[Original Article]

Geographical distribution of *Heterodera trifolii* in eastern Japan

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The geographical distribution of the clover cyst nematode *Heterodera trifolii* in eastern Japan on white clover (*Trifolium repens*) and greenhouse carnation (*Dianthus caryophyllus*) was surveyed in 2012. A total of 195 and eight soil samples were collected from the rhizospheres of white clover and greenhouse carnation in eastern Japan, respectively. Second-stage juveniles (J2s) of cyst nematodes were detected in 57 of the 195 samples of white clover (29.2%) and five of the eight samples of greenhouse carnation (62.5%) by the Baermann funnel method. The cyst nematodes were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA (rRNA) gene by using digestion patterns of *Alu*I, *Mse*I, and *Rsa*I. Consequently, *H. trifolii* was detected from 56 of the 195 samples of white clover (28.7%) and five of the eight samples of greenhouse carnation (62.5%). Our results showed that *H. trifolii* is widely distributed in eastern Japan and can be potentially a serious threat to carnation production. Nematol. Res. 46(1), 1–8 (2016).

Key words: carnation, clover cyst nematode, *Heterodera elachista*, PCR-RFLP analysis, white clover

INTRODUCTION

The clover cyst nematode, *Heterodera trifolii* Goffart is a monosexual parthenogenetic species distributed throughout Europe, Africa, Asia, Australia, New Zealand, North America, South America, and Hawaii (Mulvey and Anderson, 1974; Anon., 2003). This cosmopolitan species has a broad host range including species of Amaranthaceae, Capparaceae, Caryophyllaceae, Chenopodiaceae, Fabaceae, Geraniaceae, and Polygonaceae (Raski and Hart, 1953; Mulvey, 1959; Holtzmann and Aragaki, 1963; Norton and Isely, 1967; Mowat, 1974; Riggs, 1982; Wang and Riggs, 1999; Wang *et al*., 2001; Subbotin *et al*., 2010). This nematode is one of the most harmful parasitic nematodes to white clover (*Trifolium repens* L.) causing growth suppression and yield reduction (Subbotin *et al*., 2010). This nematode also causes severe damage to greenhouse carnation (*Dianthus caryophyllus* L.) in Japan (Momota *et al*., 1990; Toyoshima *et al*., 1992; Momota and Mizukoshi, 2000). This damage includes plant stunting, wilt, leaf curling, and delays in flower bud appearance (Momota *et al*., 1990; Momota and Mizukoshi, 2000).

In Japan, *H. trifolii* was first reported in 1961 from white clover, red clover (*T. pretense* L.), common chickweed (*Stellaria media* (L.) Vill.), and curly dock (*Rumex japonicus* Houtt.) in Hokkaido Prefecture (Inoue, 1961; Sakurai *et al*., 1961; Yamada *et al*., 1961; Yuhara *et al*., 1961). *Heterodera trifolii* was subsequently detected from white clover in Nagano Prefecture (Kureha, 1962). Recently, this pest was reported from greenhouse carnation in both Hokkaido Prefecture (Momota and Mizukoshi, 2000) and Nagano Prefecture (Momota *et al*., 1990; Toyoshima *et al*., 1992). Although distributional surveys revealed the widespread occurrence of *H. trifolii* on wild white clover in Hokkaido Prefecture (Mizukoshi, 2000) and greenhouse carnation in Nagano Prefecture (Toyoshima *et al*., 1992), the distribution of the species in other areas of Japan still remains unknown.

The internal transcribed spacer (ITS) region of the nuclear ribosomal RNA (rRNA) gene is the most generally used genetic marker for plant-parasitic nematode species identification in Japan (Orui, 1996; Orui and Mizukubo, 1999a, 1999b; Uehara, *et al*., 2005, 2006; Uesugi *et al*., 2009). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the ITS of the rRNA gene has proven to be most useful for *Heterodera* species identification (Subbotin *et al*., 2000, 2003, 2010; Amiri *et al*., 2002; Tanha Maafi *et al*., 2003; Madani *et al*., 2004). This technique requires the combination of the patterns of PCR products obtained after digestion with a series of restriction enzymes. As for Japanese *Heterodera* species,

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Orui (1997) reported that a combination of the digestion patterns of *Rsa*I with one of three restriction endonucleases, *Alu*I, *Mse*I, or *Tha*I, was effective for the discrimination of five species, including *H. elachista* Ohshima, *H. glycines* Ichinohe, *H. trifolii*, *Heterodera* sp. parasitic on tobacco, and *Globodera rostochinensis* (Wollenweber).

The aim of the present study is to determine the distribution of *H. trifolii* in eastern Japan by PCR-RFLP analysis of the ITS of the rRNA gene for species identification.

Fig. 1. Geographical distribution of *Heterodera trifolii* and *H. elachista* in eastern Japan. Numbers correspond to those in Tables 3 and 4.

MATERIALS AND METHODS

In 2012, a total of 195 soil samples were collected from the rhizospheres of white clover grown mainly in pastures in eastern Japan (Table 1): 76 samples were collected in Hokkaido Prefecture, 16 in Aomori Prefecture, 30 in Iwate Prefecture, three in Akita Prefecture, nine in Fukushima Prefecture, six in Ibaraki Prefecture, nine in Tochigi Prefecture, two in Gunma Prefecture, four in Tokyo Prefecture, 11 in Niigata Prefecture, two in Yamanashi Prefecture, 18 in Nagano Prefecture, and nine in Shizuoka Prefecture. In addition, eight soil samples were collected from the rhizospheres of greenhouse carnation in eastern Japan (Table 2): one sample in Hokkaido Prefecture and seven in Nagano Prefecture. At each site, a trowel was used to collect soil samples from more than three points at depths of approximately 5–15 cm around the roots of white clover and greenhouse carnation. Then, approximately 1 kg of soil in total was mixed well in a plastic bag, labelled, and kept in a refrigerator at 10℃ for further studies.

Nematodes were extracted from 2 × 20 g soil samples by the Baermann funnel method for three days at room temperature. In the first extraction, we looked for the presence of second-stage juveniles (J2s) of cyst nematodes, and if any were detected, one J2 for each soil

Table 1. Numbers of soil samples of the rhizosphere of white clover in eastern Japan with *Heterodera* detection status.

Region	Prefecture	Number of soil samples	*Heterodera* positive	Species	
				H. trifolii	*H. elachista*
Hokkaido	Hokkaido	76	26	26	0
Tohoku	Aomori	16	8	8	0
	Iwate	30	7	7	0
	Akita	3	1	1	0
	Fukushima	9	3	3	0
Kanto	Ibaraki	6	0	0	0
	Tochigi	9	1	1	0
	Gunma	2	1	1	0
	Tokyo	4	0	0	0
Chubu	Niigata	11	2	1	1
	Yamanashi	2	2	2	0
	Nagano	18	4	4	0
	Shizuoka	9	2	2	0
Total		195	57	56	1

Table 2. Numbers of soil samples of the rhizosphere of greenhouse carnation in Hokkaido Prefecture and Nagano Prefecture with *Heterodera* detection status.

Region	Prefecture	Number of soil samples	*Heterodera* positive	Species
				H. trifolii
Hokkaido	Hokkaido	1	1	1
Chubu	Nagano	7	4	4
Total		8	5	5

sample was identified to the species level by PCR-RFLP analysis. Subsequently, to confirm species identification, an additional extraction was performed for those soil samples from which J2s had been detected in the first extraction, and five J2s of cyst nematodes were then identified. Consequently, a total of six J2s were identified in each soil sample. If fewer than six J2s were thus extracted, then all of them were identified. There were the following exceptions among the white clover samples: in two samples (sample codes: H48 and H62), only five J2s were identified in the first extraction without additional extraction; in one sample (H51), three J2s in the first extraction and three J2s in an additional extraction were identified; in one sample (T35), we identified five of six extracted J2s without additional extraction, because we failed to extract DNA from one J2 specimen; in one sample (FU2), two J2s in the first extraction and four J2s in additional extraction were identified. A total of 264 and 30 J2s of cyst nematodes were identified in the white clover and greenhouse carnation samples, respectively (Tables 3, 4). For white clover samples, six extracted J2s were identified in 33 samples, five J2s in six samples, four J2s in two samples, three J2s in four samples, two J2s in four samples, and one J2 in eight samples (Table 3). For greenhouse carnation samples, six extracted J2s were identified in all five samples (Table 4).

DNA was extracted from individual J2s. A single nematode was placed into a drop of sterile distilled water on a clean glass slide. After the water dried, the nematode was crushed with a small sterile filter-paper chip under a stereo microscope using forceps (Iwahori *et al*., 2000). The paper chip was then dropped into a 1.5 ml plastic tube containing 4 µl of 0.1% SDS lysis buffer consisting of 10 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 500 µg/ml Proteinase K (Takara Bio, Shiga, Japan), and 0.1% SDS, and then the tube was incubated first at 50℃ for 2 h, followed by 95℃ for 10 min (Sakai, 2010). After the incubation, 196 µl of sterile distilled water was added to yield 200 µl of lysate for each specimen, which was then stored at –20℃ until used as the template for PCR amplification. PCR amplification was performed in a final volume of a 20 µl reaction mixture consisting of 10 µl of 2 × Quick Taq® HS DyeMix (Toyobo, Osaka, Japan), 0.4 µl (10 pmol/µl) of each primer, 5 µl of DNA template, and 4.2 µl of distilled water. The ITS region of the rRNA gene was amplified by using the F194 (5'-CGT AAC AAG GTA GCT GTA G-3') and F195 (5'-TCC TCC GCT AAA TGA TAT G-3') primers, described by Ferris *et al*. (1993). The amplification conditions were as follows: a single step of pre-denaturation at 94℃ for 2 min, followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 50℃ for 30 sec, and extension at 72℃ for 1 min. PCR products were separated by 1.5% agarose gel (Agarose-ME, Classic Type, Nacalai Tesque, Kyoto, Japan) electrophoresis with 0.5 × TBE buffer, stained by GelRed™ (Biotium, San Francisco, CA, USA), and visualized by illumination with UV light.

For the species identification, *Alu*I, *Mse*I, and *Rsa*I digestion patterns were examined for each extracted cyst nematode. These three endonucleases were used as standard endonucleases for PCR-RFLP analysis in Orui (1997). Four µl of the PCR products were digested with 2–3 U of restriction enzyme in a total volume of 10 µl at 37℃ for 3 h. The RFLP patterns were analyzed by 2.0% agarose gel electrophoresis in 0.5 × TBE buffer.

RESULTS AND DISCUSSION

For the soil samples collected from the rhizosphere of white clover, J2s of cyst nematodes were detected in 57 of 195 samples examined (29.2%) (Fig. 1; Tables 1, 3). A total of 264 J2s of cyst nematodes were identified, among which 263 were *H. trifolii* and one was *H. elachista* (Table 3). *Heterodera trifolii* was detected from white clover in 26 of 76 samples (34.2%) in the Hokkaido region, 19 of 58 samples (32.7%) in the Tohoku region, two of 21 samples (9.5%) in the Kanto region, and 10 of 40 samples (25.0%) in the Chubu region (Table 1). For the soil samples collected from the rhizosphere of greenhouse carnation, J2s of cyst nematodes were detected in five of eight samples examined (62.5%) and a total of 30 J2s of cyst nematodes were all identified as *H. trifolii* (Fig. 1; Tables 2, 4). In the past distributional surveys of *H. trifolii* conducted in Hokkaido Prefecture and Nagano Prefecture, species identification was only

Table 3. Species of *Heterodera* identified from soil samples of the rhizosphere of white clover in eastern Japan.

No.	Region	Prefecture	Locality	Sample code	*n*[1]	Species	
						H. trifolii	*H. elachista*
1	Hokkaido	Hokkaido	Sapporo City	HA23/22	6	6	0
2			Sapporo City	HA23/61	6	6	0
3			Sapporo City	HA35	3	3	0
4			Sapporo City	HA29	1	1	0
5			Sapporo City	HA11	1	1	0
6			Sapporo City	HA9	6	6	0
7			Sapporo City	HU1	5	5	0
8			Sapporo City	HU2	6	6	0
9			Sapporo City	HU3	6	6	0
10			Sapporo City	HU4	3	3	0
11			Memuro Town	H3	2	2	0
12			Memuro Town	H4	6	6	0
13			Makubetsu Town	H10	6	6	0
14			Makubetsu Town	H13	1	1	0
15			Toyokoro Town	H15	6	6	0
16			Otofuke Town	H21	6	6	0
17			Rikubetsu Town	H31	6	6	0
18			Oketo Town	H32	4	4	0
19			Kitami City	H34	6	6	0
20			Kitami City	H38	6	6	0
21			Bihoro Town	H43	6	6	0
22			Shari Town	H48	5	5	0
23			Shari Town	H51	6	6	0
24			Shibecha Town	H58	3	3	0
25			Teshikaga Town	H61	6	6	0
26			Teshikaga Town	H62	5	5	0
27	Tohoku	Aomori	Kuroishi City	T20	6	6	0
28			Towada City	T23	6	6	0
29			Shichinohe Town	T25	6	6	0
30			Shichinohe Town	T26	6	6	0
31			Tohoku Town	T29	1	1	0
32			Oirase Town	T32	6	6	0
33			Hachinohe City	T35	5	5	0
34			Hashikami Town	T36	6	6	0
35		Iwate	Tono City	T4	6	6	0
36			Tono City	T6	4	4	0
37			Takizawa City	T8	2	2	0
38			Takizawa City	T11	6	6	0
39			Hachimantai City	T13	6	6	0
40			Morioka City	T50	6	6	0
41			Morioka City	T52	6	6	0
42		Akita	Odate City	T19	6	6	0
43		Fukushima	Inawashiro Town	FU2	6	6	0
44			Samegawa Village	Z2	5	5	0
45			Samegawa Village	Z4	5	5	0
46	Kanto	Tochigi	Nasu Town	TG2	2	2	0
47		Gunma	Katashina Village	G12	3	3	0
48	Chubu	Niigata	Agano City	NI8	1	0	1
49			Shibata City	NI11	1	1	0
50		Yamanashi	Oshino Village	Y6	6	6	0
51			Oshino Village	Y7	2	2	0
52		Nagano	Saku City	NW1	6	6	0
53			Saku City	NW2	6	6	0
54			Saku City	NW3	1	1	0
55			Karuizawa Town	NW6	6	6	0
56		Shizuoka	Gotemba City	S2	1	1	0
57			Gotemba City	S3	6	6	0
	Total				264	263	1

[1]*n*: numbers of J2 used for molecular identification.

Table 4. Species of *Heterodera* identified from soil samples of the rhizosphere of greenhouse carnation in Hokkaido Prefecture and Nagano Prefecture.

No.	Region	Prefecture	Locality	Sample code	*n*[1]	Species	
						H. trifolii	*H. elachista*
1	Hokkaido	Hokkaido	Nanae Town	NAN	6	6	0
2	Chubu	Nagano	Sakuho Town	SAK	6	6	0
3			Fujimi Town	FJO	6	6	0
4			Fujimi Town	FJS	6	6	0
5			Matsumoto City	MAT	6	6	0
	Total				30	30	0

[1]*n*: numbers of J2 used for molecular identification.

based on host plants and not confirmed by morphological or molecular methods (Toyoshima *et al*., 1992; Mizukoshi, 2000). Our results molecularly confirmed the wide distribution of *H. trifolii* in both prefectures as previously reported by Toyoshima *et al*. (1992) and Mizukoshi (2000). This is the first record of *H. trifolii* in nine prefectures, Aomori, Iwate, Akita, Fukushima, Tochigi, Gunma, Niigata, Yamanashi, and Shizuoka Prefectures. Thus, our survey clearly revealed that *H. trifolii* is widely distributed in eastern Japan. This is the first work that provides valuable new information on the geographical distribution of *H. trifolii* throughout eastern Japan by using PCR-RFLP analysis of the ITS of the rRNA gene.

Carnation is one of the important ornamental flowers in Japan. Three prefectures of eastern Japan including Nagano Prefecture, Hokkaido Prefecture, and Chiba Prefecture, are the first, third, and fourth carnation producers, respectively, and account for approximately 45% of the total carnation production area of Japan (MAFF, 2014). Severe damage to greenhouse carnation caused by *H. trifolii* has been reported from Hokkaido Prefecture (Mizukoshi, 2000; Momota and Mizukoshi, 2000) and Nagano Prefecture (Momota *et al*., 1990; Toyoshima *et al*., 1992). Momota and Mizukoshi (2000) detected *H. trifolii* from wild white clover around greenhouses and pointed out that areas of wild white clover around greenhouses could be potential sources of *H. trifolii* infection in the greenhouse carnation. Considering its wide distribution on wild white clover in eastern Japan, *H. trifolii* could potentially be an even more serious threat to carnation production. It is most important to prevent the introduction of *H. trifolii* into greenhouses and to use nematicides appropriately for nematode control in carnation production areas. Because there are also carnation production areas in western Japan, *e.g*., Aichi, Hyogo, Fukuoka, and Nagasaki Prefectures (MAFF, 2014), the distribution of *H. trifolii* should be surveyed in western Japan in the future.

The Japanese cyst nematode, *H. elachista*, was detected from one soil sample of wild white clover collected from a fallow rice field in Niigata Prefecture (Fig. 1; Tables 1, 3). This nematode species is widely distributed from the Tohoku region to the Kyushu region in Japan and is mainly detected from upland rice (*Oriza sativa* L.) (Shimizu and Momota, 1992). Although rice is a good host of *H. elachista*, white clover is a non-host of this nematode (Subbotin *et al*., 2010). In the fallow rice field in which that soil sample was collected, volunteer rice was also growing around the white clover. Therefore, it is most likely that *H. elachista* parasitizing volunteer rice in the fallow rice field was accidentally detected.

In the present study, we identified the detected cyst nematodes by using PCR-RFLP analysis of the ITS of the rRNA gene, because this is a less time-consuming procedure than morphological methods or other molecular methods such as sequencing analysis (Uesugi *et al*., 2009). We used the primer sets of Ferris *et al*. (1993) and three endonucleases, *Alu*I, *Mse*I, and *Rsa*I, in accordance with Orui (1997). Consequently, we could clearly distinguish the two detected species, *H. trifolii* and *H. elachista*, by the digestion patterns of *Alu*I, *Mse*I, and *Rsa*I (Fig. 2). No intraspecific variation was observed in the digestion patterns of these three enzymes. Our results confirmed that practical identification in a field survey was successfully conducted by PCR-RFLP analysis with a combination of digestion patterns of *Rsa*I with one of two restriction enzymes, *Alu*I or *Mse*I.

ITS heterogeneity, which has been previously reported for several *Heterodera* species, contributes to the complexity of the restriction digestion patterns (Orui, 1997; Szalanski *et al*., 1997; Subbotin *et al*., 2000; Waeyenberge *et al*., 2009). For example, although the *Rsa*I digestion pattern of *H. trifolii* consisted of three bands of *ca*. 820 bp, *ca*. 580 bp, and *ca*. 230 bp, the total combined size of the restriction fragments is

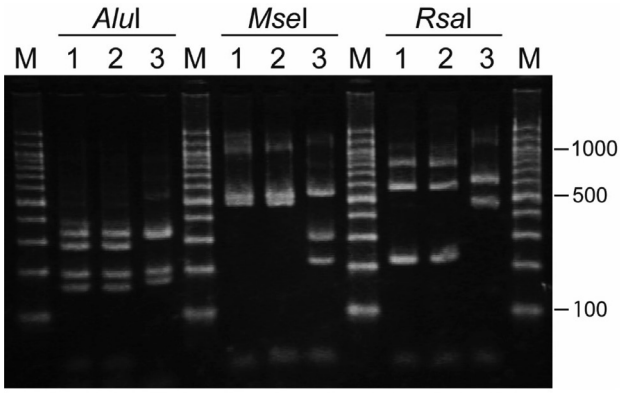

Fig. 2. PCR-RFLP profiles of *Heterodera trifolii* and *H. elachista*, digested with three restriction endonucleases, *Alu*I, *Mse*I, and *Rsa*I. Lane 1: *H. trifolii* from white clover in Hokkaido Prefecture (sample code: HU1); Lane 2: *H. trifolii* from greenhouse carnation in Nagano Prefecture (SAK); Lane 3, *H. elachista* from white clover in Niigata Prefecture (NI8); M, 100 bp DNA ladder (Nacalai Tesque).

approximately one and a half times larger than the PCR product size (Orui, 1997). This phenomenon indicates that ITS heterogeneity exists in *H. trifolii*. Amiri *et al.* (2002) pointed out that the *Rsa*I digestion pattern of the *H. trifolii* population from New Zealand differed from those of the European population studies by Subbotin *et al*. (2000). The results of these studies were not comparable, however, because Amiri *et al.* (2002) and Subbotin *et al.* (2000) used different primer sets from those used by Orui (1997) and this study. Nevertheless, intraspecific variations resulting from ITS heterogeneity might be detected when examining *H. trifolii* populations having more diverse geographical origins.

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