A TECHNIQUE FOR THE EXTRACTION AND RESTRICTION ENDONUCLEASE DIGESTION OF TOTAL DNA FROM *GLOBODERA ROSTOCHIENSIS* AND *GLOBODERA PALLIDA* SECOND STAGE JUVENILES

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Faced with the conservative morphology of many plant nematode groups, taxonomy has focused much attention on a biochemical approach. Biochemical systematics is dependant on stable, subtle molecular differences which underlie taxonomic variation. Characterisation of carbohydrate, lipid, and especially proteins, have all found applications; however, like serological investigations these have met with mixed degrees of success (Hussey, 1979). Recent advances in DNA technology now make possible the rapid and reliable analysis of the nematode genome using restriction endonucleases (Curran, Baillie & Webster, 1985). The size and number of restriction fragments formed will be a direct reflection of the genomic base sequence. This offers the potential of a unique « finger print » of a genome independent of a phenotype.

Collection of eggs and second stage juveniles

Approximately 1 000 cysts soaked in distilled water for 24 hours are crushed by rolling a glass rod over a channelled aluminium slide (2.5 × 7.5 cm) (Reid, 1955). A groove depth of 30 µm is sufficient to rupture cysts, liberating undamaged eggs and second stage juveniles (J2). These are collected in 10 ml distilled water and the suspension stirred mechanically for 1 minute to release eggs and J2. Centrifugation for 10 minutes at 9 000 RPM (rotor radius 107 mm) produces a tight pellet of nematodes and eggs, which are washed once and resuspended in 1 ml distilled water. *Globodera* eggs and egg-shells are particularly resistant to proteinase attack. It is important to remove them at this initial stage in order to improve the efficiency of DNA extraction and restriction endonuclease digestion in later steps.

Preparation of a pure second stage juvenile sample

The 1 ml of nematode suspension is transferred to a 1 ml tube homogeniser. Gentle movement of the plunger mechanically hatches eggs without damage to J2. Empty eggs can be removed by carefully pipetting the homogenised suspension onto 0.5 ml of a 50 % w/v sucrose solution in a 1.5 ml microcentrifuge tube. Centrifugation at 13 000 RPM for 1 minute sediments egg-shells while J2 and the few remaining unhatched eggs stay in the upper water layer. Second stage juveniles may then be pipetted off, washed once in distilled water and pelleted as before.

Extraction of total DNA

Approximately 100 mg wet weight of *Globodera* second stage juveniles is washed twice in DNA extraction buffer (Tris 0.1 M pH 8.5, EDTA, 0.05 M, Nacl 0.2 M, sodium dodecyl sulphate 1 %) (Curran, Baillie & Webster, 1985) pelleted in a microcentrifuge and resuspended in 1 ml extraction buffer. This is placed in a pestle and mortar, cooled to - 70° and thoroughly ground. The contents are transferred to a 1.5 ml capacity microcentrifuge tube along with 2 mg proteinase K (Sigma) and digested at 65° for 30 minutes with occasional gentle inversion of the microcentrifuge tube.

The resultant viscous solution is extracted three times with redistilled phenol and chloroform 1:1 v/v. The mixture is gently inverted for 10 minutes, spun for 2 minutes in a microcentrifuge and the aqueous layer transferred to a fresh microcentrifuge tube with a wide mouth pipette. Use of a wide mouth pipette is necessary to prevent shearing the DNA molecules.

DNA may be precipitated from the final phase by adding sodium acetate at a concentration of 0.2 M, followed by two volumes of absolute ethanol at - 70° for 30 minutes-1 hour. Precipitated DNA is spun down for 15 minutes in a microcentrifuge and the pellet dried under a stream of nitrogen prior to resuspension in 50 µl of 0.01 M Tris pH 8.0, 0.01 M EDTA buffer. RNA is removed by adding 50 µl DNAase free RNAase and incubating at room temperature for 30 minutes. This solution is then re-extracted with phenol : chloroform. The DNA is precipitated and spun down as above. The pellet is washed in 70 % ethanol at 4°, repelleted, dried under nitrogen and resuspended in 50 µl of 0.01 M EDTA buffer. DNA yields from this technique were typically in the region of 3-10 µg per 100 mg of J2.

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RESTRICTION ENDONUCLEASE DIGESTION

The technique detailed here is specifically for Eco R1 but other endonucleases could be substituted with appropriate modifications to the buffering system and incubation conditions (see suppliers' recommendations). Digestion mixtures are prepared in 0.5 ml Eppendorf microtubes. Ten units of Eco R1 (Boehringer) are added to 2 µl restriction buffer (Tris HCl pH 7.2 1.0 M, NaCl 0.5 M, $MgCl_2$ 0.1 M), 1 µg nematode DNA, and enough sterile distilled water to make a total volume of 20 µl.

As an internal standard 500 ng of λ DNA (Boehringer 100 ng/µl) may be used in place of the nematode DNA. Digestion mixtures are incubated at 37° for 1 hour and the reaction stopped by adding 2 µl loading mix (Ficol 30 %, bromophenol blue 0.25 %, Tris 0.09 M, boric acid 0.09 M, adjusted to pH 8.2 with HCl).

AGAROSE GEL ELECTROPHORESIS

Restriction fragments may be separated according to size on a 0.8 % agarose horizontal mini gel (10 cm × 6 cm) which is made up in running buffer (Tris 0.09 M, Na EDTA 2.5 mM, boric acid 0.09 M, adjusted to pH 8.2 with HCl) 2-8 µl sample of restriction digest is loaded into 5 × 1 mm slots in the gel and run at 100 V for 1 hour under running buffer. The gel is stained with ethidium bromide (2.5 µg/ml running buffer) and can then be viewed under 300 nm transmitted u/v light.

Fig. 1 shows a typical gel photographed under u/v.

Owing to the complexity of total DNA a smear is produced by electrophoresis, but restriction fragments from within repetitive DNA are seen as bright bands superimposed on the smear. Differences in band number and mobility (size) were observed between the *G. rostochiensis* and *G. pallida* populations used.

It has already been demonstrated that restriction fragment length differences (RFLDs) can differentiate nematode species (Curran *et al.*, 1985).

The technique, as reported here, is specifically tailored toward extracting and restricting DNA from two cyst nematode species. As such, it differs in a number of respects from that used by Curran (Curran, Baillie & Webster, 1985). Minor technical adaptations include : combining the phenol and chloroform during DNA extraction and thereby eliminating the need for a separate chloroform : isoamyl alcohol treatment. RNA-free DNA is given a final wash with 70 % ethanol at 4° to remove the last traces of phenol. It seems far more convenient to stop the endonuclease digestion by adding loading mix rather than heating for 10 minutes at 65°. The agarose gels are all run at 100 V for 1-1.5 hours as opposed to 15-20 V for 16 hours, this speeds up the analysis quite considerably without affecting resolution or clarity of the restriction bands. Perhaps the most significant modification of the basic technique is the need to remove *Globodera* egg-shells. Residual egg debris reduces DNA collection efficiency, but more importantly it inhibits the endonuclease. The mechanism of this inhibition is yet to be determined.

This method is almost certainly equally applicable to other cyst nematode groups and complexes. RFLDs offer the potential of a powerful new taxonomic tool in nematode systematics.

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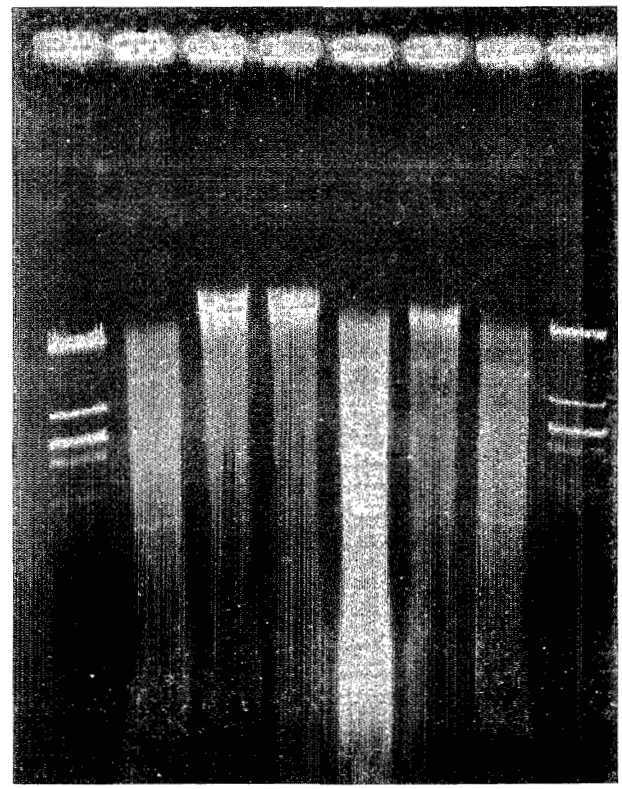

Fig. 1. Eco R1 Restriction endonuclease digest of *Globodera rostochiensis* Ro_2 second stage juveniles. Lanes 1 and 8 : λ DNA, fragment size (Kilobases) from origin 21.8, 7.52 [5.93, 5.54] (seen as a single band) 4.80, 3.41; lanes 2, 5, 6 and 7 : Ro_2 DNA EcoR1 digest showing highly repetitive restriction fragments (lanes loaded with 6, 8, 2 and 4 µl respectively); lanes 3 and 4 : unrestricted nematode DNA.