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Evidence of direct polyploidization in the mitotic parthenogenetic *Meloidogyne microcephala*, through doubling of its somatic chromosome number

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Summary - Propagation of twenty exceptionally large second-stage juveniles isolated from a diploid (2n=37) population of *Meloidogyne microcephala* resulted in the establishment of a tetraploid population with 2n=74 chromosomes. Both populations reproduced exclusively by mitotic parthenogenesis. Morphological studies demonstrated that the tetraploid population was indeed a population of *M. microcephala*, very similar to the diploid population from which it had derived. Morphological and anatomical differences involved only larger body and organ dimensions of the various life stages of the tetraploid population, and certain developmental abnormalities observed primarily in tetraploid males. It was concluded that the tetraploid population represents the first observed case of direct polyploidization of a mitotic parthenogenetic nematode, through doubling of its chromosomes. Other pathways of polyploidization in *Meloidogyne* are discussed briefly.

Résumé - ***Mise en évidence de polyploïdisation directe par doublement du nombre de chromosomes somatiques chez* Meloidogyne microcephala*, espèce parthénogénétique mitotique*** - La multiplication de vingt juvéniles de taille exceptionnelle provenant d'une population diploïde de *Meloidogyne microcephala* (2n=37) a produit une population tétraploïde (2n=74). Les deux populations se sont reproduites par parthénogenèse mitotique exclusive. Une étude morphologique a montré que cette population tétraploïde constitue bien une population de *M. microcephala*, très semblable à la population diploïde dont elle provenait. Les différences morphologiques et anatomiques consistent en une plus grande taille du corps et des organes à certains stades de la population tétraploïde ; quelques anomalies ont été observées surtout chez les mâles tétraploïdes. Il est conclu que la population tétraploïde représente le premier cas observé d'une polyploïdisation directe chez un nématode parthénogénétique, par doublement de ses chromosomes. Il est discuté brièvement d'autres processus aboutissant à la polyploïdisation chez les *Meloidogyne*.

Key-words: *Meloidogyne microcephala*, nematode, parthenogenesis, polyploidy.

A population (E-116) of root-knot nematode, originally isolated from tobacco (*Nicotiana tabacum* L.) from Thailand, was described as *Meloidogyne microcephala* Cliff & Hirschmann, 1984. Preliminary cytogenetic examination had revealed that this population reproduced by mitotic parthenogenesis and had a somatic chromosome number of about 36. These observations were confirmed in subsequent cytological examinations routinely conducted yearly to check the purity (lack of accidental contamination) of the population. In 1987, twenty second-stage juveniles of distinctly larger size were detected among the juveniles hatched from a group of about 30 egg masses. These large juveniles were used as inoculum on a tomato plant, and 45 days later, five egg-producing females were recovered from the infected roots. Juveniles obtained from the egg masses of all five females were large in size. Furthermore, cytological examination of these five females indicated a chromosome number much higher than 36, suggesting polyploidy. Since this observation would represent the first case of direct polyploidization occurring in a mitotically parthenogenetic nematode, further studies were undertaken, *i*) to verify the polyploid nature of the new form, *ii*) to confirm that the new form morphologically is indeed a form of *M. microcephala* and not a contaminant of another species, and *iii*) to document any morphological changes that may have occurred following polyploidization.

Materials and methods

Greenhouse cultures of both, the regular diploid form of *M. microcephala* and the suspected polyploid form that was derived following propagation of twenty unusually large juveniles isolated from the diploid form, were maintained on tomato (*Lycopersicon esculentum* Mill.) cv. Rutgers in a greenhouse at 22 to 28°C.

For the cytological study, young, egg-laying females were smeared on microscope slides, and the smears

were processed and stained with propionic orcein according to established procedures (Triantaphyllou, 1985*b*).

For the morphological study, adult females were handpicked from infected roots and fixed in 2% formalin. The head regions were removed and examined in 2% formalin. Perineal patterns were cut from fresh egg-laying females in 45% lactic acid and mounted in glycerin. Eggs were fixed and mounted in 2% formalin. Second-stage juveniles were hatched from egg masses in moist chambers, fixed in hot TAF, and mounted in TAF. Males were obtained by incubating washed infected roots in moist chambers. The roots were periodically rinsed with water and the males, collected from the washings, were fixed in hot TAF and mounted in TAF.

Results

Cytological studies

The process of oogenesis was found to be very similar in the normal diploid and the suspected polyploid (henceforth referred to as tetraploid) form. However, cytological observations were easier in the tetraploid form, because its chromosomes were more widely spread and, therefore, more discrete than those of the diploid form throughout the oogonial and maturation divisions of the oocytes. The following account is primarily a description of the process of oogenesis of the tetraploid form, but applies equally well to that of the diploid form.

The oogonial zone of the ovary of young females showed many mitotic divisions (Fig. 1A). Often, about ten to fifteen oogonia were at prophase or metaphase. The chromosomes at prophase were elongate and spread all over the nucleus (Fig. 1B). They were discrete, but difficult to count precisely. At metaphase, the chromosomes became highly condensed, as they oriented with their long axis on a perfect metaphase plate (Fig. 1C). They often were spread in a large metaphase plate, and for this reason, they were quite discrete and could be counted easily in spite of their large number. At least ten metaphase plates of oogonial divisions of the tetraploid form were analyzed precisely and all had 74 chromosomes. In another fifteen metaphase figures, the chromosome number was estimated to be between 72 and 76. Ten metaphase figures of the diploid form had about 37 chromosomes each.

Oocytes in the middle of the growth zone of the ovary were still in the diffuse stage. Their nuclei had transparent nucleoplasm and no visible chromosomes. Those oocytes approaching the oviduct-spermatheca region had a very large nucleus with a prominent nucleolus (Fig. 1D, arrow). Their chromosomes were condensed and appeared as elongate double threads at first, but condensed further into shorter, single threads or spheres in oocytes located at the end of the ovary (Fig. 1E). In the latter oocytes, the nucleolus had dissolved and the chromosomes were still spread all over the nucleus.

As the oocytes passed through the spermatheca into the uterus, they advanced to prometaphase. Each one of their chromosomes consisted of two discrete chromatids that were oriented parallel to each other forming dyads, typical of *Meloidogyne* chromosomes (Fig. 1F). Such prometaphase oocytes, which have not yet developed an egg shell, often break in cytological preparations. Part of their cytoplasm seeps out, and the chromosomes usually become spread in a large area and can be counted. These are the only oocytes in which the chromosome number could be determined precisely. In fifteen such oocytes, 74 univalent chromosomes (dyads) were counted (Fig. 1F, G). In slightly more advanced oocytes at metaphase I, a thin egg shell developed, the chromosomes were still discrete and arranged in a large metaphase plate but could not be counted precisely (Fig. 1H). Counting precisely the chromosomes of the diploid form was difficult because of their close arrangement on the metaphase plate. However, in twenty metaphase plates of the diploid form the chromosome number appeared to be 36 or 37 (Fig. 1I).

Morphological comparison of the tetraploid form with the diploid

Female (Fig. 2A): Body size and other measurements of the tetraploid were much larger than those of the diploid, although the ranges of most measurements overlapped to some extent (Table 1). No overlap occurred in stylet length, stylet knob width, and vulval slit length.

Except for size differences, the morphological features of the tetraploid were similar to those of the diploid. Thus, although the tetraploid had larger stylets, the shape of the stylet knobs was very similar in the two forms, except for the dorsal knob which seemed to slope more distinctly posteriad in the tetraploid (Fig. 2A). The morphology of the perineal pattern was similar to that of the diploid, although the patterns were generally larger, and the distinct lateral lines were often broader than in the diploid and, occasionally, had transverse lines.

Male (Fig. 2D-H, N, O): All dimensions of the tetraploid males were larger than those of the diploid. No overlap in size was exhibited in stylet length, esophagus length (= head end to metacorpus valve), spicule, and gubernaculum lengths (Table 1). In general, there was a much larger range in body length and the various other morphometric features of the tetraploid as compared to those of the diploid.

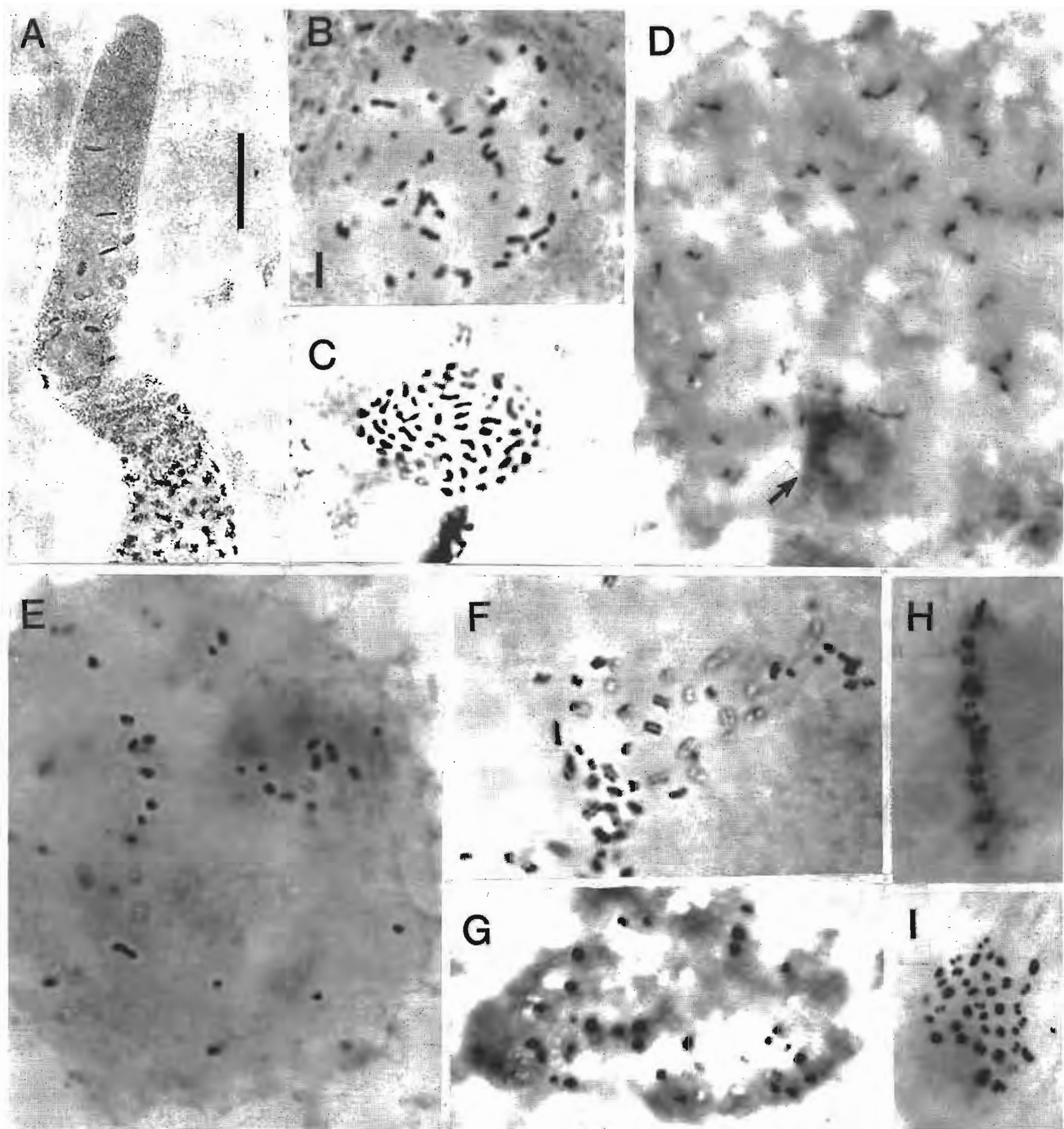

Fig. 1. *Oogenesis in* Meloidogyne microcephala, *tetraploid (A-H) and diploid (I) forms. A : Apical region of the ovary showing oogonial divisions at metaphase in side view; B : Oogonium at prophase with condensed chromosomes dispersed all over the nucleus; C : Metaphase plate of oogonial division in polar view; D, E : Early and later stages of condensation of the chromosomes in oocytes approaching the oviduct-spermatotheca region; F, G : Early and later prometaphase stages in oocytes shortly after they have passed through the spermatheca and into the uterus; H : Side view of a metaphase I plate of an advanced oocyte in the uterus; I : Polar view of a metaphase plate in an oocyte of the diploid form with about 37 chromosomes. (Scale bar = A = 50 µm; B-I = 2 µm).*

Tetraploid males exhibited striking abnormalities in most of the morphological characters (Fig. 2E-H, N, O). Frequently, they had a distinct bend in the body at the bulb region with a swelling in the body posterior of the gland region. Also, in some males the body diameter changed in width at small distances

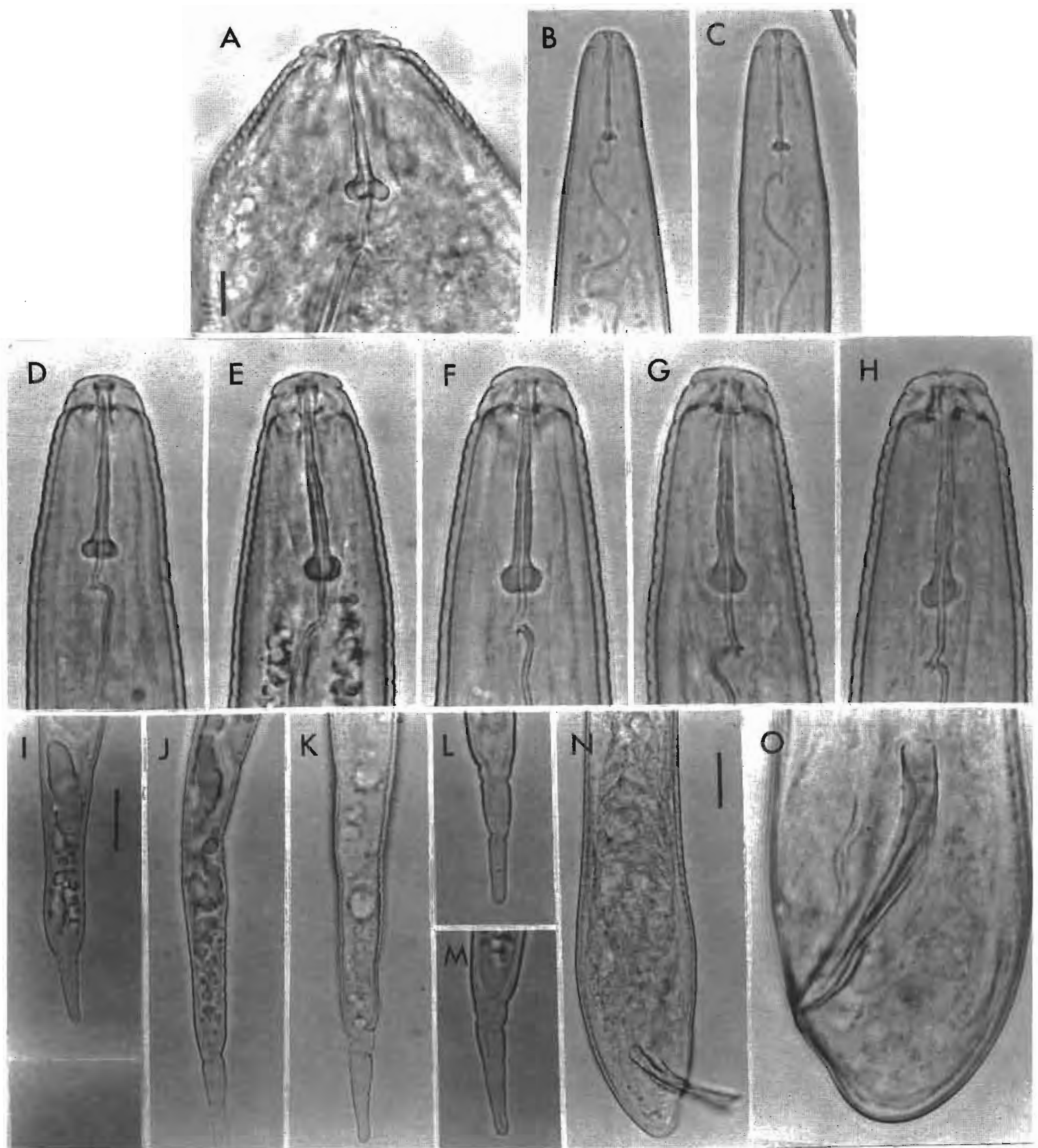

Fig. 2. Meloidogyne microcephala. *A : Head region of tetraploid female; B : Head region of diploid J2; C : Head region of tetraploid J2; D : Head region of diploid male; E-H : Head regions of tetraploid males; I : Tail of diploid J2; J-M : Tails of tetraploid J2; N, O : Tails of tetraploid males. (Scale bars 10 μm; B-H, same scale as in A; J-M, O, same scale as in I.)*

Table 1. *Morphometrics of females, males, and juveniles of the diploid and tetraploid forms of* Meloidogyne microcephalala *(All measurements in µm).*

	Females		Males		Juveniles	
	Diploid	Tetraploid	Diploid	Tetraploid	Diploid	Tetraploid
L	601.2 ± 17.63 (450.0-840.0)	794.7 ± 20.35 (482.0-960.0)	1345.5 ± 25.66 (979.0-1521.9)	2040.9 ± 55.37 (1280.0-2540.0)	457.5 ± 2.6 (415.6-471.7)	552.3 ± 4.59 (508.0-607.0)
Body width	400.8 ± 14.77 (255.0-525.0)	509.5 ± 13.42 (370.0-682.0)	30.4 ± 0.45 (25.4-35.6)	37.7 ± 0.97 (28.4-49.8)	14.2 ± 0.18 (11.9-15.8)	15.5 ± 0.18 (13.8-17.5)
a	1.5 ± 0.03 (1.1- 1.9)	1.6 ± 0.04 (1.3-2.0)	53.0 ± 0.78 (44.7-60.7)	55.3 ± 1.46 (42.1-77.5)	32.4 ± 0.39 (29.0-38.3)	36.2 ± 0.50 (29.2-40.6)
b					8.3 ± 0.07 (7.4-8.9)	7.6 ± 0.08 (6.8-8.7)
c			101.3 ± 2.36 (74.5-133.6)	128.5 ± 4.34 (74.9-177.4)	10.1 ± 0.14 (9.1-13.6)	8.6 ± 0.06 (8.0-9.3)
c'					4.5 ± 0.07 (3.4-5.0)	6.3 ± 0.07 (5.6-7.2)
Stylet	14.4 ± 0.09 (13.5-15.1)	19.2 ± 0.10 (17.8-20.4)	20.6 ± 0.11 (19.1-21.6)	26.2 ± 0.22 (23.7-28.3)	9.3 ± 0.04 (9.1-9.8)	12.4 ± 0.06 (11.8-13.0)
Stylet knob height	2.4+0.04 (2.0-2.9)	2.9 ± 0.09 (2.7-3.2)	2.4 ± 0.03 (2.2-2.9)	3.6 ± 0.07 (2.8-4.3)		
Stylet knob width	4.2 ± 0.05 (3.7-4.7)	5.2 ± 0.04 (4.8-5.6)	4.3 ± 0.05 (3.7-4.8)	5.8 ± 0.07 (4.5-6.3)		
Stylet knob width/hei-ght	1.8 ± 0.04 (1.5-2.4)	1.8 ± 0.02 (1.5-2.0)	1.8 ± 0.02 (1.6-2.0)	1.6 ± 0.03 (1.3-2.0)		
Stylet base to head end					13.6 ± 0.44 (12.8-14.3)	16.1 ± 0.06 (15.6-16.9)
DGO	3.7 ± 0.09 (2.9-4.9)	5.7 ± 0.20 (4.0-8.3)	3.5 ± 0.05 (2.9-4.0)	5.5 ± 0.20 (3.2-7.9)	3.1 ± 0.07 (2.5-3.8)	3.2 ± 0.05 (2.8-3.8)
Head end to metacorpus valve			83.8 ± 0.99 (76.4-93.2)	133.0 ± 2.43 (102.1-160.0)	55.1 ± 0.45 (50.7-62.3)	73.3 ± 0.62 (66.5-80.6)
Body length/head end to metacorpus valve			15.7 ± 0.27 (12.6-19.0)	15.4 ± 0.43 (9.4-18.6)		
Head end to excretory pore			145.7 ± 2.58 (118.2-169.1)	217.2 ± 4.10 (149.5-260.7)	83.6 ± 0.62 (74.1-89.0)	113.9 ± 0.49 (106.3-120.0)
Excretory pore (% L)			11.2 ± 0.21 (8.8-13.8)	10.8 ± 0.27 (8.1-14.1)	18.3 ± 0.15 (15.4-19.3)	20.5 ± 0.16 (19.0-22.3)
Vulval slit length	20.8 ± 0.27 (17.8-23.8)	29.0 ± 0.38 (25.8-33.2)				
Vulva-anus distance	17.9 ± 0.27 (14.3-23.5)	22.4 ± 0.45 (15.8-28.0)				
Interphasmidial distance	23.6 ± 0.53 (17.8-27.9)	31.6 ± 0.74 (21.8-42.7)				
Tail length			13.0 ± 0.18 (11.1-15.5)	16.2 ± 0.44 (12.4-19.8)	45.4 ± 0.53 (42.9-50.0)	65.1 ± 0.56 (59.2-79.0)
Spicule length			26.6 ± 0.27 (24.0-29.5)	35.0 ± 0.36 (31.6-38.6)		
Gubernaculum length			7.5 ± 0.27 (6.7-8.4)	10.3 ± 0.12 (8.8-11.5)		
Body width at anus					10.2 ± 0.09 (8.9-10.9)	10.4 ± 0.08 (9.5-11.1)

leading to a bead-like appearance of the entire body. In other males the posterior part of the tail region was swollen through the tail tip (Fig. 2N, O). The lateral field was incompletely areolated and had six to seven lines in some males and even ten lines in certain body regions of others.

The head region of the tetraploid males was very similar to that of the diploid (Fig. 2D-H). However, several specimens exhibited distinct annulations and an incompletely developed, lobed and very low head region. The cephalic framework was weakly or incompletely developed.

In many males there was much variation in size and shape of the stylet knobs (Fig. 2E-H). The two subventral stylet knobs were indented medially, or were incompletely developed with oblique and distorted outlines (Fig. 2G, H). The stylet cone was frequently broken at the middle or the tip, and the shaft and cone were not distinctly demarcated (Fig. 2G).

The metacorpus valve of the esophagus was exceptionally large, and the two large esophageal gland nuclei were situated close together in the anterior part of the gland lobe.

The reproductive system of the very large males was usually more or less normally developed and had a reflexed testis. In some males the gonad seemed much disorganized. However, mature sperm was usually present. Often, the glandular part of the *vas deferens* was not clearly defined. In some males the cytoplasmic area around the spicules seemed disorganized and the spicules themselves were malformed with smaller heads and additional sclerotizations in the shaft area.

The phasmids frequently appeared defective, or only one phasmid was developed in some males.

Second-stage juveniles (Fig. 2B, C, I-M): The body length of the tetraploid J2 was much larger and not overlapping in range with that of the diploid. Also, the ranges of stylet length, stylet base to head end, esophagus length, excretory pore distance to head end, tail length, and c' ratio (tail length/body width at anus) did not overlap (Table 1).

As compared to the diploid, the head cap of the tetraploid was well developed and the head region appeared faintly annulated on one side (Fig. 2B, C). The stylet was much larger than that of the diploid with larger stylet knobs that sloped distinctly posteriorly.

The tail was much longer and more drawn out as compared to that of the diploid (Fig. 2J, K), although the basic features of the tail were very similar in the two forms. The tail tip area resembled basically in shape that of the diploid, although it was much longer, and the constricting deep annules between tail and hyaline tail terminus were more pronounced (Fig. 2L, M). The phasmids were located a shorter distance posterior to the anal opening (20% of tail length).

The J2, when developed, had more flexures (five to seven) inside the tetraploid eggs than in the diploid eggs.

Eggs: The eggs of the tetraploid were larger than those of the diploid. Measurements of 50 eggs: Length = 95.2-118.6 (108.4 + 0.89) μm; width = 42.0-60.2 (50.8 ± 0.53) μm; length/width ratio = 1.7-2.8 (2.2 ± 0.04).

Eggs of diploid population: Length = 82.5-100.8 (91.7 + 4.4) μm; width=38.7-44.0 (39.8 ± 1.3) μm.

Discussion

Cytogenetic relationships of root-knot nematodes have been evaluated and a possible pathway of evolution of parthenogenesis and polyploidy within the group has been presented (Triantaphyllou, 1985*a*). Basically, it has been assumed that *i*) the ancestral root-knot nematodes were amphimictic animals, *ii*) parthenogenetic forms have evolved from amphimictic ones, *iii*) since the presently amphimictic forms have a haploid chromosome number of eighteen, this number may represent the basic number from which all other chromosomal forms have evolved, and *iv*) high chromosome numbers encountered in many parthenogenetic species represent various states of polyploidy, including triploidy, tetraploidy and possibly higher levels of ploidy.

Direct polyploidization of a diploid meiotic parthenogenetic form has been detected in populations of *Meloidogyne hapla* (Triantaphyllou, 1984). However, direct polyploidization of a mitotic parthenogenetic form has not been demonstrated. It has been suggested that triploid mitotic parthenogenetic forms have evolved from diploid meiotic parthenogenetic forms following accidental suppression of the meiotic process and subsequent fertilization of an unreduced (2n) egg with a normal sperm having the reduced (n) chromosome number (Triantaphyllou, 1985*a*). Tetraploid mitotic parthenogenetic forms most likely have originated from tetraploid meiotic forms following suppression of the meiotic process, or possibly through direct polyploidization of diploid mitotic parthenogenetic forms. The present study provides strong evidence that the second pathway does take place. The tetraploid population that was isolated from the diploid *M. microcephala* represents such a case, *i.e.*, development of a tetraploid form in a mitotic parthenogenetic species through doubling of the somatic chromosome number of a diploid form. This process possibly gave rise to a single tetraploid female which subsequently produced, by mitotic parthenogenesis, viable tetraploid progeny. Through isolation of the tetraploid progeny, we were able to

maintain the tetraploid polulation for many generations (maintained now in liquid nitrogen as "Population #2116-Vial 251" of the N.C. State University Nematode Collection). In preliminary tests, where mixtures of equal numbers of tetraploid and diploid juveniles were propagated on the same tomato plant for consecutive generations, fewer tetraploid females were recovered in each subsequent generation, and tetraploid females could not be detected after the sixth generation of propagation. This observation suggests that under field conditions, the tetraploid form would probably have failed to survive. It also explains why detecting tetraploid mitotic parthenogenetic forms in natural populations is extremely rare. On the contrary, triploid forms, which apparently are more competitive, are very common in many important *Meloidogyne* species, such as *M. arenaria, M. javanica* and *M. incognita* (Triantaphyllou, 1985*a*).

With respect to morphological features of the diploid and tetraploid forms, it was demonstrated that, except for size differences, the basic morphological structures of the tetraploid females, as well as those of the tetraploid second-stage juveniles were similar to those of the diploid form. The differences were mainly due to an increased size of the various structures. This fact confirmed that the tetraploid was truly a form of *M. microcephala* and not a contaminant. Only the males of the tetraploid form exhibited striking abnormalities of most morphological characters, in addition to general increases in size and larger ranges of the various structures. These abnormalities were predominantly expressed in certain features of the head region, stylet, and male reproductive system. They probably represent developmental disturbances, possibly enhanced by the process of sex reversal that is believed to be responsible for the development of many males in the genus *Meloidogyne* (Papadopoulou & Triantaphyllou, 1982).

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