



## The genome size and chromosome complement of the potato cyst nematode *Globodera pallida*

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**Summary** – The genome size of *Globodera pallida* was estimated by direct microdensitometry of stained somatic nuclei in comparison to those of *Caenorhabditis elegans*. There was no significant difference between the genome sizes of these nematodes and thus the genome of *G. pallida* is estimated to be composed of  $1 \times 10^8$  base pairs. This value is of the same order as genome sizes calculated previously for a variety of other nematode species. Chromosomes in the dissected gonads of *G. pallida* females were visualised using five staining methods: propionic acid/orcein, Feulgen, Giemsa and two fluorescent stains, DAPI (4'-6-diamidino-2-phenylindole) and Hoechst 33258 (Bisbenzimidazole). Propionic acid/orcein proved to be the best staining method and despite the small size of the chromosomes it was used to demonstrate that *G. pallida* has a diploid chromosome complement of 18 ( $n = 9$ ).

**Résumé – Taille du génome et nombre chromosomique du nématode à kystes de la pomme de terre *Globodera pallida*** – La taille du génome de *Globodera pallida* a été estimée par microdensitométrie directe de noyaux somatiques colorés, et comparée à celle de *Caenorhabditis elegans*. Elles ne se sont pas révélées significativement différentes, aussi le génome de *G. pallida* est-il estimé être composé de  $1 \times 10^8$  paires de base. Cette valeur est du même ordre que les tailles du génome déjà calculées pour d'autres espèces de nématodes. Dans les gonades disséquées, les chromosomes ont été rendus visibles par cinq techniques différentes de coloration: acide propionique/orcéine, Feulgen, Giemsa et deux colorants fluorescents, le DAPI (4'-6-diamidino-2-phénylindole) et le Hoechst 33258 (Bisbenzimidazole). La coloration à l'acide propionique/orcéine s'est révélée la meilleure et, en dépit de la faible taille des chromosomes, a pu être utilisée pour montrer que le nombre chromosomique de *G. pallida* est égal à dix-huit ( $n = 9$ ).

**Key-words** : Nematodes, *Globodera pallida*, genome size, chromosome number.

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are highly specialised and economically important pests. Although aspects of the physiology, morphology and host-parasite relationships of these parasites have been studied, little work has been attempted at the genomic level. Cytological studies and chromosome counts have been done on *G. rostochiensis* (Riley & Chapman, 1957; Cotten, 1959, 1960) but these were completed before *G. pallida* was separated from *G. rostochiensis* as a new species; there is no comparable work on *G. pallida*.

The genome size and chromosome complement are fundamental pieces of information in many molecular genetic investigations and provide a framework to support more detailed studies. As part of a continuing study to characterise the genome of *G. pallida* this paper presents results determining the genome size and chromosome number of this species.

### Materials and methods

#### NEMATODES

Cysts of *G. pallida* originated from stock cultures held at Rothamsted Experimental Station. To obtain nematodes with dividing cells in which chromosomes may be observed, sterile cultures of *G. pallida* on potato explants were established. Transformed roots of *Solanum tuberosum* (Kumar & Forrest, 1990) were cultured in Petri dishes containing Knop medium (Knop, 1860) to which sucrose and Daichin agar (Brunschwig Chemie, Amsterdam, The Netherlands) was added to give final concentrations of 1% and 0.8%, respectively. After two weeks the roots were ready for inoculation.

Cysts of *G. pallida* were soaked overnight in distilled water and crushed to release the eggs. The eggs were surface sterilised with 0.2%  $\text{HgCl}_2$ , washed in double

distilled water and retained on the surface of a 0.22 µm pore millipore filter. The filter was then smeared over the agar plate depositing the eggs on the surface of the agar. Nematodes which subsequently hatched were able to migrate to the roots and invade. The plates were incubated at 18 °C and within three weeks young females were visible on the surface of the roots. At this stage, before they started to tan, they were collected from the roots and stored in phosphate buffered saline (PBS) at -20 °C.

#### GENOME SIZE

The genome size of *G. pallida* was estimated by direct microdensitometry of stained somatic nuclei in comparison to those of *Caenorhabditis elegans*. To minimise loss of material during hydrolysis and associated staining steps, slides were subbed with a solution of 0.1 % gelatin and 0.01 % chrome alum (Sulston & Hodgkin, 1988). Slides were immersed in the solution and then air dried.

Hatched second-stage juveniles of *G. pallida* and mixed stages of *C. elegans* were cut with a scalpel at approximately mid-body and dried onto the subbed slides. The specimens were then hydrolysed with 1M HCl (as described below for preparation of chromosomes) and fixed in a solution containing ethanol, acetic acid and chloroform (6 : 3 : 1). Somatic nuclei were stained with Feulgen according to Sulston and Horvitz (1977) and the nematodes observed under glycerol.

The density of somatic nuclei from *G. pallida* and *C. elegans* was determined on a comparative basis using a Vickers M86 scanning laser microdensitometer at a wavelength of 560 nm. For each nucleus, five density readings were taken and aggregated to a single value. Areas of nematode body devoid of nuclei were scanned to determine background levels of staining.

#### CHROMOSOME NUMBER

Females of *G. pallida*, in a drop of PBS on a subbed slide, were dissected to release the gonads. The female body was removed leaving the gonads which were then spread in a smear using another slide. The smear was allowed to dry ensuring that it was firmly attached to the slide.

The standard procedure of hydrolysis in 1M HCl at 60 °C for 20 to 30 min resulted in unsatisfactory preparations with the oocytes containing large globules and the cytoplasm being overstained. Hydrolysis, by immersing the slides in 1M HCl at room temperature for 20 to 30 min, was found to improve the quality of the preparation considerably; the oocytes remained free of globules and the cytoplasm was almost clear. After hydrolysis, the slides were removed and allowed to dry before fixation. At this stage the smears were transparent and only faintly visible.

Tissues were fixed by immersing the slides in a freshly prepared solution of absolute ethanol and glacial acetic acid (3 : 1) for 60 min. During fixation the nematode material turns white and the smears are easily visible on the slide. The slides were allowed to dry before the chromosomes were stained.

Five staining methods were evaluated in preliminary tests: propionic acid/orcein (Triantaphyllou, 1975), Feulgen (Sulston & Horvitz, 1977), Giemsa (Lavanaia & Sharma, 1980) and two fluorescent stains, DAPI (4'-diamidino-2-phenylindole) and Hoechst 33258 (Bis-benzimide) (Albertson & Thomson, 1982). Propionic acid/orcein and DAPI were used for further studies. Propionic acid/orcein has been widely used for staining nematode chromosomes. It causes swelling of the chromosomes which stain very heavily in contrast to the virtually unstained cytoplasm. The two fluorescent stains gave similar results: they stained the chromatin well although the resolution between individual chromosomes was poor.

A modified propionic acid/orcein method (Triantaphyllou, 1979) has been developed specifically for the study of nematode chromosomes. The stain was prepared by combining 2 g of orcein stain and 100 ml of 45 % propionic acid, which was boiled for an hour. The stain was cooled and filtered before use. Slides were covered with approximately 2 ml of propionic acid/orcein stain for at least 3 h. After staining, the slides were washed in 45 % propionic acid. A cover-slip soaked in 45 % propionic acid was applied and the slides were sealed with glyceel.

Chromosome counts were made using a 100 × oil immersion objective on a Zeiss Universal photomicroscope equipped with a MC63 camera; a 63 × oil immersion objective was used to photograph the nuclei using Ilford PanF 50 film. A green filter ( $\lambda = 546$  nm) was used to enhance the contrast and definition of the chromosomes.

#### Results and discussion

The somatic nuclei within *G. pallida* and *C. elegans* were measured using a calibrated eyepiece graticule and found to present the same apparent surface area for density analysis (data not shown). Any differences in nuclear DNA content (genome size) between these two nematodes should thus only be a feature of the relative staining density. Density measurements [absorbance (abs) units at 560 nm] derived from somatic nuclei in *G. pallida* ( $n = 10$ , mean abs.  $1193.3 \pm 55.7$ ) and from somatic nuclei in *C. elegans* ( $n = 10$ , mean abs.  $1135.2 \pm 108.5$ ) indicate that there is no significant difference ( $P > 0.05$ ) in the size of the genomes and, thus, based on recent estimates for *C. elegans* (Hammond & Bianco, 1992), *G. pallida* has a genome size of approximately  $1 \times 10^8$  base pairs of 83 fg DNA.

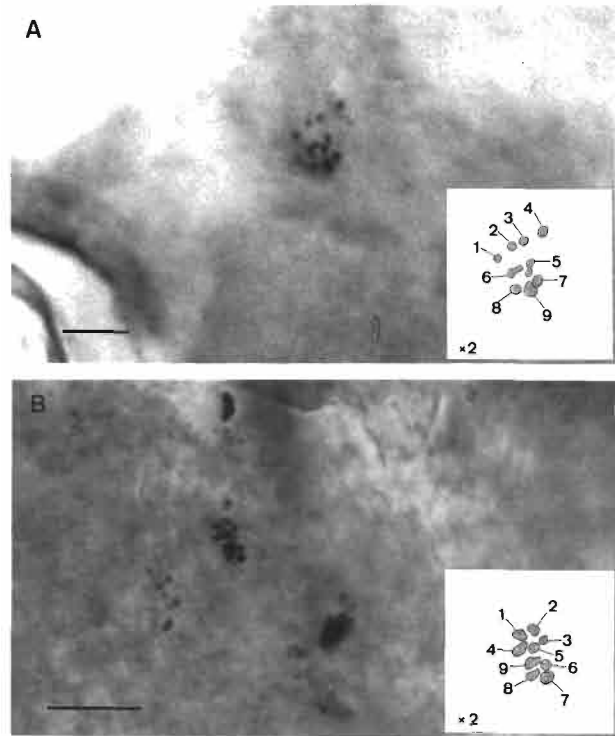
This value is of the same order as the genome sizes calculated for a variety of other nematode species (Hammond & Bianco, 1992). Little work has been done on comparative genome sizes in plant parasitic nematodes. Pableo and Triantaphyllou (1989) used DNA reassociation kinetics to determine that the haploid DNA content of *Meloidogyne* spp. is  $0.51 \times 10^8$  base pairs. The genome of *Ditylenchus dipsaci* was estimated to be  $2.4 \times 10^8$  base pairs (Palmer, 1989) which is approximately five times larger than *Meloidogyne* spp. The actual values for genome size of these two plant parasitic nematodes may be up to 20 % larger because the genome of *Escherichia coli*, to which they are both directly or indirectly compared, is now believed to be bigger than assumed previously (Knott *et al.*, 1989). The DNA content of *G. pallida* falls between the values determined for these two nematodes but it is close to that of *Meloidogyne*.

The reproductive system of *G. pallida* is a long and convoluted ribbon structure consisting of two ovaries, each connected to the vulva via separate gonoducts. Each gonoduct consists of a long, thin distal end, where the multiplication and growth of the oogonia occurs, and towards the proximal end a discrete oviduct and spherical glandular spermatheca are present leading to a cylindrical uterus terminating in the vulva.

Preliminary evaluation of the five methods used to stain chromosomes showed propionic acid/orcein to be the best. This method resulted in dark stained chromosomes against an almost clear background and also caused slight swelling of the chromosomes which aided resolution. As a consequence of this all of the subsequent chromosome observations and counts were based on tissue stained with propionic acid/orcein.

Cell division was observed over the entire length of the convoluted ovary. Mitotic cell division occurred mainly in the distal end of the ovary but cells in metaphase were only seen in side view and, thus, the chromosomes could not be distinguished. Many phases of meiosis seem to occur simultaneously in the ovary but the only stages when the chromosomes could be counted were at late prophase and metaphase of the first meiotic division.

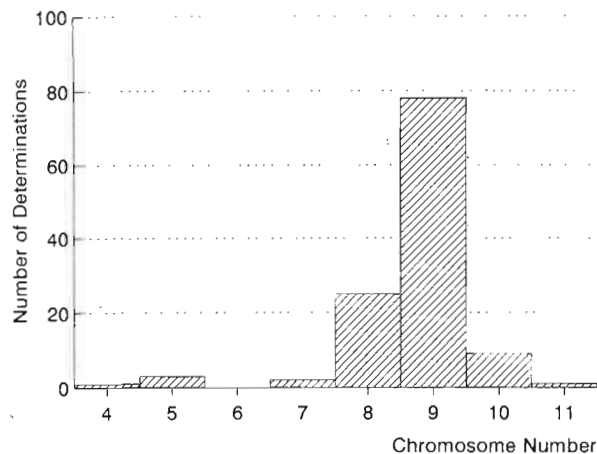
Within the ovary the most suitable regions to observe and count the chromosomes were where the oocytes approach the oviduct-spermatheca region. In that area and in the proximal end of the uterus, some cells in prometaphase and metaphase were observed; chromosome number was determined mainly from preparations showing first meiotic metaphase, but also from late prophase. Within the spermatheca area, cells in prometaphase and metaphase, stained with propionic acid/orcein, were examined (Fig. 1 A, B). At prometaphase, the chromosomes were generally not well separated but nine pairs of small rod shaped bivalents could be distinguished (Fig. 1 A). In a few cells the chromosomes in metaphase were more widely spread on the preparation



**Fig. 1.** Chromosomes within the oviduct-spermatheca region of gonads dissected from *Globodera pallida* and stained with propionic acid/orcein. A: Chromosomes in metaphase; B: Chromosomes in prophase. Insets in A and B are diagrammatic representations of the stained chromosomes. (Scale bar = 10  $\mu$ m).

(Fig. 1 A) and in these cases nine pairs of bivalent chromosomes were counted more readily. The meiotic chromosomes are less than 1  $\mu$ m in length and lack any visible constriction at metaphase. It was not possible to detect clear evidence of morphological differences between chromosomes because of their small size. However, at prometaphase one pair of chromosomes appeared bigger than the others (Fig. 1 B, chromosome pair number 9) and observations on some cells in metaphase indicated that three of the chromosome pairs seem to be bigger than the others; this has also been observed by Riley and Chapman (1957) in *G. rostochiensis* chromosomes. No sex chromosomes were distinguished in the female karyotype.

Results of chromosome counts of 121 individual cells each from different females (Fig. 2) shows that, although variations do occur, in the majority of observations (66 %) the diploid chromosome number was eighteen ( $n = 9$ ) observed as nine pairs. This is in agreement with the data from Cotten (1960) on *Heterodera* (= *Globodera*) *rostochiensis*. The chromosome condition  $n = 9$  is common among many groups of amphimictic cyst nematodes including members of the *G. tabacum* complex (Triantaphyllou, 1975). The variation in chromosome numbers observed in this study (Fig. 2) is probably due



**Fig. 2.** Chromosome counts of 121 individual cells from gonads of *Globodera pallida* stained with propionic acid/orcein. Most observations (66 %) showed a chromosome complement of  $n = 9$ .

in part to observational error, although some biological variation, such as aneuploidy, is considered to be a contributory factor. Some polyploid cells seem to be present but the difficulty in counting the chromosomes in these cells prevents any conclusion about polyploidy in *G. pallida*.

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