

Identification of *Xiphinema vuittenezi* by Polymerase Chain Reaction

SHESH KUMARI, JIBAN K. KUNDU and JAROSLAV POLÁK

Department of Virology, Division of Plant Medicine, Research Institute of Crop Production,
Prague-Ruzyně, Czech Republic

Abstract

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So far, the identification of the nematode species *Xiphinema vuittenezi* relied mainly on time-consuming morphological and morphometrical studies. Therefore, a polymerase chain reaction (PCR) protocol was optimised that both reliably and rapidly identifies *X. vuittenezi*. The internal transcribed spacer (ITS) species-specific primer of ribosomal DNA gene of *X. vuittenezi* was used. Nine populations of this species from Central Bohemia were investigated by means of PCR.

Keywords: nematodes; *Xiphinema vuittenezi*; PCR; ITS primer

Xiphinema vuittenezi Luc, Lima, Weischer & Flegg, 1964, is one of the most widespread migratory ectoparasitic nematodes in Europe but is particularly prevalent in Central Europe (BROWN & TAYLOR 1987). The species is regarded as one of the economically most important *Xiphinema* species for fruit tree and nut production (EVANS *et al.* 1993), it is characterised by a wide host range (LUC *et al.* 1964) and is adapted to a wide range of soil textures.

Identification of plant-parasitic nematodes based on morphology and morphometrics is time-consuming and difficult due to overlapping of many characteristics and their plasticity. The identification of species thus requires considerable experience and skill. Techniques based on DNA detection provide an attractive solution to problems associated with identification. Recently, polymerase chain reaction (PCR)-based methods have also been developed and successfully applied for nematode diagnostics (FULLAONDO *et al.* 1999; ZIJLSTRA 2000; SUBBOTIN *et al.* 2001; AMIRI *et al.* 2002; WANG *et al.*

2003). Ribosomal DNA, specifically from internal transcribed spacer (ITS) regions are often used for detection purposes (FERRIS *et al.* 1993; WILLAMSON *et al.* 1997; ZIJLSTRA *et al.* 1997). The species-specific PCR tools have also utilised areas outside the ITS region, including mitochondrial DNA (DAUTOVA *et al.* 2002) and oesophageal gland protein (RAY *et al.* 1994; TESAŘOVÁ *et al.* 2003). Compared to traditional diagnostic methods, PCR offers high specificity, sensitivity and a more rapid means of identifying large numbers of nematode samples. The development and use of a PCR method for specific identification of *X. vuittenezi*, found in Central Bohemia (KUMARI 2003), is presented in this contribution.

MATERIALS AND METHODS

Nematode populations

Populations of *X. vuittenezi* were extracted from soil by Cobb's sieving and decanting method as

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described by KUMARI (2003) from nine orchards and vineyards (apple orchards at Bříství, Chrást, Horoměřice, Slaný; peach orchard at Kutná Hora; pear orchard at Slaný and vineyards at Karlštejn, Prague-Troja, Prague-Vinohrady). Live nematodes were stored at 4°C for more than 6 months in 1M NaCl.

DNA preparation from nematodes

DNA preparation by Triton X-100. DNA templates from single nematodes were prepared according to KUNDU (2003), modified for nematode DNA preparation. A single nematode was transferred from 1M NaCl to a 2 ml Eppendorf tube containing 20 µl sterile water, and directly homogenised with a hand homogeniser (Sigma-Aldrich, Germany) for 2–3 min. The homogenate was centrifuged at 10 000 rpm for 5 min, and the supernatant was collected and transferred to a new 0.5 ml Eppendorf tube containing 1 µl Triton X-100. After incubation for 10 min at 60°C in a water bath (with mixing from time to time), the tubes were chilled on ice for 5 min. At this stage, either the PCR reaction was performed or DNA was stored at –20°C for further study.

Total DNA extraction. A phenol-chloroform procedure as described by KUNDU *et al.* (2003) was applied for total DNA extraction. Ten nematodes were crushed with a hand homogeniser (Sigma-Aldrich, Germany) in extraction buffer 100mM Tris-HCl pH 8.0 containing 50mM EDTA and 1% SDS, extracted twice with phenol:chloroform:isoamylalcohol (25:24:1), precipitated with ethanol (absolute) in the presence of LiCl and dissolved in sterile deionised water.

PCR primer. The internal transcribed spacer 1 (ITS1) region spanning the 18S and 5.8S ribosomal DNA genes was used for the PCR amplification.

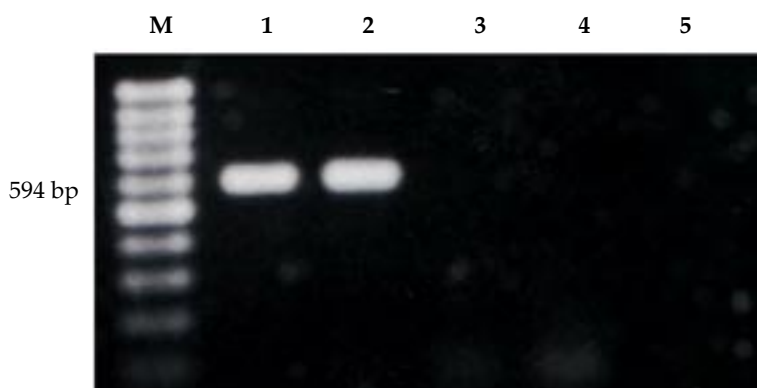
Species-specific sense primer V18 (5'-GTGGAAC-GAAAAGACCTC-3') and antisense primer located in the first 40 bp of the 5.8S gene A-ITS1 (3'-GAAT-AGCCACCTAGTGAGCCGAGCA-5'), amplified 591 bp fragment were used (WANG *et al.* 2003).

Amplification of DNA. The PCR was performed in a 25 µl reaction volume with the following mastermix: 2.5 µl 10x Qiagen PCR buffer (containing 15mM MgCl₂), 1.0 µl Q solution 5x (Qiagen), 0.5 µl 10mM dNTP, 0.5 µl Taq polymerase (5 units/µl) (Taq PCR Core Kit, Qiagen, Germany), 0.5 µl each primer (5 pmol/µl) (synthesised by Generi Biotech, Czech Republic). Five microliter DNA template (prepared by Triton X-100) or 1 µl total DNA was added to the reaction and adjusted to 25 µl by sterile deionised water. The reaction was carried out in a thermocycler (MJ research) as follows: one step at 94°C for 3 min (initial denaturation), 39 cycles of three steps: 94°C for 45 s (denaturation), 55°C for 1 min (annealing), 72°C for 1 min 20 s (polymerization), and a final step at 72°C for 10 min (elongation).

Agarose gel (1.5 %) electrophoresis. Aliquots of PCR products were electrophoresed in TAE buffer (90mM Tris-borate and 2mM EDTA) at 80 V for 45 min. The bands were stained with ethidium bromide (0.5 µg/ml) and visualised and photographed under a UV transilluminator. A 100 bp ladder (Fermentas, MBI) was used as DNA size marker.

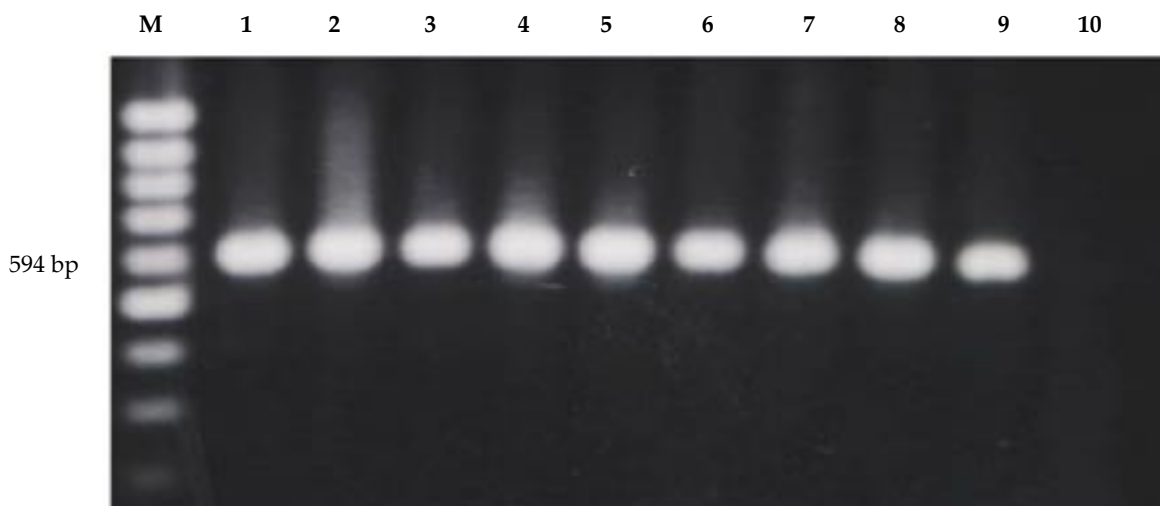
RESULTS AND DISCUSSION

PCR was performed on single females of *X. vuittenezi* from nine localities of Central Bohemia with an incidence of nematodes. Morphological and morphometrical characteristics of each nematode population from these nine localities from the Central Bohemia have been studied by KUMARI (2003). The identification of individual nematodes



Lane M – 100 bp DNA ladder; lane 1 – *X. vuittenezi* (phenol-chloroform procedure); lane 2 – *X. vuittenezi* (Triton X-100 isolated DNA); lane 3 – water control; lane 4 – *L. leptcephalus*; lane 5 – *X. diversicaudatum*

Figure 1. Electrophoresis of the amplified products from DNA isolated from bulk nematodes – *Xiphinema vuittenezi*, *Longidorus leptcephalus* and *X. diversicaudatum*



Lane M – 100 bp DNA ladder; lane 1 to 9 – *X. vuittenezi* (1 – Bříství, 2 – Horoměřice, 3 – Chrást, 4 – Karlštejn, 5 – Kutná Hora, 6 – Slaný – apple, 7 – Slaný – pear, 8 – Prague-Troja, 9 – Prague-Vinohrady), lane 10 – sample without nematode DNA

Figure 2. Electrophoresis of the amplified products from single females of *Xiphinema vuittenezi* from nine localities

of *X. vuittenezi* from all localities was reliably confirmed using ITS primers of ribosomal DNA of *Xiphinema* spp. (WANG *et al.* 2003). The ITS primers have recently been widely used for nematode identification, as this sequence is in high abundance in the genome. The inter-specific variability of the ITS1 fragment allows to design a species-specific primer for *X. vuittenezi* (591 bp) together with *X. index*, *X. italiae* and *X. diversicaudatum* without occurrence of cross-amplification (WANG *et al.* 2003). A single fragment of approximately 591 bp was amplified for all studied individuals of *X. vuittenezi* (Figure 2). No PCR products were obtained in the negative water control or the DNA templates of *Longidorus leptcephalus* and *X. diversicaudatum* (Figure 1). The DNA template preparation using Triton X-100 showed a novel approach for the PCR protocol presented in this contribution. In comparison with total DNA, the DNA prepared by Triton X-100 had similar PCR efficiency in *X. vuittenezi* identification. Triton X-100 has previously been successfully used elsewhere for RNA and DNA preparation (Ryšánek *et al.* 2000; KUNDU 2002). The PCR assay described here is a reliable and rapid means of identification of *X. vuittenezi*. The assay allowed to verify the results of a recent morphological and morphometrical study of this nematode species in Central Bohemia (KUMARI 2003). Hence, the procedure could be used for the routine identification of *X. vuittenezi* from fruit orchards.

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Souhrn

KUMARI S., KUNDU J. K., POLÁK J. (2004): **Určování nematody *Xiphinema vuittenezi* pomocí polymerázové řetězové reakce.** Plant Protect. Sci., **40**: 1–4.

Určování nematod druhu *Xiphinema vuittenezi* se provádí pomocí časově náročné morfologické a morfometrické studie. Proto byla optimalizována metoda PCR (polymerázová řetězová reakce), která je spolehlivá i rychlá pro identifikaci *X. vuittenezi*. Byl používán druhově specifický ITS primer z genomu ribozomální DNA *X. vuittenezi*. Pomocí PCR jsme diagnostikovali devět populací *X. vuittenezi* ze středních Čech.

Klíčová slova: nematody; *Xiphinema vuittenezi*; PCR; ITS primer

Corresponding author:

Dr. SHESH KUMARI, Výzkumný ústav rostlinné výroby, odbor rostlinolékařství, oddělení virologie,
161 06 Praha 6-Ruzyně, Česká republika
tel.: + 420 233 022 494, fax: + 420 233 311 592, e-mail: kumari@vurv.cz
