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*Short note*

# Molecular methods confirming the presence of *Globodera rostochiensis* (Wollenweber, 1923) in Yugoslavia

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Yugoslavia (a federation of Serbia and Montenegro) is an important European potato producing country with an annual production of nearly one million tons (Marks & Rojancovski, 1998). The economic significance of potato cyst nematodes (PCN) is well known worldwide in regions where potato is an important crop. As in other European countries, PCN are quarantine pests in Yugoslavia, with an appropriate legislation for import/export, internal production and marketing of seed potatoes. During regular inspections, PCN were recently detected in two remote potato fields in Serbia (Krnjaic *et al.*, 2000). The cysts were multiplied on potatoes in a greenhouse, and identified as *Globodera rostochiensis* on the basis of their morphology. The present work complements the morphological diagnosis by utilising molecular methods that provide unequivocal identification of PCN species. Problems of efficiency of current molecular methods for PCN diagnostics are discussed.

**Nematodes.** Samples originating from near Pear, UTM CP 96 (sample 1) and Krupanj, UTM CQ 61 (sample 2) and containing about 30 cysts per population were analysed. The cysts were about two years old, and of relatively low viability. Consequently, nematodes from sample 2 were cultivated on susceptible potatoes in a greenhouse before being analysed. Cysts from a *G. rostochiensis* population from Belgium (Assenede) and a *G. pallida* population from The Netherlands (Rookmaker) were used for comparison in the tests. All procedures were performed with single cysts.

**PCR-RFLP-rDNA.** DNA was extracted and amplified as described by Subbotin *et al.* (1999). The ITS regions of rDNA were amplified using universal primers TW81 and AB28 in a GeneE DNA thermal cycler (New Brunswick Scientific). Five to seven µl of PCR product were restricted with *Alu*I or *Hin*fI in the corresponding buffer according to the manufacturers instruction. Resulting fragments were run on a 1.5% agarose gel, stained with ethidium bromide, visualised on a UV transilluminator and photographed.

**PCR with specific primers.** PCR diagnostics with PCN specific primers were carried out in a PCR-100 Programmable Thermal Controller (MJ Research, Inc) with PITSr3, PITSp4 and the universal primer ITS5 as described by Bulman & Marshall (1997), or with four species specific primers described by Fullaondo *et al.* (1999).

**Results of identification.** PCR using the primers TW81 and AB28 yielded a single fragment of nearly 960 bp from all cysts examined. Restriction patterns obtained after the digestion of PCR products by *Alu*I and *Hin*fI are shown in Figs. 1A and B, respectively. Comparison of the RFLP profiles with those obtained for the reference *G. rostochiensis* and *G. pallida* populations, and with the RFLPs published by Subbotin *et al.* (1999) revealed that the cysts from Yugoslavia were of *G. rostochiensis*.

The results of PCR with PITSr3, PITSp4 and ITS5 primer combination are shown in Fig. 1C.

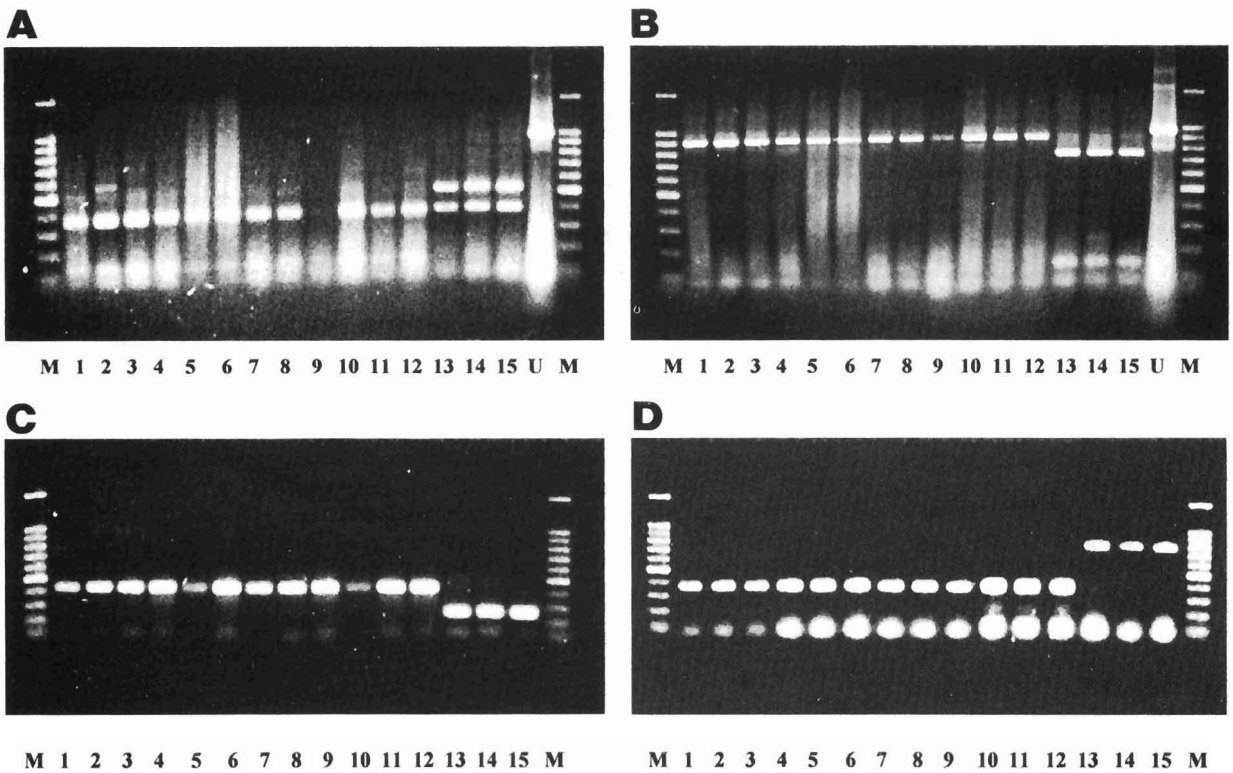


**Fig. 1.** Identification of potato cyst nematodes from Yugoslavia using molecular methods. A: Restriction fragments of amplified ITS regions digested by *Alu*I; B: Restriction fragments of amplified ITS regions digested by *Hin*fI; C: Products of PCR amplification with primers developed by Bulman & Marshall (1997); D: Products of PCR amplification with primers developed by Fullaondo *et al.* (1999). Lanes 1-3: cysts from near Pear (Yugoslavia); lanes 4-9: cysts from near Krupanj (Yugoslavia); lanes 10-12: *Globodera rostochiensis* (Assenede, Belgium), and lanes 13-15: *G. pallida* (Rookmaker, the Netherlands); M: 100 bp DNA marker; U: unrestricted PCR fragment.

Comparison of the fragment length obtained from Yugoslavian cysts with that from the reference populations and data published by Bulman & Marshall (1997) shows that the Yugoslavian samples belong to *G. rostochiensis*. The multiplex PCR according to Fullaondo *et al.* (1999) yielded for each of the Yugoslavian samples a single fragment of about 315 bp (Fig. 1D). Even after optimising the original PCR protocol, only about half of the cysts examined yielded a PCR product characteristic for *G. rostochiensis*. The optimal PCR mixture contained 2 µl DNA extract, 2.5 µl 10X Qiagen PCR buffer, 0.5 µl dNTPs (200 M each dNTP), 1U *Taq* Polymerase (*Taq* PCR Core Kit, Germany), 0.5 µM of each primer and double distilled water to a final volume of 25 µl.

PCR-based identification of nematodes with species specific primers requires optimal PCR conditions. Even under optimal conditions, PCR efficiency largely depends on properly designed primer sequences. We observed that multiplex PCR with specific primers developed by Bulman & Marshall (1997) gave clearer results then those obtained with the four primer sets designed by Fullaondo *et al.* (1999).

It is not surprising that *G. rostochiensis* is the first PCN species to be found in Yugoslavia, as it is dominant in Europe. Recognition of the presence of PCN in Yugoslavia requires a thorough survey of their distribution and determination of the pathotypes, as necessary prerequisites for developing efficient systems for PCN control.

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