Discrimination of three cyst-forming nematodes of the genus *Globodera* (Nematode: Heteroderidae) from Japan based on PCR-RFLP of ribosomal DNA

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Abstract

The ITS1-5.8S-ITS2 region of rDNA sequences from three *Globodera* species found in Japan, *G. rostochiensis*, *G. tabacum* and *G. hypolyasi*, were sequenced and compared. Sequence lengths for *G. rostochiensis*, *G. tabacum* and *G. hypolyasi* were 1,001, 1,003 and 1,016 bp, respectively. Sequence divergence within the three *Globodera* species ranged from 3.8 to 14.7%. Eight restriction enzymes (*Afa*I, *Alu*I, *Fsp*I, *Hha*I, *Hinf*I, *Mva*I, *Pma*CI and *Sac*II) were selected on the basis of sequence information. These three species were easily distinguished according to species-specific patterns by digesting the amplified products with *Alu*I or *Pma*CI. The *Fsp*I-digested pattern distinguished *G. rostochiensis* from the other *Globodera* species.

Key words: *Globodera rostochiensis*; *Globodera tabacum*; *Globodera hypolyasi*; identification, RFLP

INTRODUCTION

Three species of cyst-forming nematodes of the genus *Globodera* have been reported in Japan. *G. rostochiensis* was first found in Japan in 1972, in a potato field of Makkari Village in Hokkaido (Yamada et al., 1972). It subsequently spread to other parts of Hokkaido. Infection with this species had been restricted to Hokkaido, the northernmost island of Japan, for about 20 years. However, in 1992, *G. rostochiensis* was found in Nagasaki Prefecture of Kyushu Island, the southern island of Japan (Nakasuga and Nakasono, 1996). In 2003, it was also found in Aomori Prefecture of Honshu, the main island of Japan. In 1976, cysts of *Globodera*, which are different from those of *G. rostochiensis* in morphological characteristics, were found in a potato growing area in Nagasaki Prefecture, and the nematode was described as *G. hypolyasi* (Ogawa et al., 1983). Inoculation tests have proved that mugwort *Artemisia princeps* is a host of this species and that the potato is not a host. *G. hypolyasi* was also found in the east part of Hokkaido (Yamada and Takakura, 2003). The occurrence of *G. tabacum* was recorded for the first

time in Japan in 1998 (Sumiya et al., 2002). *G. tabacum* was found in a greenhouse of eggplants in Kochi Prefecture. The infected area is currently under surveillance and being subjected to control programs. Given the great importance of the potato cyst nematode (*G. rostochiensis*) as a parasite and quarantine species, the presence of other *Globodera* species complicates the work of identifying cyst-forming nematodes in Japan. Identifying cyst-forming nematodes based on differences in morphological and morphometric characteristics requires a great deal of skill, is time-consuming and is often inconclusive for individuals. PCR-based diagnostics provide an attractive solution to these problems. Comparative analysis of coding and non-coding regions of the ribosomal DNA (rDNA) is becoming a popular tool for species and subspecies identification, and for evaluating the phylogenetic relationships among plant-parasitic nematodes from many genera (Vrain et al., 1992; Ferris et al., 1993; Iwahori et al., 1998; Orui and Mizukubo, 1999; Mizukubo et al., 2003). RFLP analysis of the internal transcribed spacers (ITS) was useful for the identification of *Globodera* species (Thiéry and Mugniéry, 1996; Subbotin et al., 2000). In the pres-

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ent study, we performed a DNA sequencing analysis for the ITS regions of the rDNA of Japanese isolates of the three *Globodera* species, examined the sequence variations, and applied PCR-RFLP analysis for the discrimination of three *Globodera* species in Japan based on the sequences of ITS regions.

MATERIALS AND METHODS

Nematodes and DNA extraction from a single juvenile. Sixty-four isolates of *G. rostochiensis*, two isolates of *G. hypolysi*, and one isolate of *G. tabacum* were used in this study (Table 1). A cyst presoaked in sterile water was crushed and the juveniles were collected. Crude lysate of DNA was prepared using a modified method of Uehara et al. (1998). A single juvenile nematode was cut with a needle in 14 μ l of worm lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 60 μ g/ml proteinase K, 0.45% NP40, 0.45% Tween20 and 0.01% gelatin) on a glass slide under a dissecting microscope. The crude lysate was transferred to a new tube. After freezing at -80°C for 20 min, the

solution was incubated at 65°C for 70 min and then at 95°C for 15 min and kept at -20°C until use.

PCR, cloning and sequencing. Three isolates of *G. rostochiensis* (Izumi, Makkari, Hokkaido), *G. tabacum* (Yasu, Kochi) and *G. hypolysi* (Obihiro, Hokkaido) were used for sequencing. The ITS regions of rDNA (ITS-rDNA) were amplified using the primers of Ferris et al. (1993). The primer sequences were 5'-CGTAACAAGGTAGCTGTAG-3' and 5'-TCCTCCGCTAAATGATATG-3'. PCR was performed in a 25- μ l reaction mix containing about 4 μ l of crude lysate, 1 unit Takara *Ex Taq*TM (Takara-bio), 0.2 mM dNTPs, 0.2 μ M of each primer, and 10 $\times$ *Ex Taq* buffer (20 mM Mg²⁺ Plus) (Takara-bio). The PCR amplification profile consisted of 4 min at 94°C , 35 cycles of 30 s at 94°C , 30 s at 55°C and 30 s at 72°C , followed by a final step of 5 min at 72°C . Five microliters of the PCR product was analyzed by electrophoresis in a 3.0% TAE-buffered agarose gel (Sigma Type II-A). PCR products were cloned into pCR2.1 (Invitrogen). Three clones of each isolate were used for DNA sequencing. Plasmid inserts were sequenced by dideoxynucleotide chain termination using an ABI

Table 1. Origins of *Globodera* species used in this study

Species	Locality in Japan		
	Prefecture	City, Town or Village	Area
<i>G. rostochiensis</i>	Hokkaido	Bifuka	Niupu
		Hakodate	Furukawa, Ishikawa, Toyohara
		Kimobetsu	Aikawa, Fukuoka, Hiraoka, Rusan, Shiribetsu, Suzukawa
		Kiyosato	Kamishari-higashi, Kamishari-naka, Kamui, Kouyo
		Koshimizu	Mizukami
		Kucchan	Fujimi, Fuso, Kotohira, Midori, Mizuho, Suehiro, Takami, Takasago, Tatsumi
		Kyogoku	Kawanishi, Koushin, Kyogoku, Misaki
		Makkari	Hikari, Izumi*, Kamisato, Midorioka, Miharashi, Sakuragawa
		Nakashibetsu	Musa
		Niseko	Fujikawa, Higashiyama, Kondo, Niseko, Nishiyama, Satomi, Tomikawa
		Obihiro	Taisyō
		Rusutsu	Izumikawa, Rusutsu, Toyooka
		Shari	Fuji, Ikushinakita, Ikushinaminami, Koshikawa, Misaki, Nakashari, Raiun, Toyokura, Toyosato, Syuennishi
	Aomori	Kanagi	Ashino, Shichisekino
	Nagasaki	Misawa	Sabishirota, Shiogama, Shikanaka, Shimono
		Kazusa	
<i>G. tabacum</i>	Kochi	Yasu*	
<i>G. hypolysi</i>	Hokkaido	Obihiro*	
	Nagasaki		

* These three isolates were used for sequencing.

PRIZM ready reaction kit (Applied Biosystems) in an Applied Biosystems 377 automated sequencer. DNA sequences were analyzed using DNASIS (Version 3.5) software.

RFLP. The PCR products were digested separately with each of the restriction enzymes (*Afa*I, *Alu*I, *Fsp*I, *Hha*I, *Hin*fI, *Mva*I, *Pma*CI and *Sac*II). The restriction enzymes were selected on the basis of their sequence information. For six isolates of *G. rostochiensis* (Kamishari-naka, Kiyosato, Hokkaido; Obihiro, Hokkaido; Izumi, Makkari, Hokkaido; Furukawa, Hakodate, Hokkaido; Ashino, Kanagi, Aomori; Kazusa, Nagasaki), two isolates of *G. hypolyasi* (Obihiro, Hokkaido; Nagasaki) and one isolate of *G. tabacum* (Yasu, Kochi), each PCR product was digested with the eight restriction enzymes. For all of the 64 *G. rostochiensis* isolates, each PCR product was digested with the two restriction enzymes (*Alu*I and *Fsp*I). Digestion was conducted in a mixture of 5 μ l of the PCR product, 0.5 μ l of restriction enzyme, 1.0 μ l of 10 $\times$ buffer and 4.0 μ l of double-distilled water. The digested DNA fragments were separated by electrophoresis on a 3.0% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

RESULTS

Sequence analysis

The approximate size of the ITS-rDNA PCR products was 1,000 bp for all species (Fig. 1). The aligned ITS-rDNA sequences from the clones of *G. rostochiensis*, *G. tabacum* and *G. hypolyasi* are shown in Fig. 2. Sequence data are available from the DDBJ/EMBL/GenBank databases under the accession numbers AB207271, AB207272 and AB207273. Sequence lengths of *G. rostochiensis*, *G. tabacum* and *G. hypolyasi* were 1,001, 1,003 and 1,016 bp, respectively. Sequence divergence within the three *Globodera* species ranged from 3.8 to 14.7%. The ITS-rDNA sequences of *G. rostochiensis* and *G. tabacum* were similar to each other (sequence similarity: 96.2%). Sixteen nucleotide differences were observed between *G. rostochiensis* and *G. tabacum*. Sequence similarity between *G. rostochiensis* and *G. hypolyasi* and that between *G. tabacum* and *G. hypolyasi* were 85.2% and 85.8%, respectively.

Fig. 1. Amplification of the rDNA ITS regions of *Globodera* species. Nematodes: 1, *G. rostochiensis* (Kamishari-naka, Kiyosato, Hokkaido); 2, *G. rostochiensis* (Obihiro, Hokkaido); 3, *G. rostochiensis* (Izumi, Makkari, Hokkaido); 4, *G. rostochiensis* (Furukawa, Hakodate, Hokkaido); 5, *G. rostochiensis* (Ashino, Kanagi, Aomori); 6, *G. rostochiensis* (Kazusa, Nagasaki); 7, *G. hypolyasi* (Hokkaido); 8, *G. hypolyasi* (Nagasaki); 9, *G. tabacum* (Kochi). The size marker on both edge lanes is a 100-bp DNA ladder.

RFLP analysis of the ITS region

*Afa*I, *Alu*I, *Fsp*I, *Hha*I, *Hin*fI, *Mva*I, *Pma*CI and *Sac*II cut the PCR products into several fragments in at least one *Globodera* species used. The restriction patterns are shown in Fig. 3, and the fragment sizes based on sequence data except for the *Hha*I-digested pattern are shown in Table 2. In the case of *Hha*I, the fragment sizes based on sequence data disagree with the sizes of fragments separated by electrophoresis. *Alu*I cut the PCR products of the three *Globodera* species and provided species-specific restriction patterns. *Pma*CI cut the PCR products of *G. rostochiensis* and *G. tabacum* and provided species-specific restriction patterns. *Pma*CI did not cut the PCR products of *G. hypolyasi*. Therefore, the three species were easily discriminated. *Fsp*I cut only the PCR product of *G. rostochiensis* into two fragments. The *Fsp*I-digested pattern distinguished *G. rostochiensis* from the other *Globodera* species. *Sac*II cut only the PCR product of *G. tabacum* into two fragments. The *Mva*I-digested pattern showed polymorphism between the *G. rostochiensis* isolates and also indicated the occurrence of heterogeneity in the ITS regions. As for the five restriction enzymes, *Afa*I, *Hha*I, *Hin*fI, *Pma*CI and *Sac*II, intra-specific variation was not found among the six isolates of *G. rostochiensis* examined by PCR-RFLP analysis. Moreover, as for the two restriction enzymes, *Alu*I and *Fsp*I, no

<i>G. rostochiensis</i> -Izumi	:CGTAACAAGGTAGCTGAGGTGAACCTGCTGCTGGATCATTACCCAAGTGATACCAATTACCACTACCTGCTGTCAGTTGAGTCAGTGTGGGCAACA	: 100
<i>G. tabacum</i> -Kochi	:.....T.....	: 100
<i>G. hypolysi</i> -Hokkaido	:.....GT.....A.....A.....	: 98
<i>G. rostochiensis</i> -Izumi	:CCACATGCTCCGTTTGTGTTGACGGACACATGCCCGCTGTGTATGGGCTGGCACATTGACCAACAA--TGTACGGACAGCGCCCTGTGGGCACATGAG	: 198
<i>G. tabacum</i> -Kochi	:.....	: 198
<i>G. hypolysi</i> -Hokkaido	:...T.....T.....T..G..T.....T.....GT..A.....A.....	: 198
<i>G. rostochiensis</i> -Izumi	:TGTTGGGGTGAACCGATGTTGGTGGCCCTATGGGTGAGCCGACGATTGCTGCTGCTCGGGTGGCTGCGCCAACGGAGGAAGCACGCCACAGGGGCAC	: 298
<i>G. tabacum</i> -Kochi	:.....	: 298
<i>G. hypolysi</i> -Hokkaido	:...T.....T.....A.....TC..G..C..A.....A.....T..T.....G.....	: 298
<i>G. rostochiensis</i> -Izumi	:CCTAACGGCTGTGCTGGCTCTGTGCGTCGTT-GAGCGGTTGTTGCGCCTTGCAGATATGCTAACATGGAGTG--TAG-CTGCTACTCCATGTTGTAC	: 394
<i>G. tabacum</i> -Kochi	:.....G.....G.....T.....G.....T.....	: 395
<i>G. hypolysi</i> -Hokkaido	:.....T.....T.....G.....GT..G..T..T..C.....	: 398
<i>G. rostochiensis</i> -Izumi	:GTGCCGTACCTTGCGGCATGCTGCGCTTGTGTGCTAGCTCC-GTGGCCGTGATGAGACGACGTGTTAGGACCCGCTGGCATTGGCACGTGGTTTA	: 493
<i>G. tabacum</i> -Kochi	:.....CC.....A.....	: 493
<i>G. hypolysi</i> -Hokkaido	:...A.....G.....G.....T.....C.....	: 497
<i>G. rostochiensis</i> -Izumi	:AGACTTGATGAGTGCCCGCAGGACCGCCAGC-TTTTCCCATTTTTATTTATTTT-A-TGCAATTGATTGC-TAAATATTTCTAGTCTTATCGGTG	: 589
<i>G. tabacum</i> -Kochi	:.....T.....T.....T.....	: 590
<i>G. hypolysi</i> -Hokkaido	:.....G.....G.....T.....C.A.....T.....ACG.....C.....	: 597
<i>G. rostochiensis</i> -Izumi	:GATCACTCGGCTCGTGGATCGATGAAGAAGCAGCCCACTGGCATAATTAGTGTGAAGTCAGAAACCTTGAACACAGAACTTTTGAATGCATTTGCG	: 689
<i>G. tabacum</i> -Kochi	:.....	: 690
<i>G. hypolysi</i> -Hokkaido	:.....C.....	: 697
<i>G. rostochiensis</i> -Izumi	:CATTGGAGTGACATCCATTGGCAGCCTGGTTGAGGTCGTAACCAAAAAATGCATGCTGTCGCTGTTT-ATTTGCAAGATCAGC---CTTGGGCG	: 785
<i>G. tabacum</i> -Kochi	:.....T..C.....	: 785
<i>G. hypolysi</i> -Hokkaido	:.....A..AAG..G.....G.....GT.....A..C..T..T.....	: 797
<i>G. rostochiensis</i> -Izumi	:TGTTCTTGCAT-ACTATTGAATGCTACGCTGTGTAGCG-TTGGACGTGCTGGCGGAAATGTGTTGTCATTGCGGCTTTACAGACCGTAATTTAGGCAC	: 883
<i>G. tabacum</i> -Kochi	:.....	: 885
<i>G. hypolysi</i> -Hokkaido	:.....A..AAG..G.....G.....GT.....A..C..T..T.....	: 897
<i>G. rostochiensis</i> -Izumi	:GCCCTTCG-TTCACATGCGATAGCTGAATGCCTCGCCAAATAGGCATTTGCAATTGAACATTTGACCTGAAGTACAGACGTGAACACCCGCTGAACCTTAAG	: 982
<i>G. tabacum</i> -Kochi	:.....	: 984
<i>G. hypolysi</i> -Hokkaido	:.....G.....G.....A.....	: 997
<i>G. rostochiensis</i> -Izumi	:CATATCATTTAGCGGAGGA	: 1001
<i>G. tabacum</i> -Kochi	:.....	: 1003
<i>G. hypolysi</i> -Hokkaido	:.....	: 1016

Fig. 2. Sequences of ITS1, 5.8S and ITS2 and partial sequences of 18S and 26S gene regions of rDNA from *Globodera rostochiensis* (Izumi, Makkari, Hokkaido), *G. tabacum* (Kochi) and *G. hypolysi* (Hokkaido). Sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB207271, AB207272 and AB207273.

intra-specific variation in the restriction patterns was found among any of the *G. rostochiensis* isolates examined in this study.

DISCUSSION

The potato cyst nematode (*G. rostochiensis*) is a pest of worldwide importance, causing serious damage to potato crops. In Japan, seed-potato production is not allowed in infected fields even if other production conditions are favorable. In 2003, *G. rostochiensis* was found in the Tokachi region of Hokkaido, which is the best production area for seed-potatoes in Japan. The species *G. hypolysi*, collected from *Artemisia princeps* in potato fields, was described by Ogawa et al. (1983). *G. hypolysi* parasitic Asteraceae may assume importance even though they cause no economic damage themselves. If their cysts are found in a field for seed-potatoes and are mistakenly identified as those of the potato cyst nematode, no seed production is allowed in the infected field. The occurrence of *G. tabacum* was recorded for the first time in Japan in 1998.

The purpose of the present study was to obtain

PCR-RFLP profiles and information on the exact sizes of the restriction fragments for the three *Globodera* species. As for the two restriction enzymes, *AhaI* and *FspI*, no intra-specific variation in the restriction patterns was found among any of the *G. rostochiensis* examined in this study. The present work confirmed that PCR-RFLP enables clear differentiation of the three *Globodera* species. Amplification and analysis of ITS-rDNA have many advantages. The rapidity with which the profiles can be obtained and the clarity of the results allow for the easy identification of species. Sequence information of ITS-rDNA is useful for comparison with published sequence data. The sequences determined in this study were compared with previously published sequences for *G. rostochiensis* (Gr-Ja1) and *G. tabacum solanacearum* (Gt-Sol1) (Subbotin et al., 2000). *G. rostochiensis* (Izumi, Makkari, Hokkaido) and Gr-Ja1 were very similar (99.6%), and *G. tabacum* (Yasu, Kochi) and Gt-Sol1 were also very similar to each other (99.7%). Three nucleotide differences were observed between *G. rostochiensis* (Izumi, Makkari, Hokkaido) and Gr-Ja1, and two nucleotide differences were observed between *G. tabacum* (Yasu, Kochi) and Gt-Sol1.

Fig. 3. Restriction fragments of the amplified ITS regions of the rDNA of *Globodera* species. Endonuclease: A, *AfaI*; B, *AluI*; C, *FspI*; D, *HhaI*; E, *HinfI*; F, *MvaI*; G, *PmaCI*; H, *SacII*. Nematodes: 1, *G. rostochiensis* (Kamishari-naka, Kiyosato, Hokkaido); 2, *G. rostochiensis* (Obihiro, Hokkaido); 3, *G. rostochiensis* (Izumi, Makkari, Hokkaido); 4, *G. rostochiensis* (Furukawa, Hakodate, Hokkaido); 5, *G. rostochiensis* (Ashino, Kanagi, Aomori); 6, *G. rostochiensis* (Kazusa, Nagasaki); 7, *G. hypolyssi* (Hokkaido); 8, *G. hypolyssi* (Nagasaki); 9, *G. tabacum* (Kochi). The size marker on both edge lanes is a 100-bp DNA ladder.

Table 2. Restriction fragment sizes (bp) of rDNA-ITS regions of *Globodera* species based on sequence data

Species	Enzyme						
	<i>AfaI</i>	<i>AluI</i>	<i>FspI</i>	<i>HinfI</i>	<i>MvaI</i>	<i>PmaCI</i>	<i>SacII</i>
<i>G. rostochiensis</i>	601, 221, 170, 9	381, 362, 149, 97, 12	653, 348	918, 83	(715), 471, 286, 244	518, 483	1,001
<i>G. hypobysi</i>	612, 282, 113, 5, 4	907, 97, 12	1,016	1,016	407, 247, 205, 88, 69	1,016	1,016
<i>G. tabacum</i>	602, 222, 170, 9	511, 383, 97, 12	1,003	920, 83	472, 287, 244	483, 413, 107	597, 406

() : An intra-specific variation pattern.

These findings are in good agreement with results of morphological and morphometrical studies. Gr-Ja1 and Gt-Sol1 were estimated to show species-specific patterns by digesting the amplified products with *AluI* or *PmaCI* based on the sequence information. Sequence divergences of the *G. tabacum* complex were insufficient to distinguish these subspecies.

Orui (1997) reported the distinction of *G. rostochiensis* and four *Heterodera* species from Japan. We have reported here the distinction of three *Globodera* species. Twelve species of cyst-forming nematodes (*Heterodera*, *Globodera*, *Cactodera* and *Afenestrata*), including hitherto undescribed species, have been found in Japan. Cyst-forming nematodes include important agricultural pests, such as *G. pallida* and *H. schachtii*, which have not been found in Japan. Subbotin et al. (1999) reported that *AfaI*, *AluI*, *Bsp143I* and *HinfI* generated polymorphism and clearly distinguished between *G. rostochiensis* and *G. pallida*. RFLP profiles and the sequences of ITS regions are particularly useful for determining the identities of quarantine nematodes. It is necessary to obtain RFLP profiles in the ITS-rDNA of more species. The *MvaI* digestion pattern showed polymorphism among the *G. rostochiensis* populations in this study. Subbotin et al. (2000) reported that *StyI* generated polymorphism among *G. rostochiensis* isolates. Sequence variations in ITS-rDNA in populations of *G. rostochiensis* and *G. pallida* have been studied (Thiéry and Mugniéry 1996; Blok et al., 1998). Furthermore, the range of intra-specific variation should be studied to apply PCR-RFLP as a routine tool for the identification of cyst-forming nematodes in any institution.

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