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Chromosome number of the potato cyst nematode *Globodera rostochiensis*

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Summary – The chromosome number of *Globodera rostochiensis* was determined by investigating : *i)* bivalent configurations at metaphase I, *ii)* meiotically reduced cells during oogenesis and *iii)* somatic mitosis at early embryonic cleavage divisions. A diploid chromosome number of 2n = 18 was found. Chromosome numbers were readily determined in polar bodies using the fluorochrome Hoechst 33258. An improved squashing method enabled observations at early cleavage divisions.

Résumé – ***Nombre de chromosomes du nématode à kyste de la pomme de terre* Globodera rostochiensis** – Le nombre de chromosomes de *Globodera rostochiensis* a été déterminé par l'analyse *i)* de leurs configurations bivalentes au stade métaphase I, *ii)* de cellules au stade de réduction méiotique pendant l'oogenèse, et *iii)* de cellules en mitose somatique au cours de divisions embryonnaires prématurées. Le nombre de chromosomes diploïde est 2 n = 18. L'utilisation du fluorochrome Hoechst 33258 a facilité la détermination du nombre de chromosomes dans les globules polaires. Les premières divisions ont été observées en utilisant une méthode d'écrasement améliorée.

Key-words : Chromosome number, embryogenesis, *Globodera*, holocentric behaviour, oogenesis.

The potato cyst nematode species *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone are obligate plant parasites which are serious pests in potato crops. Populations of both species are classified into pathotypes as defined by their ability or inability to reproduce on a standard set of potato clones (Kort *et al.*, 1977). One of these clones, *Solanum tuberosum* ssp. *andigena* CPC 1673, harboring the H_1-resistance gene, is used to define the *G. rostochiensis* pathotypes Ro_1, and Ro_4. A gene-for-gene relationship was demonstrated for the interaction between *G. rostochiensis* and the H_1-resistance gene. Virulence is inherited at a single locus and is recessive to avirulence (Janssen *et al.*, 1991). This gene-for-gene interaction is used as a model to unravel the molecular basis of virulence in the potato cyst nematode *G. rostochiensis*. Currently we are tracing molecular markers linked to the avirulence gene in backcross populations (Rouppe van der Voort *et al.*, 1994). Progress is restrained by serious gaps in the knowledge in the genetic constitution of this bisexually-reproducing cyst nematode species.

Data on the chromosome number of potato cyst nematodes are scarce and conflicting. Riley and Chapman (1957) observed nine pairs of chromosomes at first metaphase of meiosis (2n = 18) in *Heterodera rostochiensis*. It is noted that until 1970 (Jones *et al.*, 1970; Stone, 1973) both potato cyst nematode species were considered pathotypes of a single species, *H. rostochiensis*. Variations in the basic chromosome number of *H. rostochiensis* were reported by Cotten (1959, 1960). He observed diploid chromosome numbers of 20 and 22. Occasionally, 19, 23 and 24 chromosomes were found. Although nearly indistinguishable morphologically, the two potato cyst nematode species are discriminated by 70 % of their polypeptides as resolved by two-dimensional gel electrophoresis (Bakker & Bouwman-Smits, 1988). These data indicate that *G. rostochiensis* and *G. pallida* diverged millions of years ago. Hence, the discrepancy in chromosome numbers as reported by the afore-mentioned papers can be explained by the possibility that a mixture of different species were analysed. Recently, the chromosome complement of one of the two species, *G. pallida*, was determined to be 2n = 18 although considerable variation was observed (Grisi *et al.*, 1995). The authors stated that part of this variation could be due to observational errors.

In this report, the chromosome number of *G. rostochiensis* was investigated. Using the fluorochrome Hoechst 33258 and an improved squashing method, the chromosome number was established by : *i)* observation of bivalent configurations at metaphase I, *ii)* the reduction process of the diploid complement during oogenesis and *iii)* determination of the re-established somatic number at early embryonic cleavage divisions.

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Materials and methods

Populations and lines

The *G. rostochiensis* population " Mierenbos ", classified as Ro_1 was obtained by the Plant Protection Service, Wageningen and multiplied in our laboratory. A virulent (Ro_5-22) and an avirulent (Ro_1-19) inbred line of *G. rostochiensis* were derived from controlled single matings and selected as described previously (Janssen *et al.*, 1990).

The *G. rostochiensis* lines were reared on the susceptible clone *S. tuberosum* ssp. *tuberosum* L. cv. Eigenheimer. Potato plants were inoculated with approximately 200 cysts and placed in a growth chamber at 18 °C and 16 h daylength. To harvest adult females, root balls were inspected daily. Young females outside the roots were collected from the potato roots with a small brush.

Fixation

Collected females were immediately fixed in cold 3 : 1 (v/v) ethanol-acetic acid and stored at – 20 °C for at least 5 days. This treatment caused increased permeability of the egg wall for cytological stains and facilitated squash preparations of eggs. Fixation was followed by either Hoechst 33258 or Feulgen staining.

Squash preparations and Hoechst staining

Microscope slides were pre-treated in a 1 % gelatin solution and subsequently air-dried. White females were placed on the slides, the adhering fixative was removed with tissue paper and a drop of 45 % (v/v) acetic acid was added. Females were opened with fine needles to release the body content. Gonads were squashed under a cover-slip (24 × 50 mm) and the egg contents were spread. Slides were dipped in liquid nitrogen and the cover-slip was then removed using a razor blade. The slides were air-dried, rinsed in 95 % (v/v) ethanol and fixed overnight in 3 : 1 (v/v) ethanol-acetic acid. Before staining, the slides were air-dried again and placed for 5 min in PBS (Sulston & Hodgkin, 1988). Preparations were stained for 5 min in 1 μg/ml Hoechst 33258 (bisbenzimide, Sigma) dissolved in PBS. Finally, the slides were rinsed in bidistilled water.

Feulgen staining

After removing the adhering fixative, young females were incubated in 5N HCl for 25 min at room temperature. This results in a hydrolysis of the purine-deoxyribose bonds. Reactive aldehydes were demonstrated by Schiff's reagents (Feulgen & Rossenbeck, 1924). The incubation time was 3 h at 4 °C. Thereafter, females were rinsed in tap water until the water remained clear.

Up to three stained females were placed on a slide, water was removed and a drop of 45 % (v/v) acetic acid was added. The females were opened with fine needles and the body contents was smeared on the slide. A cover slip was placed over it and pushed gently.

Microscopy

A Leitz Wetzlar Orthoplan microscope with a 100 × oil immersion objective was used to examine the Hoechst 33258 stained slides. The filter combination BP 355-425 / LP 460 was used to visualize the nuclei. Objects were photographed with a Kodak Ektachrome P 800/1600 film adjusted at 400 ISO. The Feulgen stained nuclei and eggs were visualized with a 100 × oil immersion objective and photographed with Agfa Ortho film (25 ISO). Chromosome lengths were determined in Feulgen stained nuclei using a stage micrometer (Leitz Wetzlar).

Results

The potato cyst nematode, *G. rostochiensis* did not show intraspecific variation in chromosome behaviour during maturation divisions and embryogenesis among the populations tested. The data presented here are representative for the Ro_1-Mierenbos population and the lines Ro_1-19 and Ro_5-22.

Chromosome pairing at metaphase I

Chromosome pairing was clearly observed at diakinesis/metaphase I. At this stage the nuclear membrane disappeared. Nine bivalents of different sizes became discernable (Fig. 1A). The chromosomes were rod-shaped and lack visible constrictions. Each chromosome consisted of two separated chromatids which were arranged side by side, apparently perpendicular to the equatorial plane.

Reduction of the diploid complement during oogenesis

The first maturation division resulted in two haploid nuclei with a chromosome number of n = 9; the egg nucleus and the first polar nucleus, both with condensed chromosomes. The spindle was situated perpendicular to the cell membrane and the first polar nucleus ended near the periphery of the oocyte. The egg nucleus proceeded to anaphase II (Figs 1 B, 2 A) whereas the first polar nucleus maintained its telophase I configuration (Fig. 2 B). The chromatids separated by parallel disjunction during anaphase II, and no centromere was visible (Fig. 1 B). The second maturation division resulted in the formation of a second polar body and the egg pronucleus.

The amount of fluorescence reflected also the reduction of the diploid complement into the haploid condition (n = 9), which was accompanied with a change from 4 C to 2 C in the first meiotic division and to 1 C in the second division (Fig. 1A, B, C). The sperm pronucleus, which was localized at one pole of the oocyte (Fig. 2 C), moved towards the egg pronucleus and they fused to form the zygote nucleus. Polyspermy within one egg was never observed.

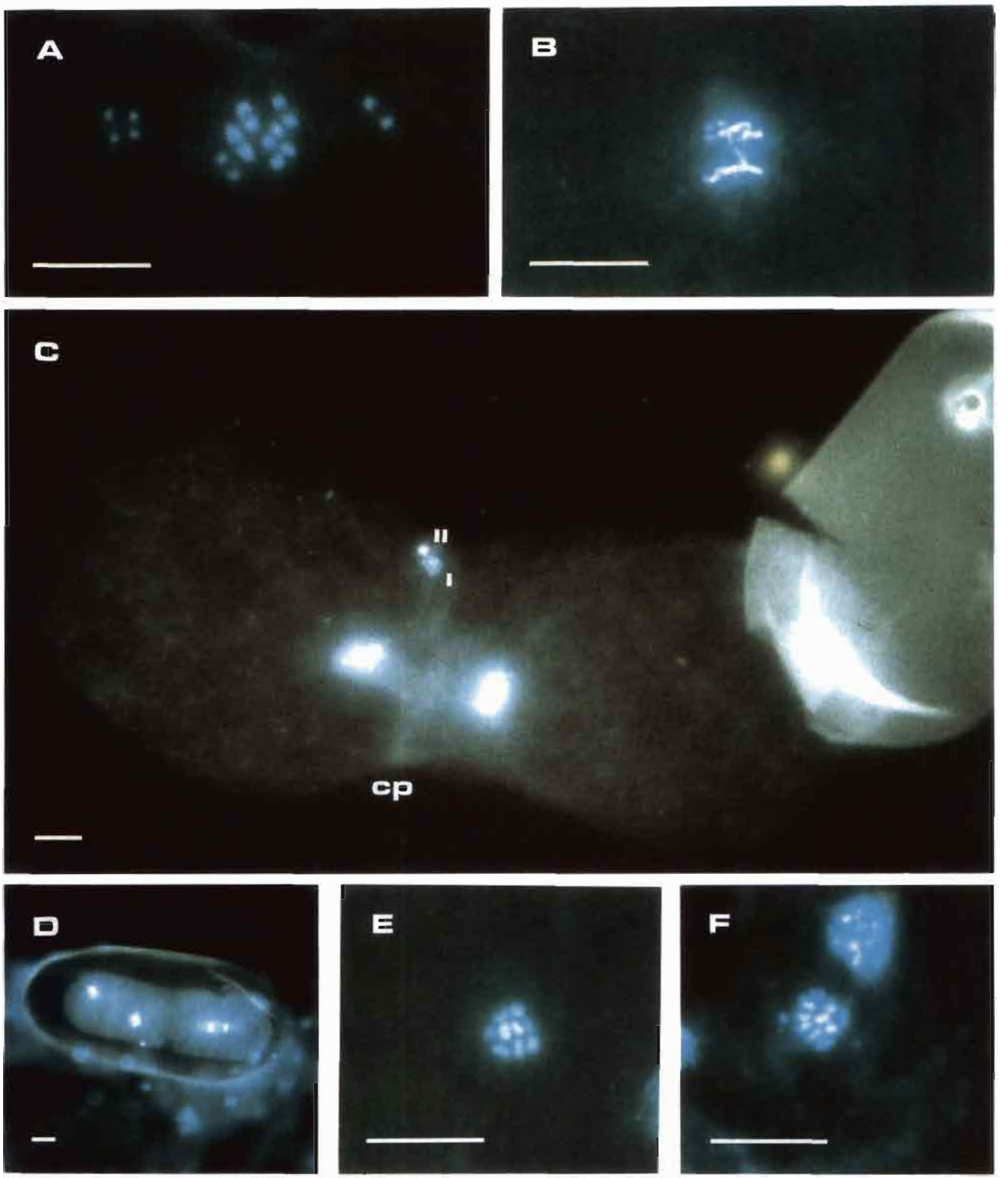

Fig. 1. *Chromosomes and nuclei of* Globodera rostochiensis *stained with the fluorochrome Hoechst 33258. A : Metaphase I with nine bivalents; B : Anaphase II; C : First cleavage division [At both sides of the cleavage plate (cp) two somatic nuclei are discernible; the first (I) and the second polar body (II) are indicated]; D : Second cleavage division; E : First polar nucleus, n = 9; F : First polar nucleus, n = 10.* (Scale bar = 10 μm.)

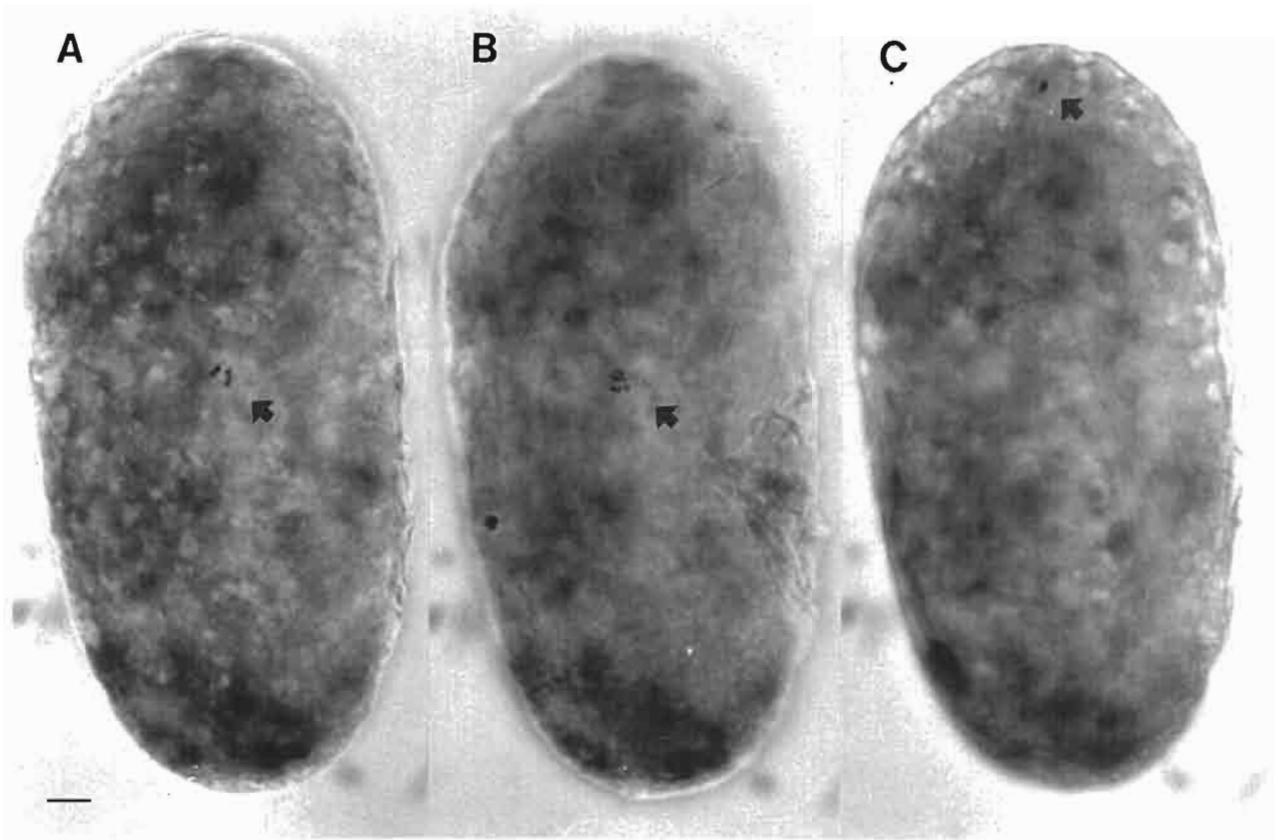

Fig. 2. *A single oocyte at three focal planes with A : Anaphase II; B : First polar nucleus; C : Sperm pronucleus* (Feulgen staining; only the objects marked with an arrow are Feulgen stained. Scale bar = 10 μm).

Somatic chromosome number at early embryonic cleavage divisions

Early cleavage divisions showed the restored somatic number of eighteen chromosomes (Fig. 3). These mitotic chromosomes were much more elongated than the meiotic chromosomes and showed no primary constrictions. Both polar bodies remained visible during early embryonic development on top of the yolk, close to the egg wall (Fig. 1 C). While the first polar body, still with recognizable chromosomes persisted almost until completion of embryogenesis, the second polar body disintegrated soon after the first divisions. Different mitotic phases within one embryo indicated that daughter cells did not divide simultaneously from the first cleavage division onwards (Fig. 1 D).

Determination of chromosome number

The diploid chromosome number of *G. rostochiensis* was determined to be 2n = 18. Most observations of chromosomes were made on haploid nuclei of the first polar body (Fig. 1 E). The chromosomes of these nuclei were free from cytoplasm and were most definite as compared to other nuclear stages. Seven chromosomes varying in length from 0.8 to 1.2 μm could usually be distinguished from a small chromosome (0.6 μm in length) and a large chromosome (1.5 μm in length). In

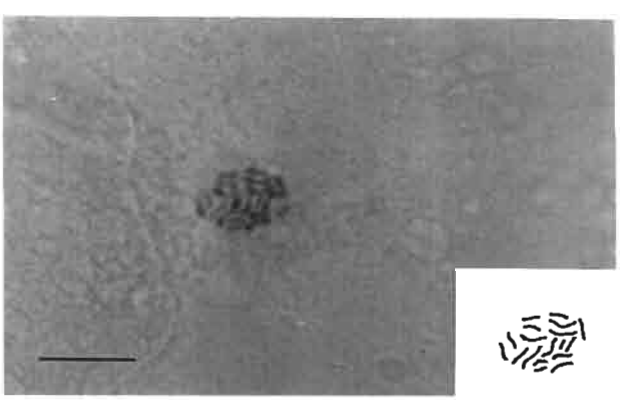

Fig. 3. *Mitotic chromosomes at metaphase in a young embryo stained with Feulgen.* (The inset is a diagrammic representation. Scale bar = 10 μm.)

only two out of the 200 polar nuclei examined, one extra chromosome was observed (Fig. 1 F).

Discussion

Despite the extensive divergence at the molecular level between *G. rostochiensis* and *G. pallida* (Bakker & Bouwman-Smits, 1988; De Jong *et al.,* 1989; Folkertsma *et al.,* 1994) both species share a similar chromosome complement of 2n = 18. This chromosome number is analogous to karyotypes found in other cyst nematodes (genera *Globodera, Heterodera*), with the exception of *H. betulae* (Triantaphyllou, 1975). The karyotypic uniformity between *Globodera* species helps to explain the capacity of these extensively diverged species to produce interspecific hybrids. Matings between *G. rostochiensis* and *G. pallida* resulted in viable, although not fertile, second stage juveniles (Mugniéry, 1979; Mugniéry *et al.,* 1992).

Variation in the chromosome number of *G. rostochiensis* was much lower than the variation reported by Cotten (1959, 1960) for *G. rostochiensis* and by Grisi *et al.* (1995) for *G. pallida.* Only one percent of the polar nuclei showed a deviant chromosome number. Triantaphyllou (1975) observed in a small number of females of two *H. schachtii* populations an extra chromosome which was transmitted to 50 % of the progeny of these females. The extra chromosome divided normally during maturation divisions and was considered to be a supernumerary chromosome. However, because of the low frequency of an extra chromosome and because this extra chromosome can be the result of non-disjunction during anaphase II, it seems unlikely that supernumerary chromosomes are common in the *G. rostochiensis* populations studied.

The use of fluorochrome Hoechst 33258 facilitated the counting of potato cyst nematode chromosomes. Hoechst 33258 interacts specifically with DNA. Its fluorescence increases the microscopic resolution and the chromosomes were mostly observed within one plane. The first polar nuclei are especially suitable for karyotype determination. The absence of cytoplasmic counterstaining and the persistence of polar nuclei during embryogenesis enable unequivocal determinations of the haploid chromosome numbers. This could be used for karyotype analyses in other nematode species.

For a number of nematode species including *Caenorhabditis elegans, Meloidogyne hapla, Parascaris equorum,* and *P. univalens,* chromosomes lacking a localized centromere are found (White, 1973; Goldstein & Triantaphyllou, 1980; Albertson & Thomson, 1982; Pimpinelli & Goday, 1989). Although by no means proven, the side by side arrangement of the chromatids at metaphase I, the parallel segregation at anaphase II, and the absence of distinct centromeric constrictions suggest a holocentric nature of *Globodera* chromosomes.

It has been argued that the holocentric nature of nematode chromosomes may have been evolved to prevent cell death due to chromosome breakage (Pimpinelli & Goday, 1989). Characteristic for nematode genomes is their mosaic embryonic development in which each cell passes through a determined number of mitotic divisions and cannot be replaced by another cell. Breakages in monocentric chromosomes would be lethal to the cell and would thus affect the whole embryo. Holocentric chromosomes are characterized by a diffuse centromere which extends along the length of the chromosome. Fragmented holocentric chromosomes may still contain a spindle attachment site and remain through cell division. Holocentric chromosomes may provide nematodes a strategy to circumvent lethal effects of chromosome fragmentation.

Finally, it is concluded that the chromosome number of the *G. rostochiensis* populations studied is 2n = 18 and constant. From strict bivalent pairing, observed in metaphase I, Mendelian segregation of maternal and paternal alleles is expected. This is of importance for a genetic analysis of the potato cyst nematode with molecular markers.

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