Identification of *Pratylenchus penetrans* (COBB) by PCR Using ITS-Based Species-Specific Primers

Taketo UEHARA¹, Takayuki MIZUKUBO², Atsuhiko KUSHIDA¹
and Yoji MOMOTA¹

For identifying *Pratylenchus penetrans*, species-specific primers (PP1 and PP2) were developed, based on the sequences of the interscribed spacer regions (ITS1 and ITS2) of the rDNA. When the purified DNA from seven *Pratylenchus* species was subjected to PCR using these primers, the 462 bp fragment was generated only for *P. penetrans*. The same fragment was obtained with DNA from the crude lysate of single juvenile and of adult (male and female) *P. penetrans*.

Jpn. J. Nematol. 28(1/2), 1-7 (1998).

Key words: identification, ITS-rDNA, PCR, *Pratylenchus penetrans*.

Among all *Pratylenchus* species inhabiting in Japan (19), *P. penetrans* is one of the most important root lesion nematodes, and damages roots of many plants (5, 7). Namely, it is a major pest of radish, carrot, burdock, lettuce, butterbur, and strawberry. The nematode-induced primary damage is a death of plant root cells around the nematodes, leading to a local lesion formation. Stunting, chlorosis, root pruning, and sparseness are successive symptoms (12).

For a successful management of nematode pests, a rapid and reliable identification of the causal species is critically important. Although a nematode identification is primarily based on morphological characteristics, it is difficult even for taxonomists due to variations in diagnostic features.

Isozyme analysis has been used to distinguish some *Pratylenchus* species (11), but it requires a large amount of nematode homogenate. Therefore, it is not suitable for nematode identification at organismic level. Using species-specific DNA probes, dot-blot analysis has been used to identify such nematodes as *Globodera* (2), *Meloidogyne* (1, 16) and *Bursaphelenchus*, but it is time-consuming and requires radioactive detection facilities.

The development of polymerase chain reaction (PCR) technology opened a way to a plant pathogen diagnosis (8). In this paper, we report newly developed *P. penetrans*-specific primers which based on the internal transcribed spacer (ITS) regions of the rDNA.

¹Laboratory of Plant Nematology, Hokkaido National Agricultural Experiment Station, Hitsujigaoka-1, Toyohira-ku, Sapporo 062-8555, Japan.

²Laboratory of Plant Nematology, Kyushu National Agricultural Experiment Station, Suya 2412, Nishigoshi, Kikuchi-gun, Kumamoto, 861-1192, Japan.

MATERIALS AND METHODS

Nematode

Pratylenchus penetrans, *P. coffeae*, *P. vulnus*, *P. loosi*, *P. brachyurus*, *P. zaeae*, and *P. crenatus* were used. These nematodes were isolated from different geographical locations and hosts (Table 1). They were monoxenically maintained on alfalfa callus on Krusberg medium (15) and active nematodes were harvested by the Baermann-funnel method.

DNA extraction

Nematodes were crushed with mortar and pestle and then incubated at 37°C for 3 h in 200 μ l of extraction buffer (10 mM Tris-HCl: pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.5% SDS, and 200 μ g/ml proteinase K). The lysate thus obtained was extracted with phenol/chloroform/isoamyl alcohol (25: 24: 1) (17). Nucleic acids were precipitated with ethanol and then dissolved in TE buffer (10 mM Tris-HCl: pH 8.0, 1 mM EDTA).

The crude lysates were also prepared by crushing a single juvenile or adult nematode with a needle in 15 μ l of lysis buffer (50 mM KCl, 10 mM Tris-HCl: pH 8.0, 2.5 mM MgCl₂, 60 μ g/ml proteinase K, 0.45% NP 40, 0.45% Tween 20, and 0.01% gelatin) (3). The lysate was incubated at 65°C for 1 h and then at 95°C for 10 min.

PCR

The ITS1 region of the rDNA was amplified using the 18S primer (5'-TTGATTACGTCCCT-

Table 1. *Pratylenchus* populations used in a PCR specificity test using the newly developed primers PP1 and PP2.

Species	Code	Location	Host	PCR*
<i>P. penetrans</i>	PH1	Hokkaido	Carrot	+
<i>P. penetrans</i>	PH2	Hokkaido	Soybean	+
<i>P. penetrans</i>	PI	Ibaraki	Radish	+
<i>P. penetrans</i>	PC	Chiba	Carrot	+
<i>P. penetrans</i>	PA	Aichi	Cabbage	+
<i>P. penetrans</i>	PH	Hyougo	Radish	+
<i>P. penetrans</i>	PS	Saga	Japanese iris	+
<i>P. coffeae</i>	CI	Ibaraki	Taro	—
<i>P. coffeae</i>	CN	Nagasaki	Taro	—
<i>P. coffeae</i>	CKU	Kumamoto	Buckwheat	—
<i>P. coffeae</i>	CKA	Kagoshima	Taro	—
<i>P. vulnus</i>	VSA	Saitama	Strawberry	—
<i>P. vulnus</i>	VS1	Saga	Strawberry	—
<i>P. vulnus</i>	VS2	Saga	Strawberry	—
<i>P. loosi</i>	LS1	Shizuoka	Tea	—
<i>P. loosi</i>	LS2	Shizuoka	Tea	—
<i>P. brachyurus</i>	BO	Okinawa	<i>Prunus campanulata</i>	—
<i>P. crenatus</i>	CH1	Hokkaido	Soybean	—
<i>P. crenatus</i>	CH2	Hokkaido	Carrot	—
<i>P. zaeae</i>	ZK	Kagoshima	Sugarcane	—
<i>P. zaeae</i>	ZO	Okinawa	Rhodesgrass	—

* + : fragment positive
— : fragment negative

GCCCTTT-3') (23) and the 5.8S R primer (5'-ACGAGCCGAGTGATCCACCG-3'). The ITS2 region of the rDNA was amplified using the 5.8S F primer (5'-CGGTGGATCACTCGGCTCGT-3') and the 26S primer (5'-TTTCACTCGCCGTTACTAAGG-3') (23). The 5.8S R primer and 5.8S F primer were constructed based on the 5.8S rRNA gene of *Heterodera avenae* (6). PCR was performed in a 25 μ l reaction mix containing about 20 ng of DNA or 5 μ l of crude lysate, 1 unit of AmpliTaq Gold (Perkin-Elmer), 0.2 mM dNTPS, 0.2 μ M of each primer, and 1 $\times$ reaction buffer (10 mM Tris-HCl: pH 8.3, 50 mM KCl, 1.5 mM MgCl₂). PCR was performed in a Perkin-Elmer 2400 thermal cycler. An initial preincubation step at 95°C for 12 min was followed by 45 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The final extension step was for 7 min at 72°C. Then, 3 to 10 μ l aliquots of the PCR products were electrophoresed in 1.0% agarose gel, stained with ethidium bromide, and visualized with UV light.

Cloning PCR amplified ITS fragments and DNA sequencing

The plasmid pUC119 was cut with *Hinc*II to yield blunt ends, and then treated with *Taq* polymerase and dTTP for T-A overhang cloning (18). Amplified DNA was cloned into the pUC119-T vector using T4 DNA ligase, transformed into *E. coli* JM109 cells, and selected on

Fig. 1. Schematic diagram and nucleotide sequences of rDNA. The *P. penetrans*-specific primers PP1 and PP2 were selected from ITS1 and ITS2 regions, respectively. Underlined sequences indicate PP1 and PP2. The coding region of 3' end of the 18S and the 5.8S, and 5' end of the 26S are outlined.

ampicillin medium containing X-Gal and IPTG. Plasmid DNA was prepared by the SDS-alkaline lysis method and purified by precipitation with polyethylene glycol (17). Plasmid DNA templates were sequenced on both strands with the dye-primer cycle using an Applied Biosystems 373A DNA sequencer (Perkin-Elmer). The sequences were analyzed using DNASIS software (Hitachi Software Engineering).

Development of *P. penetrans*-specific primers

Based on the ITS1 and ITS2 sequences, a primer set was designed and designated as the primers PP1 and PP2 specific for *P. penetrans* (Fig. 1). These primers were synthesized by BRL Life Technologies, Inc. PCR amplification of DNA obtained from seven *Pratylenchus* species was performed using these primers and AmpliTaq Gold (Perkin-Elmer). An initial preincubation step at 95°C for 12 min was followed by 40 cycles at 95°C for 30 s and at 66°C for 30 s, and finally incubated at 72°C for 5 min.

RESULTS

Irrespective of a combination of the primers examined (18S and 26S, 18S and 5.8S R, and 5.8S F and 26S), PCR amplification produced a single fragment for every *Pratylenchus* species examined, although the length of the ITS regions from purified genomic DNA were slightly different among the species (Fig. 2A-C).

The sequence alignments of *P. penetrans* was consisted of 290 bp from the ITS1 and 189 bp from the ITS2 (Fig. 1). The PCR primers, PP1 and PP2, were successfully designed based on the

Fig. 2. PCR profile obtained by using four different combinations of primers: A, 18S / 26S; B, 18S / 5.8S R; C, 5.8S F / 26S; D, PP1 / PP2. Lanes 1 and 17, 100 bp ladder; lanes 2 to 16, PH2, PI, PC, PA, PS, CI, CN, CKU, VSA, VS1, LS1, LS2, BO, CH1, and ZK. Refer Table 1 for the explanation of these codes.

Fig. 3. Detection of DNA amplified from a single individuals of *P. penetrans* in different developmental stages. Lanes 1 and 14, 100 bp ladder; lanes 2 to 4, second stage juvenile; lanes 5 to 7, fourth stage juvenile; lanes 8 to 10, male adult; lanes 11 to 13, female adult.

ITS1 and ITS2 sequences of *P. penetrans*, respectively. These primers had 50% or higher GC content and lacked in an obvious secondary structure. The PP1 sequence was 5'-ATGATG-GAAGTGTCGCCT-3' and the PP2 was 5'-CCCAACGACGGTCAAAAGG-3'. These primers did not amplify any purified genomic DNA from *P. coffeae*, *P. vulnus*, *P. loosi*, *P. crenatus*, *P. brachyurus* and *P. zaei*, but did a 462 bp fragment from the purified genomic DNA of the seven tested isolates of *P. penetrans* (Fig. 2D). Essentially the same result was obtained when the crude lysates of these *Pratylenchus* species were used as DNA templates. The primers, PP1 and PP2, amplified the 462 bp fragment too from a single individuals of the second- and fourth-stage juveniles and of male and female adults of *P. penetrans* (Fig. 3).

DISCUSSION

In the genus *Pratylenchus*, *P. penetrans* is one of the best known species which distributes widely (5, 13) and damages a wide variety of crops. For a successful crop management, accurate identification of *P. penetrans* is critically important. The morphological characteristics, which are used to separate *P. penetrans* from other species in the same genus, are variable even in a population originating from a single gravid female (22). In order to identify *P. penetrans*, we developed a simple and efficient method which was realized by the use of the species-specific two primers for PCR.

Eukaryotic ribosomal DNA contains two internal transcribed spacers (ITS1 and ITS2) which separate the three mature rRNA sequences (5.8S, 18S, and 26S). Sequence analyses of rDNA from diverse organisms revealed highly conserved sequences in the rRNA genes and highly variable ones in the ITS (9). In fact, the sequences of the rRNA and of the ITS of *P. penetrans* were similar to and considerably different from those of the other *Pratylenchus* species, respectively (6). Consequently, our ITS sequence-based primers for PCR amplified the *P. penetrans*-specific fragments.

Restriction pattern of the amplified ITS regions have been compared to discriminate nematode species (10, 14, 20, 23, 24, 26). However, restrictive digestion of the amplified products is too laborious. Contrarily, the method we developed is simple and easy, since agarose gel electrophoresis alone is required after PCR. Recently a random amplified polymorphic DNA

(RAPD) analysis has been employed to discriminate nematode species (4, 25), but its reproducibility is a subject of discussion. Many researchers are seeking for species-specific probes which yield reliable results. The method we developed in the present study is easy and reliable for the identification of *P. penetrans* juveniles and adults at organismic level.

ACKNOWLEDGMENTS

We thank Mr. H. Yoshida, Mr. K. Saito and Dr. A. Kato, Hokkaido National Agriculture Experiment Station, for their useful suggestions, Mr. M. Yoshida, National Institute of Agro-Environment Science, for his technical advice, and Dr. R. Takahashi and Dr. J. G. Dubouzet, Hokkaido National Agriculture Experiment Station, for critical reading of the manuscript.

LITERATURE CITED

- 1) BAUM, T. J., LEWIS, S. A. & DEAN, R. A. (1994) Isolation, characterization, and application of DNA probes specific to *Meloidogyne arenaria*. *Phytopathology* **84**, 489-494.
- 2) BURROWS, P. R. & PERRY, R. N. (1988) Two cloned DNA fragments which differentiate *Globodera pallida* from *G. rostochiensis*. *Rev. Nematol.* **9**, 441-445.
- 3) CASTAGNONE-SERENO, P., ESPARRAGO, G., ABAD, P., LEROY, F. & BONGIOVANNI, M. (1995) Satellite DNA as a target for PCR-specific detection of the plant-parasitic nematode *Meloidogyne hapla*. *Curr. Genet.* **28**, 566-570.
- 4) CENIS, J. L. (1993) Identification of four major *Meloidogyne* spp. by random amplified polymorphic DNA (RAPD-PCR). *Phytopathology* **83**, 76-80.
- 5) CHIKAOKA, I. (1983) Studies on damage of crops by the root lesion nematode, *Pratylenchus penetrans* COBB, and control measures especially by the utilization of marigold (*Tagetes* spp.). *Bull. Agric. Res. Inst. Kanagawa Prefect.* **125**, 1-72. (in Japanese with English summary)
- 6) FERRIS, V. R., FERRIS, J. M., FAGHIHI, J. & IREHOLM, A. (1994) Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. *J. Nematol.* **26**, 144-151.
- 7) GOTOH, A. (1974) Geographic distribution of *Pratylenchus* spp. (Nematoda: Tylenchidae) in Japan. *Bull. Kyushu Agric. Exp. Stn.* **17**, 139-224. (in Japanese with English summary)
- 8) HENSOM, J. M. & FRENCH, R. (1993) The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* **31**, 81-109.
- 9) HILLS, D. M. & DIXON, T. D. (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66**, 411-453.
- 10) IBRAHIM, S. K., PERRY, R. N., BURROWS, P. R. & HOOPER, D. J. (1994) Differentiation of species and populations of *Aphelenchoides* and of *Ditylenchus angustus* using a fragment of ribosomal DNA. *J. Nematol.* **26**, 412-421.
- 11) IBRAHIM, S. K., PERRY, R. N. & WEBB, R. M. (1995) Use of isoenzyme and protein phenotypes to discriminate between six *Pratylenchus* species from Great Britain. *Ann. appl. Biol.* **126**, 317-327.
- 12) JATALA, P. & BERGE, J. (1990) Nematode parasites of root and tuber crops. In: *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture* (LUC, M., SIKORA, R. A. & BRIDGE, J., eds.), CAB International Institute of Parasitology, 137-180.
- 13) JENKINS, M. R. & TAYLOR, D. P. (1967) *Plant Nematology*, Reinhold, New York, 68-77.
- 14) JOYCE, S. A., BURNELL, A. M. & POWERS, T. O. (1994) Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA genes. *J. Nematol.* **26**, 260-270.
- 15) KRUSBERG, L. R. & BLICKENSTAFF, M. L. (1964) Influence of plant growth regulating substances on reproduction of *Ditylenchus dipsaci*, *Pratylenchus penetrans*, and *Pratylenchus zeae* on alfalfa tissue cultures. *Nematologica* **10**, 145-150.
- 16) PIOTTE, C., CASTAGNONE-SERENO, P., BONGIOVANNI, M., DALMASSO, A. & ABAD, P. (1995) Analysis

- of a satellite DNA from *Meloidogyne hapla* and its use as a diagnostic probe. *Phytopathology* **85**, 458-462.
- 17) MANIATIS, T., FRITSCH, E. J. & SAMBROOK, J. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd Edition, Cold Spring Harbor, New York.
- 18) MARCHUK, D., DRUMM, M., SAULNO, A. & S. COLLIN, F. (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Research* **19**, 1154.
- 19) MIZUKUBO, T. (1992) The classification, morphology and distribution of the genus *Pratylenchus* in Japan. In: *Progress in Nematology* (NAKASONO, K., ed.). Jpn. Nematol. Soc., Tsukuba, 40-47. (in Japanese)
- 20) ORUI, Y. (1996) Discrimination of the main *Pratylenchus* species (Nematoda: Pratylenchidae) in Japan by PCR-RFLP analysis. *Appl. Entomol. Zool.* **31**, 505-514.
- 21) TARES, S., LEMONTEY, J. M., DE GUIRAN, G. & ABAD, P. (1994) Use of species-specific satellite DNA from *Bursaphelenchus xylophilus* as a diagnostic probe. *Phytopathology* **84**, 294-298.
- 22) TARTE, R. & MAI, W. F. (1976) Morphological variation in *Pratylenchus penetrans*. *J. Nematol.* **8**, 185-195.
- 23) VRAIN, T. C., WAKARCHUK, D. A., LEVESQUE, A. C. & HAMILTON, R. I. (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundam. appl. Nematol.* **15**, 563-573.
- 24) WENDT, K. R., VRAIN, T. C. & WEBSTER, J. M. (1993) Separation of three species of *Ditylenchus* and some host races of *D. dipsaci* by restriction fragment length polymorphism. *J. Nematol.* **25**, 555-563.
- 25) XUE, B., BAILLIE, D. L. & WEBSTER, J. M. (1993) Amplified fragment length polymorphisms of *Meloidogyne* spp. using oligonucleotide primers. *Fundam. appl. Nematol.* **16**, 481-487.
- 26) ZIJLSTRA, C., LEVER, A. E. M., UENK, B. J. & VAN SILFHOUT, C. H. (1995) Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology* **85**, 1231-1237.

Accepted for publication: March 30, 1997.

和文摘要

種特異的プライマーを用いたキタネグサレセンチュウの同定

植原 健人・水久保隆之・串田 篤彦・百田 洋二

キタネグサレセンチュウのPCR法による同定を目的として、種特異的プライマーの作成を試みた。まず、rDNAのITS領域の塩基配列を決定し、ITS1およびITS2の塩基配列に基づいて、プライマーPP1およびPP2を作成した。この2つのプライマーを用いて、*Pratylenchus*属線虫7種のDNAを鋳型としてPCRを行ったところ、キタネグサレセンチュウでのみ462 bpの種特異的な増幅産物が得られた。同一の増幅産物は、実験に供したキタネグサレセンチュウの7地域個体群からも得られた。このプライマーセットを用いた検出精度は高く、1頭の2期幼虫、4期幼虫、雄成虫または雌成虫から抽出したDNAを用いてPCRを行った結果、いずれの発育ステージからも種特異的な増幅産物が得られた。以上の結果より、本実験で用いた一対のプライマー (PP1とPP2) は、キタネグサレセンチュウの同定に有効と結論された。