BZ1	Bladen Bridge, Toledo, Belize	–	5
BZ2	Big Creek, Belize	–	5
BZ3	Stann Creek, Belize	–	5
CM1	Cameroon	–	5
CM2	Island 5, Sanga River, Cameroon	–	5
CR1	Coyles, Costa Rica	–	5
CR2	Guanacoste, Costa Rica	–	5
CR3	West Reventazon River, Costa Rica	–	5
CR4	Pococi, Costa Rica	–	5
CR5	Sixaola, Costa Rica	–	5
CR6	Sixaola, Costa Rica	–	5
CR7	unknown, Costa Rica	–	5
CR8	Coyoles, Costa Rica	–	5
CU1	Villa Clara, Cuba	–	5
DR1	Hato Viejo, Dominican Republic	–	5
FL1	Lake Wales, Florida	+	5
FL10	Frostproof, Florida	+	5
FL11	Frostproof, Florida	+	5
FL2	Orlando, Florida	+	5
FL3	Clermont, Florida	+	5
FL4	Lake Alfred, Florida	+	5
FL5	Orlando, Florida	–	5
FL7	Avon Park, Florida	+	5
FL8	Frostproof, Florida	+	5
FL9	Frostproof, Florida	+	5
GD1	Neuf Chateau, Guadeloupe	–	5
GN1	Balikoure, Guinea	–	5
GT1	Yuma Farm, Guatemala	–	5
GT2	Creek Farm, Guatemala	–	5
GT3	Lanquin Farm, Guatemala	–	5
HI1	Panaewa, Hawaii	–	5
HI10	Hilo, Hawaii	–	5
HI11	Pelekunu Preserve, Molokai, Hawaii	–	5
HI12	Keeau, Hawaii	–	5
HI13	Hilo, Hawaii	–	5
HI14	Watts Panawea (Hawaii)	–	5
HI2	Pahoa, Hawaii	–	5
HI3	Panaewa, Hawaii	–	5
HI4	Keeau, Hawaii	–	5
HI5	Pahoa, Hawaii	–	5
HI6	Keeau, Hawaii	–	5
HI7	Waimanalo, Oahu, Hawaii	–	5
HI9	Punaluu, Oahu, Hawaii	–	5
HN1	Sula Valley, Honduras	–	5
HN2	Coyoles, Honduras	–	5
IC1	Ivory Coast	–	5
NI1	IITA, Onne Station, Nigeria	–	5
PR1	Puerto Rico	–	5
PR2	Puerto Rico	–	5
SA1	Hectorspruit, Mpumalanga, South Africa	–	5
SA2	Hazyview, Mpumalanga, South Africa	–	5
UG1	IITA, Namulonge Station, Uganda	–	5

[a] + : Median values of 100 to 1,200 nematodes/plant *R. similis* detected in >95% of test plants; –: Median values of 0 to 30 nematodes nematodes/plant *R. similis* detected in less than 5% of the test plants. NT: not tested (Kaplan, 1994a; Kaplan and Opperman, 1997; Kaplan et al., 1997; Kaplan et al., 2000). [b]Karyotype determined for 10 eggs of each *R. similis* isolate.

for 30 seconds to pelletize nematodes and eggs. After the supernatant was removed, the pellet was incubated in 200 µl of Carnoy's solution (ethanol: acetic acid: chloroform; 6:3:1, v/v/v) for 5 minutes. After removal of the fixative, the pellet was incubated in 100% methanol for 20 minutes at room temperature. The nematodes and eggs were rinsed twice with phosphate-buffered saline (PBS) for 5 minutes. Nematodes and eggs were then incubated in a buffer (0.15M Nacl, 0.03M KCl, 0.01M KH_2PO_4, pH 7.0) for 10 minutes to reduce RNA staining and then rinsed sequentially in PBS and distilled water. Specimens were stained with Hoechst 33258 (2′-[4-hydroxyphenyl]-5-[4-methyl-1-peperazinyl]-2, 5′-bi-1H-benzimidazole) (Sigma Chemical Company, St. Louis, MO) or DAPI (4′, 6-diamidino-2-phenylindole) stain (Sigma Chemical Company, St. Louis, MO) (1 µg/ml) for 5 minutes. Specimens were rinsed five times with water to remove excess stain and transferred in 20 µl of water to acid-washed microscope slides. The water on the slide was allowed to evaporate until only a few microliters of water covered the nematodes and eggs. Specimens were then covered with 2–3 µl of 2% n-propyl gallate in 80% glycerol and 22 × 22-mm number 0 cover glass. Polar bodies, ovaries, and testes were viewed with a video-enhanced Nikon fluorescent microscope (×620 or ×1,550) or a Zeiss light photo-microscope (×400 or ×1,000). Digital images were captured with a Snappy Video Snapshot (Play, Rancho, Cordova, CA) or directly on 35-mm film.

Results

Staining burrowing nematode eggs with DAPI or Hoechst 33258 enabled us to count chromosomes in polar bodies without difficulty. All of the 55 burrowing nematode isolates in our collection and the hybrid progeny had the haploid karyotype, $n = 5$ (Table 1, Fig. 1).

During determination of karyotype, we also observed strong fluorescence in the spermatheca of all adult females that had been stained with DAPI or Hoechst 33258. We examined specimens that had not been stained to ensure that we were not observing auto-fluorescence. Using video-enhanced Nomarski light microscopy, we verified that rod-shaped structures were present in all spermathecas. These structures were identical to spermatids observed in the male testis (Fig. 2).

The female reproductive system appeared to be didelphic ovatestes with outstretched arms that were often recurved in mature nematodes. A cap cell and three somatic cells were present at the tip of each arm. Proximal to these cells, a series of nuclei appeared to be present in a syncytium. A transition occurred where the nucleic acid became less condensed and cells appeared to form with cytoplasm surrounding each nucleus. Each arm terminated proximally at a spermatheca that joined the uterus. The uterus narrowed in the proximity of the vagina, suggesting the presence of two independent uteri.

Gametogenesis in young females differed from that observed in mature females. Sper-

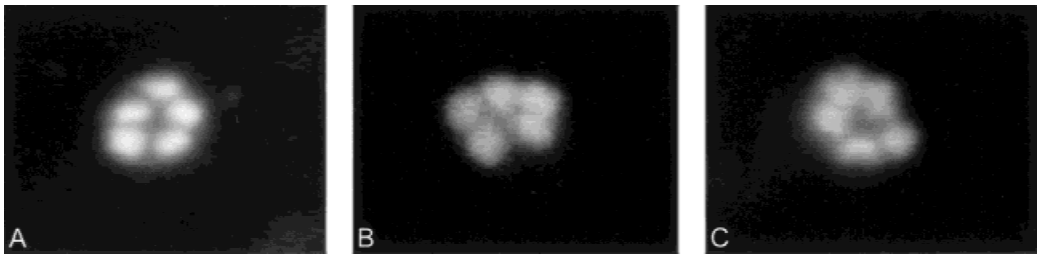

Fig. 1. Karyotype of hybrid progeny and the two parental lines from a controlled mating of a citrus-parasitic male with a non-citrus-parasitic female of *Radopholus similis*. A) Citrus-parasitic male line (FL1). B) Non-citrus-parasitic female line (FL6). C) Hybrid progeny.

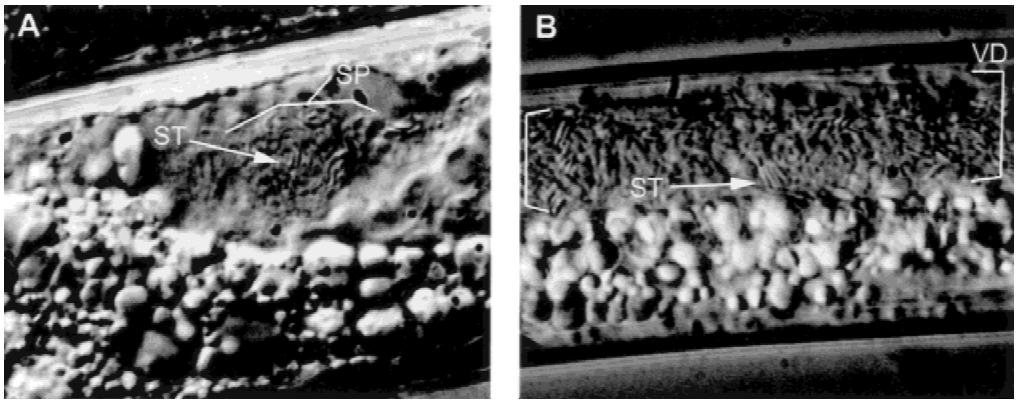

FIG. 2. Rod-shaped spermatids in *Radopholus similis*. A) Rod-shaped spermatids in spermatheca. B) Rod-shaped spermatids in the male testis. SP = spermatheca, ST = spermatid, VD = vas deferens.

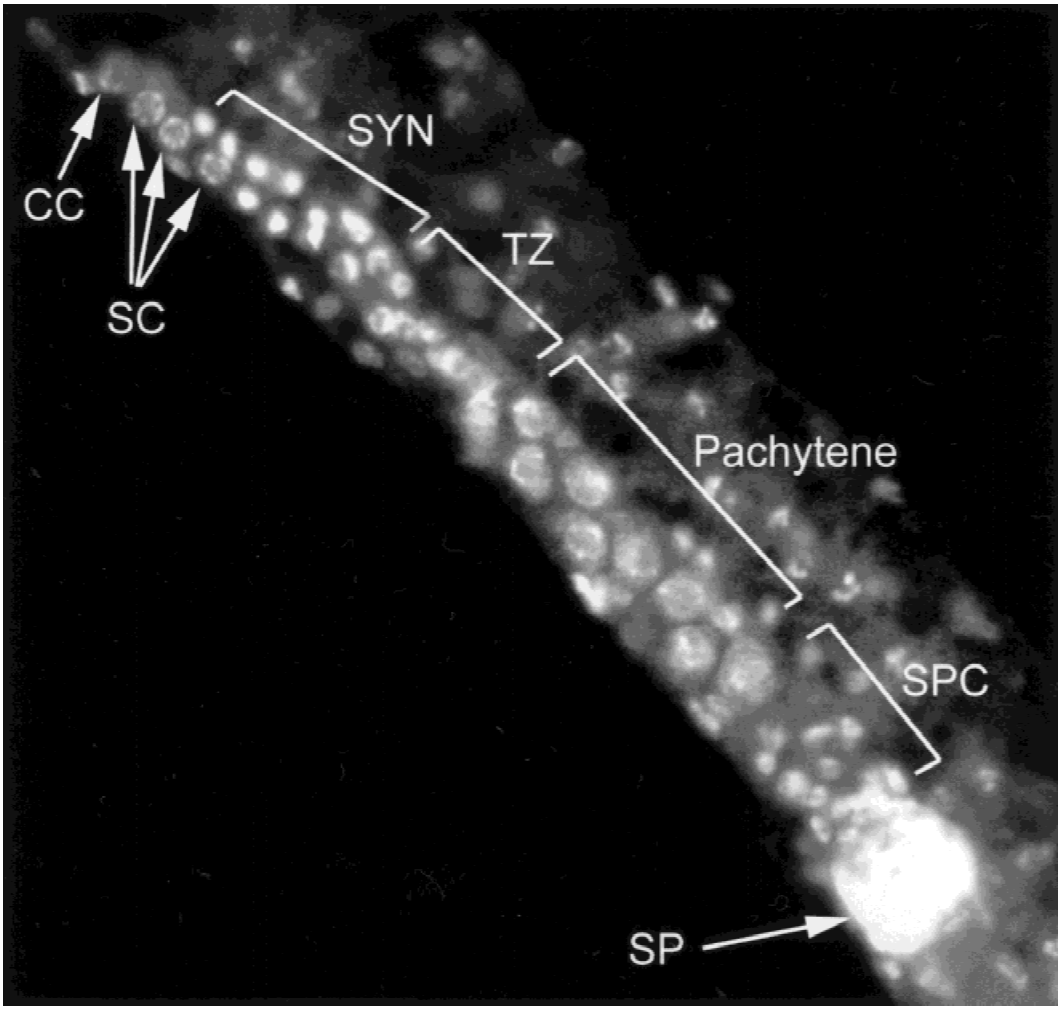

FIG. 3. Ovatestis in young female hermaphrodite of *Radopholus similis*. Gametocytes located in the proximal end are reduced in size, having undergone meiosis, and accumulate in the spermatheca; they produce a strong fluorescent signal. SP = spermatheca, CC = cap cell, SC = somatic cells, SPC = spermatocytes, SYN = syncytium, TZ = transition zone.

matogenesis occurred in young females (Fig. 3). Mitosis occurred in the syncytial area of the distal portion of the ovatestis proximal to cells that advanced through meiosis I. A number of smaller cells (spermatocytes) were observed at the proximal end of the ovatestis. These cells entered the spermatheca and appeared to become rod-shaped spermatids. A second meiotic division required to complete spermatogenesis likely occurred within the spermatheca or uterus at a later time.

Oogenesis occurred after spermatogenesis. The distal cap cell and two or three germ cells that likely functioned as stem cells at the terminus of each ovatestis were visible. More proximally, the chromatin of the next 10 to 15 cells was highly condensed. Ova then enlarged, cytoplasm was apparent, and nuclei entered prophase, but development appeared to become arrested when each cell reached pachytene. Meiosis resumed when ova reached the spermatheca (Fig. 4).

In the mature ovatestis, ova enlarged greatly and developed into a single row of 5 to 7 cells, and their nuclei became well-defined. Each ovum gradually increased in size as it approached the spermatheca. Sperm apparently penetrated the ova in the spermatheca or uterus. Polar bodies could be seen in eggs in the uterus. For purposes of karyotyping, chromosomes were easier to resolve after eggs had been laid.

Discussion

Results of prior mating studies in which reproductively viable hybrid progeny were produced when citrus-parasitic and non-citrus-parasitic parent lines were mated suggested that the two parent lines had the same karyotype (Kaplan et al., 1997). Using

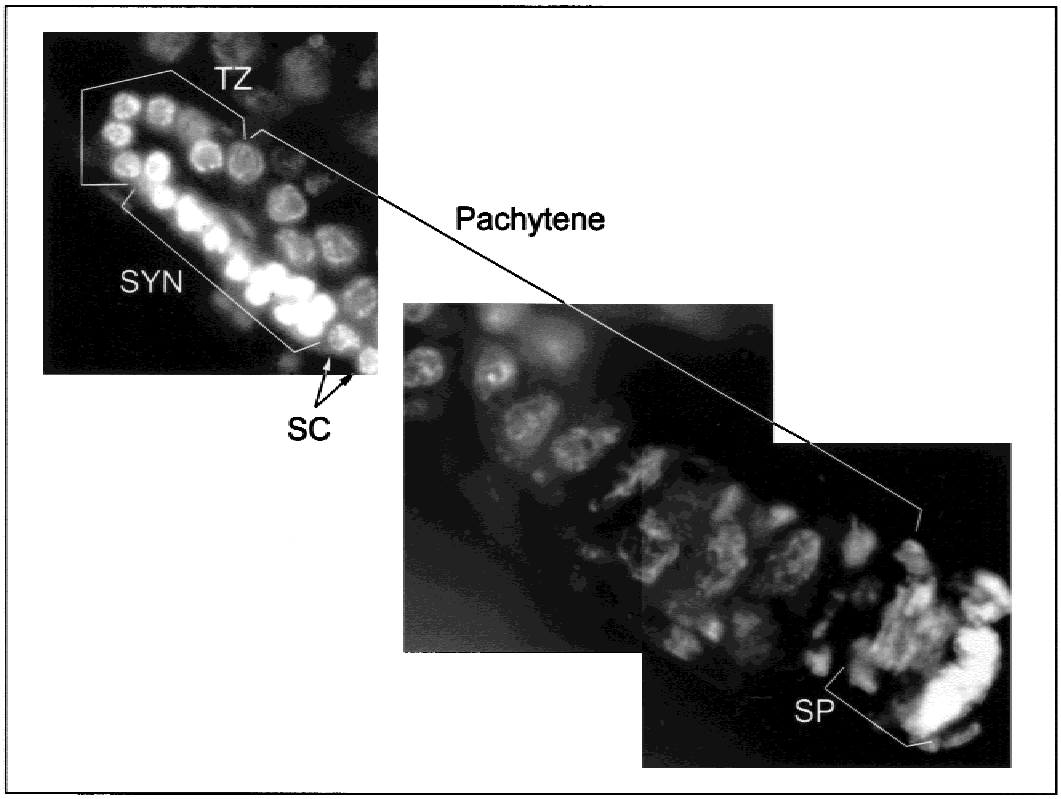

Fig. 4. Ovatestis in mature female hermaphrodite of *Radopholus similis*. Oocytes enter meiosis after leaving the transition zone following an initial round of mitosis, but remain in pachytene until they reach the spermatheca. SP = spermatheca, SC = somatic cells, SYN = syncytium, TZ = transition zone.

fluorescent nucleic acid stains, we have substantiated prior claims that karyotype in *Radopholus* could be determined more reliably from polar bodies or eggs (Hahn et al., 1996). Furthermore, our results indicated that putative differences in karyotype that had been reported to exist between citrus-parasitic and non-citrus-parasitic burrowing nematodes could not be supported (Huettel and Dickson, 1981).

Differences in karyotype would have been difficult to rationalize, given the apparent high degree of genome similarity among all burrowing nematodes that resemble *R. similis* (Kaplan et al., 1996). The *R. similis* genome was reported to be highly conserved (Fallas et al. 1996; Hahn et al., 1994; Kaplan, 1994b; Kaplan et al., 1996, 1997; Kaplan and Opperman, 1997; Marin et al., 1999). Significantly, the nucleic acid sequences of the rDNA ITS1 and the D2/D3 expansion segment of the 28S rDNA repeat, respectively, also are identical for all 58 burrowing nematode isolates (Kaplan et al., 2000). This lack of variation probably is due to the fact that burrowing nematodes have been dispersed widely from a single origin within the roots of crops during relatively recent times (Marin et al., 1998; O'Bannon, 1977).

Individual burrowing nematode isolates may differ from others based on their relative effects on crops (host range, relative aggressiveness), but the genetic basis for differences have not been identified (Fallas et al., 1996; Hahn et al., 1994; Kaplan, 1994b; Kaplan et al., 1996, 1997; Kaplan and Opperman, 1997. Fallas et al. (1996) began to address this issue by analyzing two groups of *R. similis* differing in their aggressiveness on banana; however, these groups could not be distinguished by RAPD or isozyme analyses. Analysis of the two internal transcribed spacer regions ITS1 and ITS2 and the 5.8S rDNA repeat by RFLP suggested that all isolates were *R. similis* (Fallas et al., 1996). We determined that three isolates included in that study (courtesy of J. L. Sarah) each had a haploid karyotype of $n = 5$. Further analyses of these and other isolates that vary with respect to aggressiveness may provide insight into the genetic basis of parasitism in *R. similis*.

Based on our results, we believe that *R. similis* is a syngonic hermaphrodite. Self-fertilization takes place ca. 50 to 60 days after the fourth molt in females that have not mated with a male. Thus, we propose that hermaphroditism is an alternative reproductive strategy for *Radopholus*. Evidence in support of this hypothesis includes the following observations. Strong fluorescence was detected for all spermathecas when stained with DAPI. This fluoresence was associated with the presence of rod-shaped spermatids in the spermatheca. These structures were considered to be spermatids because they resembled spermatids in the male testis and because we observed that when sperm were released from the vas deferens, they were spherical (amoeboid) (D. T. Kaplan, unpubl.). In addition, round fluorescent bodies were observed in the uterus of mated females. Thus, the rod-shaped structures in the spermatheca did not resemble sperm and did not result from mating; they were spermatids produced by the ovatestis of the hermaphroditic female.

Previous interpretations that led to the description of *R. similis* as a parthenogen were clearly inaccurate. Cobb (1915), Sher (1968), and Thorne (1949) all concluded that rod-shaped structures in the spermatheca were sperm based on the assumption that *Radopholus* was an obligate amphigonic species. Further, rod-shaped or elongated spermatids have been reported for some nematodes, but rod-shaped sperm are not known in the Nematoda. Prior studies misinterpreted the *Radopholus* reproductive system and gametogenesis. One report claimed to illustrate oogenesis but appears to have described spermatogenesis (Huettel and Dickson, 1981).

We believe that inconsistent results obtained by various investigators who attempted to establish colonies from individual burrowing nematode juveniles demonstrate an inherent delay in population growth when self-fertilization occurs. It has been known for some time that individual juveniles could establish colonies, but to

achieve success experiments had to be maintained more than 80 days. This period of time far exceeds the life cycle for burrowing nematodes that reproduce via amphigony (DuCharme and Price, 1966; Kaplan and Davis, 1990). Brooks and Perry (1962) first demonstrated that individual juveniles could produce colonies in experiments that were maintained for long periods of time and proposed that burrowing nematodes might reproduce via parthenogenesis. Loos (1962) and Rivas and Roman (1985) reported that, for burrowing nematode isolates from banana and plantain, they were unable to establish colonies from single juveniles over a 60-day period and concluded that *R. similis* reproduced solely by amphigony. Rivas and Roman (1985) subsequently conducted experiments where individuals or pairs of second-(J2) or third-stage juveniles (J3) (presumably of opposite sex) were transferred to surface-sterilized carrot disks and detected males, females, and juveniles 60 days after inoculation more frequently in the later treatment. Huettel and Dickson (1981) demonstrated that population development from individual juveniles required >93 days at 25 °C. Recently, we determined that 110 to 120 days was required for small populations of *R. similis* to emerge from carrot disks after being inoculated with individual J3 or J4 (D. T. Kaplan, unpubl. data). These findings suggested that individual nematodes that have not mated could reproduce, but there appeared to be a significant increase in the amount of time required to do so.

Hermaphroditism appears to afford the burrowing nematode with a reproductive strategy that enables individual nematodes to produce progeny without males. Ability to reproduce in the absence of males has likely contributed to the survival of burrowing nematodes in sites that had been extensively treated to eradicate the nematode (Brooks and Perry, 1962). Further, this ability could also play a role in the dispersal of *R. similis* from its point of origin.

The reproductive system in the *R. similis* female hermaphrodite is similar to that described for *C. elegans* (Hirsh et al., 1976; Schedl, 1997). The cap cell and three contiguous somatic cells are present in the distal portion of the ovatestis for both nematodes. Following these cells is a syncytial region containing nuclei in the *R. similis* ovatestis similar to that seen in the distal portion of the *C. elegans* ovatestis. Next is a transition zone where the chromatin condenses into discrete chromosomes, and mitosis is completed, and meiosis is initiated. This series of events typically is found at the reflex in the mature *C. elegans* ovatestis, but in *R. similis* the ovary is generally outstretched. We did not determine if the bend observed in the ovatestis of older *R. similis* routinely coincided with the transition zone; however, this was the case for the nematode used for Fig. 4. Both *C. elegans* and mature *R. similis* have a single row of oocytes that progressively increase in size as they approach the proximal end. These oocytes remain in late pachytene until they reach the spermatheca, where they become fertilized.

Several other *Radopholus* spp. may be hermaphrodites. These species, in contrast to *R. similis,* are not widespread, probably because they were not associated with banana or other crops that are vegetatively propagated and widely disseminated. Sher (1968) was unable to explain the presence of ''spermatized'' females (females containing sperm in the spermatheca) in the apparent absence of males for *R. inaequalis, R. magniglans, R. trilineatus,* and *R. rotundisemenus.* To date, males have not been reported for these species. All of these species, except *R. rotundisemenus,* were also described as having rod-shaped sperm in their spermathecas.

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