Characterization of Japanese populations of the clover cyst nematode, *Heterodera trifolii*, and the Korean cyst nematode, *H. koreana*

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Contents

Abbreviations

AIC	Akaike information criterion
ANOVA	analysis of variance
BI	Bayesian inference
bp	base pair
COI	cytochrome c oxidase subunit 1
df	degree of freedom
DIC	differential interference contrast
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ESS	effective sample size
EU	European Union
HARC	Hokkaido Agricultural Research Center
HSD	honestly significant difference
Hsp90	heat shock protein 90
ITS	inter transcribed spacer
J2	second-stage juveniles
LM	light microscopy
MAFF	Ministry of Agriculture, Forestry and Fisheries
MS	mean square
mtDNA	mitochondrial DNA
NARO	National Agriculture and Food Research Organization

PCR	polymerase chain reaction
PP	posterior probabilities
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
S.D.	standard deviation
S.E.	standard error
SDS	sodium dodecyl sulfate
TBE	Tris-borate-EDTA
UV	ultraviolet

Abbreviations used for nematode measurements

L = body length.

a = body length/greatest mid-body diameter.

b = body length/distance from anterior end to pharyngo-intestinal valve.

b′ = body length/distance from anterior end to posterior end of pharyngeal glands.

c = body length/tail length.

c′ = tail length/body diameter at anus.

DGO = distance from the dorsal pharyngeal gland orifice to the stylet base.

General Introduction

Nematodes are the most abundant and ubiquitous multicellular organisms on earth. They are successfully adapted to a greater variety of habitats than any other group of multicellular animals and occur worldwide in all environments (Hunt *et al.*, 2005). In spite of their various habitats, nematodes have a similar external morphology, with a bilaterally symmetrical, cylindrical, unsegmented, worm-like body (Perry and Mones, 2011). Although most nematodes are microscopic (less than 1 mm in length), the animal-parasitic nematode species are often considerably larger (*e.g.*, *Placentonema gigantissimum* (Gubanov, 1951) which can grow up to eight meters in whale) (Perry and Mones, 2011). Nematodes belong to the phylum Nematoda comprising over 25,000 described species (Hugot *et al.*, 2001). Many species are free-living, feeding on bacteria or fungal spores, whereas others are predatory or parasites of animals, including other nematodes, and a wide variety of algae, fungi, and higher plants (Hunt *et al.*, 2005). Nematodes are generally amphimitic with an egg stage, four juvenile stages, and adult stage. Some species lack males and reproduce either by parthenogenesis or hermaphrodism (Decraemer and Hunt, 2013).

Plant-parasitic nematodes are major agricultural pests causing severe damage and economic losses worldwide in many crops. There are over 4,100 species of plant-parasitic nematodes described to date (Decraemer and Hunt, 2013). They have a stylet, which can be protruded and used to penetrate plant cells like a hypodermic needle. Plant-parasitic nematodes include several economically important species, *e.g.*, the root-knot nematodes (*Meloidogyne* spp.), the cyst nematodes (*Heterodera* and *Globodera* spp.), the lesion nematode (*Pratylenchus* spp.), and the dagger nematodes

(*Xiphinema* spp.). Annual crop yield losses caused by plant-parasitic nematodes are estimated at 8.8–14.6% of total crop production and $100–157 billion worldwide (Sasser and Freckman, 1987; Koenning *et al.*, 1999; Abad *et al.*, 2008; Nicol *et al.*, 2011). Although many plant-parasitic nematodes feed on plant roots, some species feed on the aerial parts of plants, including stems, leaves, buds, flowers, and seeds (Perry and Mones, 2011).

The cyst nematodes of the genus *Heterodera* Schmidt, 1871 are one of the major groups of plant-parasitic nematodes and of great economic importance in many countries throughout the world (Turner and Subbotin, 2013). They are sedentary endoparasites having a characteristic definite survival stage, called cyst, which is the lemon-shaped and hardened dead female body with eggs (Bridge and Starr, 2007). The life cycle of cyst nematode of the genus *Heterodera* is shown in Figure 1. The protective cyst enables them to withstand desiccation and persist for many years in the soil in the absence of a host, that makes *Heterodera* species particularly threatening as invasive pests (Waeyenberge *et al.*, 2009; Subbotin *et al.*, 2010b; Turner and Subbotin, 2013). Consequently, many species have attracted considerable attention as quarantine pests. *Heterodera* species cause serious damage to many important crops by restricting root growth and uptake nutrients, leading considerable yield losses. Some of these species, *e.g.*, the European cereal cyst nematode, *H. avenae* Wollenweber, 1924, the soybean cyst nematode, *H. glycines* Ichinohe, 1952, and the sugar beet cyst nematode, *H. schachtii* Schmidt, 1871, are recognized as the most important agricultural pests of crops because of their wide distribution and impact on world agriculture. For example, *H. schachtii* has been recognized as a serious pest of sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) and some other crops, with a broad host range that includes many species

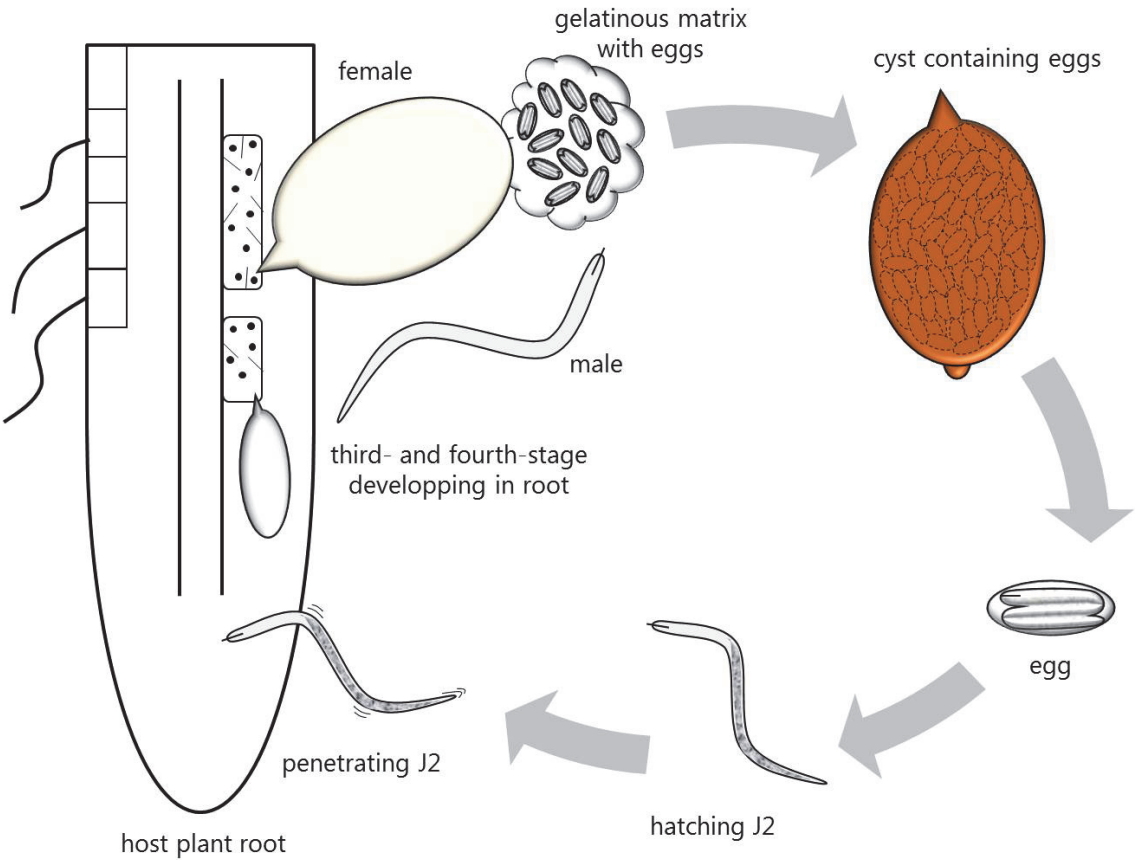

Fig. 1. Life cycle of cyst nematode of the genus *Heterodera*.

from different plant families, such as Amaranthaceae and Brassicaceae (Franklin, 1972). This nematode is found in all major sugar beet production areas of the world, favouring temperate regions but apparently tolerating a broad range of climates (Baldwin and Mundo-Ocampo, 1991). Annual yield losses in EU countries caused by *H. schachtii* was estimated in 1999 at up to €90 million (Müller, 1999). This species is an important regulated pest in South America and parts of Asia and is also included on the quarantine pest list in Japan (Hockland *et al.*, 2013; Plant Protection Station, MAFF, 2016). These countries are all concerned about the introduction of *H. schachtii*.

Heterodera is the type genus of the family Heteroderidae proposed by Schmidt (1871). This family was established for species having sexual dimorphism: the females become swollen whilst the males remain threadlike, lacking caudal alae (Schmidt, 1871). Measurements fron the cysts and J2 of the genus *Heterodera* are shown in Figures 2 and 3. This is also the largest genus of the subfamily Heteroderinae, including 85 valid species distributed throughout the world (Subbotin *et al.*, 2010b; Wang *et al.*, 2013; Zhuo *et al.*, 2013, 2014; Subbotin, 2015; Kang *et al.*, 2016). These species were originally considered to occur in temperate regions, but many species are now known to be present in tropical and subtropical regions (Evans and Rowe, 1998). On the basis of morphological and molecular data, most of the *Heterodera* species have been divided into the following seven species groups: *afenestrata* (7 species), *avenae* (12), *cyperi* (16), *goettingiana* (16), *humuli* (6), *sacchari* (6), and *schachtii* (16). However, the following six species are not assigned to these species group: *H. bifenestra* Cooper, 1955, *H. salixophila* Kirjanova, 1969, *H. skohensis* Kaushal, Sharma & Singh, 2000, *H. sojae* Kang, Eun, Ha, Kim, Park, Kim & Choi, 2016, *H. spinicaudata* Wouts, Shoemaker, Sturhan & Burrows, 1995, and *H. zeae* Koshy, Swarup & Sethi, 1971. In

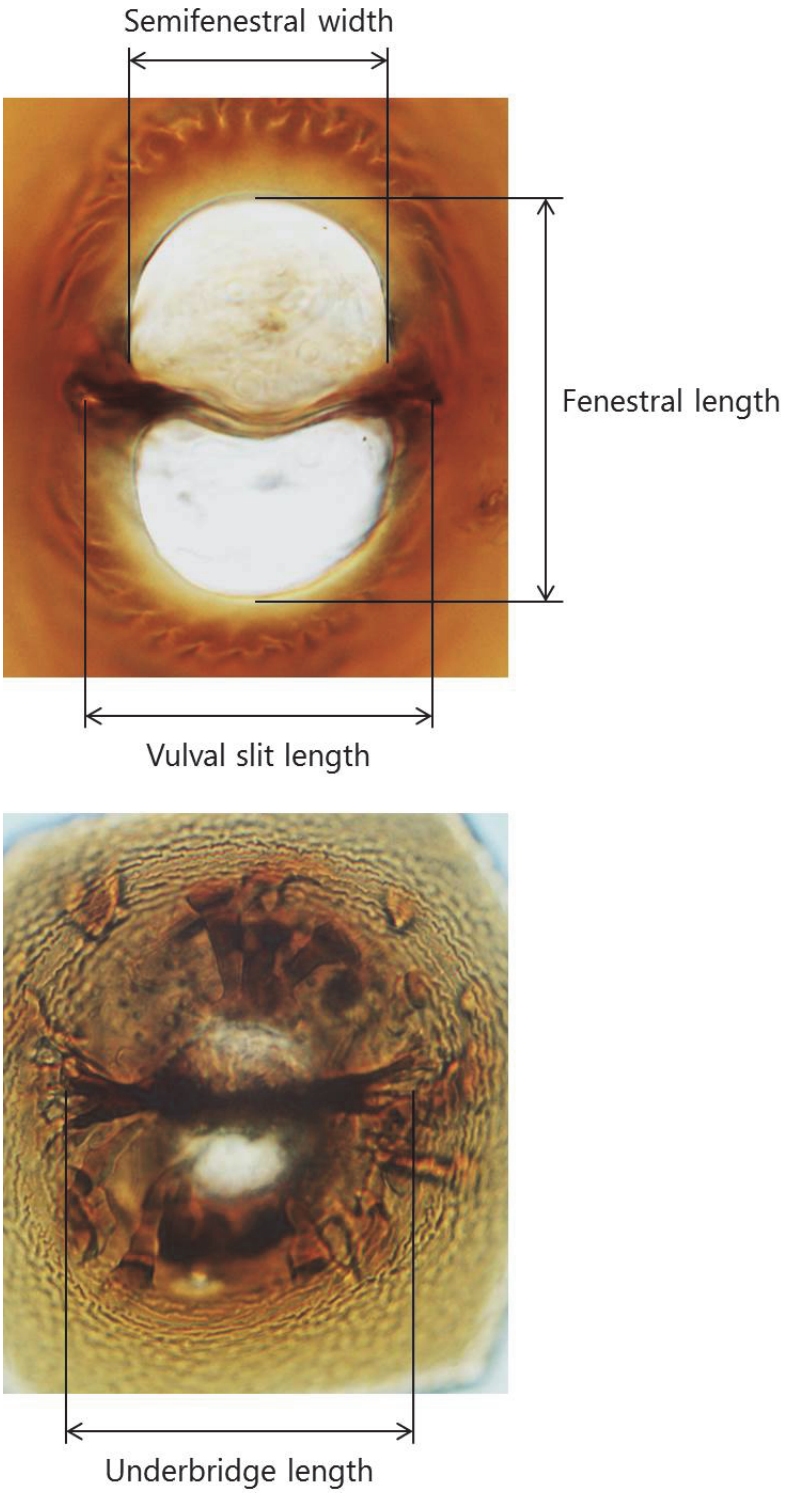

Fig. 2. Measurements of the fenestral area (fenestra and underbridge) of cyst nematode of the genus *Heterodera*, important for species and genus diagnostics.

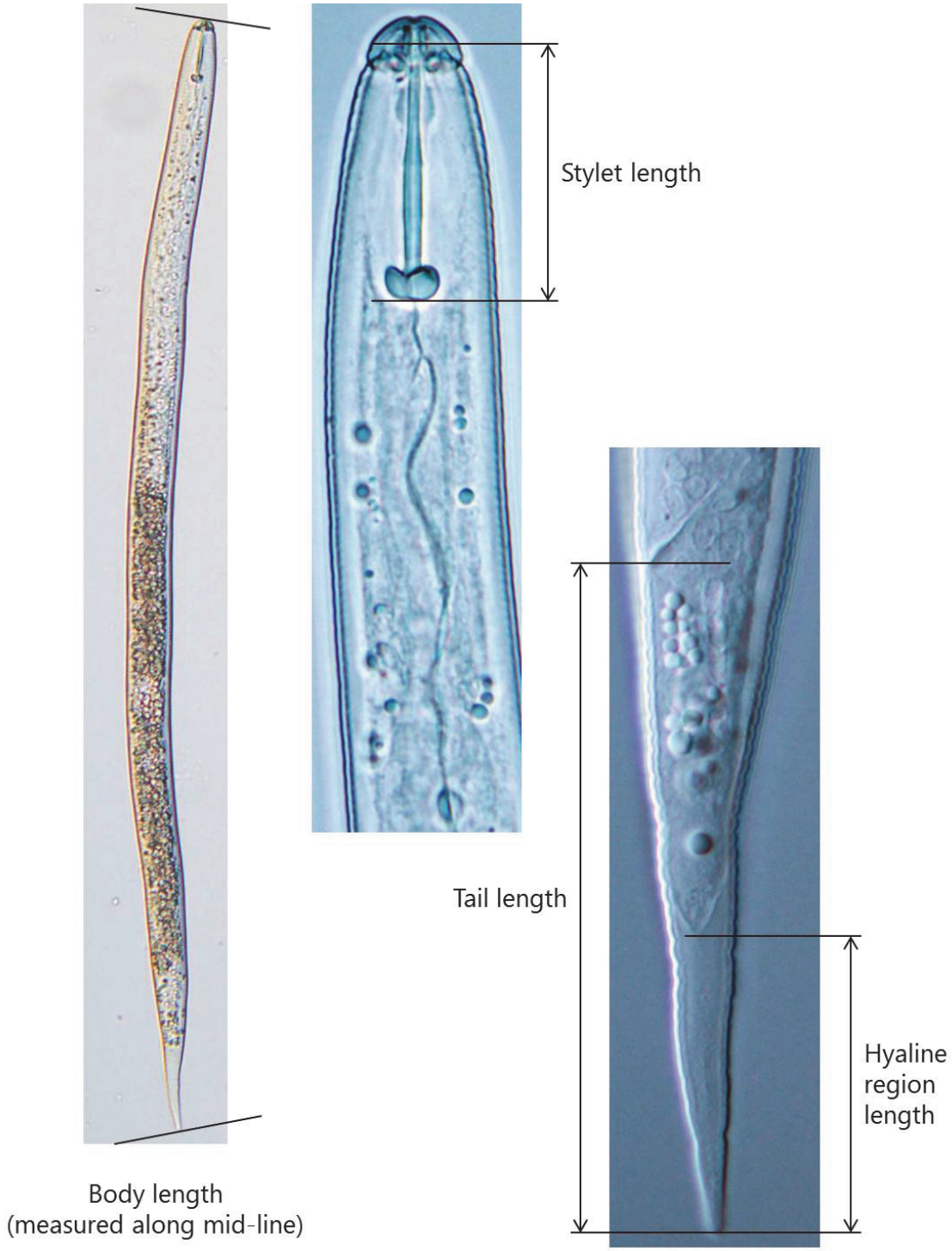

Fig. 3. Measurements of J2 of cyst nematode of the genus *Heterodera*, important for species and genus diagnostics.

Japan, eight *Heterodera* species, *H. avenae*, *H. elachista* Ohshima, 1974, *H. glycines*, *H. humuli* Filipjev, 1934, *H. latipons* Franklin, 1969, *H. trifolii* Goffart, 1932, an unidentified *Heterodera* sp. on soybean (*Glycine max* (L.) Merrill), and an unidentified *Afenestrata* sp. (= *Heterodera* sp.) on bamboo, have been recorded (Momota, 2004).

One of the Japanese species, the clover cyst nematode, *H. trifolii*, is a polyploid monosexual parthenogenetic species distributed throughout Africa, Asia, Australia, Europe, Hawaii, New Zealand, North America, and South America (Fig. 4) (Mulvey and Anderson, 1974; Anonymous, 2003). This species belongs to the *schachtii* group. Recent morphological and molecular studies revealed that *H. trifolii* is closely related to other species of the *schachtii* group, *viz.*, *H. betae* Wouts, Rumpenhorst & Sturhan, 2001, *H. daverti* Wouts & Sturhan, 1978, *H. glycines*, and *H. schachtii* (Ambrogioni and Irdani, 2001; Amiri *et al.*, 2002, 2003; Madani *et al.*, 2007; Vovlas *et al.*, 2015). In Japan, *H. trifolii* has been reported only from Hokkaido (Inoue, 1961; Sakurai *et al.*, 1961; Yamada *et al.*, 1961; Yuhara *et al.*, 1961; Mizukoshi, 2000; Momota and Mizukoshi, 2000) and Nagano Prefectures (Kureha, 1962; Momota *et al.*, 1990; Toyoshima *et al.*, 1992). This cosmopolitan species shows a broad host range, including species of Amaranthaceae, Capparaceae, Caryophyllaceae, 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). Although white clover (*Trifolium repens* L.), red clover (*T. pratense* L.), and carnation (*Dianthus caryophyllus* L.) are the most common hosts of this species (Wang and Riggs, 1999; Subbotin *et al.*, 2010b), variation in reproductive ability across different host is known among different populations of *H. trifolii* (Singh and Norton, 1970; Hirschmann and Triantaphyllou, 1979; Maas *et al.*, 1982; Wang and Riggs, 1999;

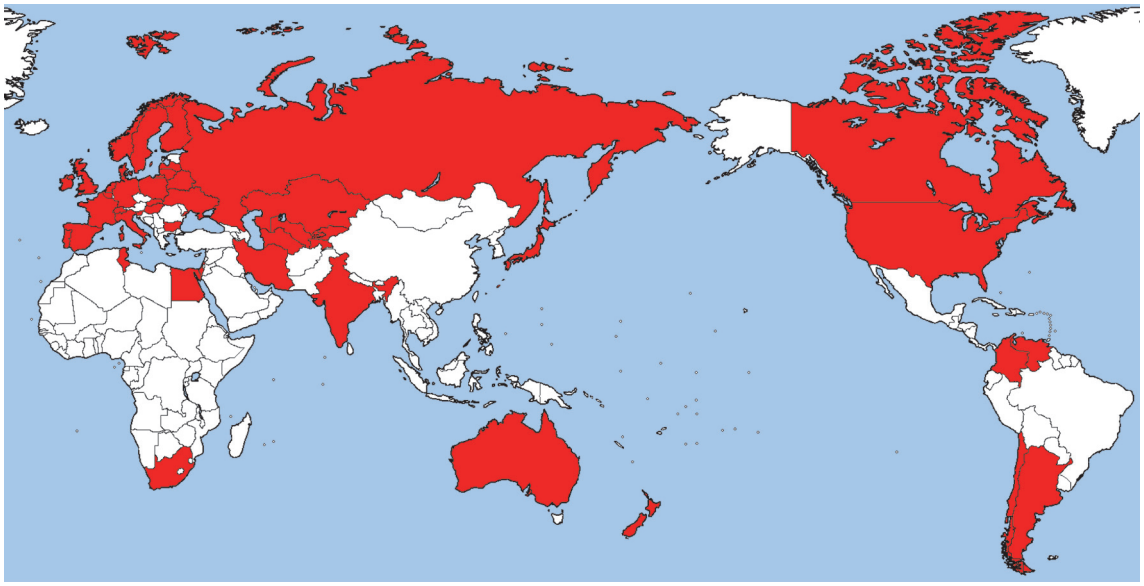

Fig. 4. Distribution map of *Heterodera trifolii*.

Wang *et al.*, 2001), indicating the presence of host and/or geographical races.

Traditionally, *Heterodera* species have been identified based on morphological and morphometric characters. DNA-based approaches using rRNA and *COI* mtDNA gene sequences have recently been utilized as useful markers for species identification, estimation of species-level phylogeny within *Heterodera*, and identification of intraspecific variations and phylogeny. Rapid and accurate identification of the *Heterodera* species is important as a first step in selecting effective and appropriate measures for control of these nematodes. Application of control measure, especially when growing resistant crops, requires accurate identification of the cyst nematodes up to species level (Waeyenberge *et al.*, 2009). Fast and reliable identification of quarantine nematodes and their close non-quarantine relatives is also essential for effective quarantine inspection and implementation of phytosanitary measures. However, there is little information available on distributional, morphological, morphometric, and biological data for most Japanese *Heterodera* species to date, except for *H. glycines* that is the most economically important species in Japan. Especially, no molecular data on the other Japanese *Heterodera* species is available.

The aims of the present thesis are: *i*) to provide distributional, morphological, morphometric, molecular, and biological data of Japanese *H. trifolii* populations; *ii*) to identify an unidentified cyst nematode species on bamboo in Japan and to provide its distributional, morphological, morphometric, and molecular data; and *iii*) to reveal phylogenetic relationships within *Heterodera* using rRNA and *COI* gene sequences. The present thesis consists of four chapters. In Chapter 1, the geographical distribution of *H. trifolii* on white clover and greenhouse carnation was investigated in eastern Japan. In Chapter 2, Japanese populations of *H. trifolii* were characterized morphologically,

morphometrically, and molecularly. In Chapter 3, reproductive ability of three Japanese populations of *H. trifolii* was examined on five crops and three carnation cultivars. In Chapter 4, *H. koreana* (Vovlas, Lamberti & Choo, 1992) Mundo-Ocampo, Troccoli, Subbotin, Del Cid, Baldwin & Inserra, 2008 was recorded from Japan for the first time, and the species was characterized morphologically, morphometrically, and molecularly.

Chapter 1

Geographical distribution of *Heterodera trifolii* in eastern Japan

1.1 Introduction

In Japan, *H. trifolii* was first reported in 1961 from white clover, red clover, common chickweed (*Stellaria media* (L.) Vill.), and Japanese dock (*Rumex japonicus* Houtt.) in Hokkaido Prefecture (Inoue, 1961; Sakurai *et al.*, 1961; Yamada *et al.*, 1961; Yuhara *et al.*, 1961). This species was subsequently detected from white clover in Nagano Prefecture (Kureha, 1962). Recently, this species was reported from greenhouse carnation in both Hokkaido (Momota and Mizukoshi, 2000) and Nagano Prefectures (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 ITS (ITS1 and ITS2) regions are the most generally used genetic markers for plant-parasitic nematode species identification in Japan (Orui, 1996, 1997; Orui and Mizukubo, 1999a, 1999b; Uehara *et al.*, 2005, 2006; Uesugi *et al*., 2009). PCR-RFLP analysis of the ITS1-5.8S-ITS2 region has proven to be most useful for *Heterodera* species identification (Subbotin *et al.*, 2000, 2003, 2010b; 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, 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*, *H. glycines*, *H. trifolii*, *Heterodera* sp. parasitic on tobacco, and *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959.

The aim of the present study is to determine the distribution of *H. trifolii* in eastern Japan by PCR-RFLP analysis of the ITS1-5.8S-ITS2 region for species identification.

1.2 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 or 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°C 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, I looked for the presence of J2 of cyst nematodes, and if any were detected, one J2 for each soil sample was identified to the species level by PCR-RFLP analysis. Subsequently, to confirm

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 and Nagano Prefectures 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

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

DNA was extracted from individual J2. 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^{-1} Proteinase K (Takara Bio, Shiga, Japan), and 0.1% SDS, and then the tube was incubated first at 50°C for 2 hr, followed by 95°C for 10 min (Sakai, 2010). After the incubation, 196 μl of sterile distilled water was added to yield

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	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

n: numbers of J2 used for molecular identification.

Table 3. (Continued.)

No.	Region	Prefecture	Locality	Sample code	n	Species	
						H. trifolii	*H. elachista*
31	Tohoku	Aomori	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

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 and Nagano Prefectures.

No.	Region	Prefecture	Locality	Sample code	n	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

n: numbers of J2 used for molecular identification.

200 μl of lysate for each specimen, which was then stored at –20°C 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 μM) of each primer, 5 μl of DNA template, and 4.2 μl of distilled water. The ITS1-5.8S-ITS2 region 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°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C 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°C for 3 hr. The RFLP patterns were analyzed by 2.0% agarose gel (Agarose-ME, Classic Type, Nacalai Tesque, Kyoto, Japan) electrophoresis in 0.5 × TBE buffer.

1.3 Results and Discussion

For the soil samples collected from the rhizosphere of white clover, J2 of cyst nematodes were detected in 57 of 195 samples examined (29.2%) (Fig. 5; Tables 1, 3). A total of 264 J2 of cyst nematodes were identified, among which 263 were *H. trifolii*

Fig. 5. Geographical distribution of *Heterodera trifolii* and *H. elachista* in eastern Japan. Numbers correspond to those in Tables 3 and 4.

and one was *H. elachista* (Table 3). *Heterodera trifolii* was detected from white clover in 26 of 76 samples (34.2%) in Hokkaido, 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, J2 of cyst nematodes were detected in five of eight samples examined (62.5%) and a total of 30 J2 of cyst nematodes were all identified as *H. trifolii* (Fig. 5; Tables 2, 4). In the past distributional surveys of *H. trifolii* conducted in Hokkaido and Nagano Prefectures, species identification was only based on host plants and not confirmed by morphological or molecular methods (Toyoshima *et al.*, 1992; Mizukoshi, 2000). The 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, this 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 ITS1-5.8S-ITS2 region.

In the present study, soil samples were collected only from the rhizospheres of white clover and carnation. However, *H. trifolii* has been recorded from other host plants, such as red clover, in Japan (Inoue, 1961; Yuhara *et al.*, 1961). Wang and Riggs (1999) and Wang *et al.* (2001) reported that curly dock (*R. crispus* L.) and common lespedeza (*Lespedeza striata* (Thunb.) Hook. & Arn.) are good hosts of *H. trifolii*. These host plants other than white clover are also widely distributed throughout Japan. Therefore, *H. trifolii* may occur on more various host plants in eastern Japan.

Carnation is one of the important ornamental flowers in Japan. Three prefectures of eastern Japan including Nagano, Hokkaido, and Chiba Prefectures, 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 (Mizukoshi, 2000; Momota and Mizukoshi, 2000) and Nagano Prefectures (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 Agano City, Niigata Prefecture (NI8) (Fig. 5; Tables 1, 3). This species is widely distributed from the Tohoku region to Kyushu 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.*, 2010b). In the fallow rice field in which the 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, I identified the detected cyst nematodes by using PCR-RFLP analysis of the ITS1-5.8S-ITS2 region, because this is a less time-consuming procedure than morphological methods or other molecular methods such as sequencing analysis (Uesugi *et al.*, 2009). I 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, I 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. 6). No intraspecific variation was observed in the digestion patterns of these three enzymes. The 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, 580, and 230 bp, the total combined size of the restriction fragments is 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 studied 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.

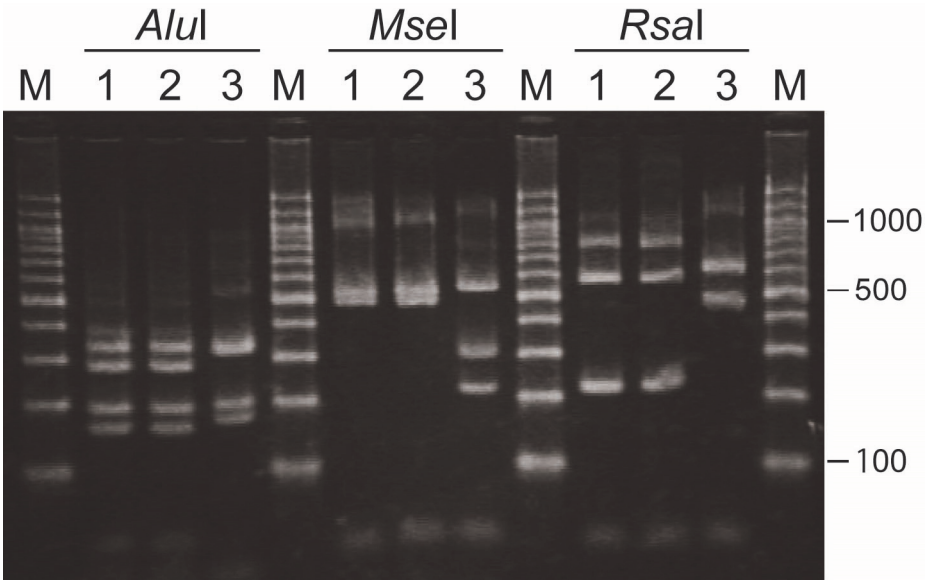

Fig. 6. PCR-RFLP profiles of *Heterodera trifolii* and *H. elachista*, digested with three restriction endonucleases, *Alu*I, *Mse*I, and *Rsa*I. Lane 1: *H. trifolii* from white clover in Sapporo City, Hokkaido Prefecture (HU1); Lane 2: *H. trifolii* from greenhouse carnation in Sakuho Town, Nagano Prefecture (SAK); Lane 3, *H. elachista* from white clover in Agano City, Niigata Prefecture (NI8); M, 100 bp DNA ladder (Nacalai Tesque, Kyoto, Japan).

Chapter 2

Characterization of populations of *Heterodera trifolii* in Japan and their phylogenetic relationships with closely related species

2.1 Introduction

Heterodera trifolii belongs to the *schachtii* group, characterized by an ambifenestrate vulval cone with a long vulval slit, well developed bullae, and a strong underbridge (Subbotin *et al.*, 2010b). The *schachtii* group contains 15 valid species: *H. betae*, *H. cajani* Koshy, 1967, *H. ciceri* Vovlas, Greco & Di Vito, 1985, *H. daverti*, *H. galeopsidis* Goffart, 1936, *H. glycines*, *H. lespedezae* Golden & Cobb, 1963, *H. medicaginis* Kirjanova *in* Kirjanova & Krall, 1971, *H. mediterranea* Vovlas, Inserra & Stone, 1981, *H. rosii* Duggan & Brennan, 1966, *H. schachtii*, *H. sonchophila* Kirjanova, Krall & Krall, 1976, *H. spiraeae* Kazachenko, 1993, *H. swarupi* Sharma, Siddiqi, Rahaman, Ali & Ansari, 1998, and *H. trifolii* (see Subbotin *et al.*, 2010b). Two of these species, the sugar beet cyst nematode, *H. schachtii*, and the soybean cyst nematode, *H. glycines*, are considered among the most economically important pests worldwide of sugar beet and soybean, respectively. In particular, *H. schachtii* is an important regulated quarantine pest in countries of South America and Asia and is also included on the quarantine pest list of Japan (Hockland *et al*., 2013; Plant Protection Station, MAFF, 2016). These countries are all concerned about the introduction of *H. schachtii*. In Japan, only two species of the *schachtii* group, *H. glycines* and *H. trifolii*, have been reported so far (Momota, 2004). However, no detailed information on morphological,

morphometric, and molecular data is currently available for Japanese populations of *H. trifolii*.

The objectives of the present study were: *i*) to perform morphological and morphometric characterization of *H. trifolii* populations from Japan; *ii*) to perform molecular characterization of *H. trifolii* and related species using the ITS regions, the D2–D3 expansion segments of 28S rRNA gene, and the partial *COI* mtDNA gene sequences; and *iii*) to study the phylogenetic relationships within *Heterodera* using rRNA and *COI* gene sequences.

2.2 Materials and Methods

Nematode populations

In 2012, eight populations of *H. trifolii* were collected from different geographical regions and different host plants in Japan: five populations from white clover and three populations from carnation (Table 5). Soil samples were collected from a depth of approximately 5–15 cm around the roots of white clover and carnation using a trowel, mixed in a plastic bag, labelled, and kept in a refrigerator at 10°C for use in further studies. Cysts were extracted from soil samples by the dry flotation-sieving method (Aiba, 2014). J2 were extracted from soil samples by the Baermann funnel method. For four soil samples (HU2, H61, T20, and FU2), additional samples were collected in 2013 from the same locality in order to obtain the necessary specimens for morphological identification. Six populations of *H. trifolii* (HU2, H61, NAN, T20, FU2, and SAK) were used for morphological examination by LM and for molecular analyses. Two populations of *H. trifolii* (T4 and MAT) were used only for molecular analyses. Several populations of three other species of the *schachtii* group, including *H. betae* and *H.*

Table 5. *Heterodera* species used in the present study.

Species	Locality	Host plant	Sample code	GenBank accession number			Source
				ITS	D2–D3 of 28S rRNA	*COI* mtDNA	
H. trifolii	Sapporo City, Hokkaido Prefecture, Japan	*Trifolium repens*	HU2	–	LC208662	LC208698	S. Sekimoto
H. trifolii	Teshikaga Town, Hokkaido Prefecture, Japan	*T. repens*	H61	LC208683, LC208684	LC208663	LC208699	S. Sekimoto
H. trifolii	Nanae Town, Hokkaido Prefecture, Japan	*Dianthus caryophyllus*	NAN	–	LC208664	LC208700	K. Takada
H. trifolii	Kuroishi City, Aomori Prefecture, Japan	*T. repens*	T20	LC208685, LC208686	LC208665	LC208701	S. Sekimoto
H. trifolii	Tono City, Iwate Prefecture, Japan	*T. repens*	T4	–	LC208666	LC208702	S. Sekimoto
H. trifolii	Inawashiro Town, Fukushima Prefecture, Japan	*T. repens*	FU2	–	LC208667	LC208703	S. Sekimoto
H. trifolii	Sakuho Town, Nagano Prefecture, Japan	*D. caryophyllus*	SAK	LC208687, LC208688	LC208668	LC208704	S. Sekimoto
H. trifolii	Matsumoto City, Nagano Prefecture, Japan	*D. caryophyllus*	MAT	–	LC208669	LC208705	S. Yokosawa
H. betae	The Netherlands	Unknown	2B	LC208689	LC208670	LC208706	G. Karssen
H. betae	The Netherlands (detected at Japanese import plant quarantine inspection)	*Beta vulgaris* ssp. *vulgaris* var. *vulgaris*	C25	LC208690, LC208691	LC208671	LC208707	J. Hisai

Table 5. (Continued.)

Species	Locality	Host plant	Sample code	GenBank accession number			Source
				ITS	D2–D3 of 28S rRNA	*COI* mtDNA	
H. schachtii	The Netherlands (detected at Japanese import plant quarantine inspection)	*Beta vulgaris* ssp. *vulgaris* var. *vulgaris*	C20	LC208692, LC208693	LC208672	LC208708	J. Hisai
H. glycines	Sapporo City, Hokkaido Prefecture, Japan	*Glycine max*	HG1	–	–	LC208709	S. Aiba
H. glycines	Shinshinotsu Village, Hokkaido Prefecture, Japan	*G. max*	HG3	LC208694, LC208695	LC208673, LC208674	LC208710	S. Aiba
H. glycines	Memuro Town, Hokkaido Prefecture, Japan	*G. max*	HG9	–	LC208675, LC208676	LC208711	A. Kushida
H. glycines	Ninohe City, Iwate Prefecture, Japan	*G. max*	HG5	–	LC208677	LC208712	S. Aiba
H. glycines	Daisen City, Akita Prefecture, Japan	*G. max*	HG4	LC208696, LC208697	LC208678	LC208713	S. Aiba
H. glycines	Kahoku Town, Yamagata Prefecture, Japan	*G. max*	HG6	–	LC208679, LC208680	LC208714	S. Aiba
H. glycines	Fukushima City, Fukushima Prefecture, Japan	*G. max*	HG7	–	LC208681, LC208682	LC208715	S. Aiba
H. glycines	Satte City, Saitama Prefecture, Japan	*G. max*	HG8	–	–	LC208716	S. Aiba

schachtii from The Netherlands, both of which have not occurred in Japan, and *H. glycines* from Japan, were also used only for molecular analyses. *Heterodera betae* was provided by Gerrit Karrsen (Wageningen University, The Netherlands), under a special import permit (permit No.: 24Y880) issued by the Minister of Agriculture, Forestry and Fisheries, Japan. DNA samples of *H. betae* and *H. schachtii*, both of which were detected at Japanese import plant quarantine inspection, were provided by Junya Hisai (Yokohama Plant Protection Station, MAFF, Japan). Eight populations of *H. glycines* were provided by Satoshi Aiba and Atsuhiko Kushida (HARC/NARO, Japan).

Morphological analysis

Morphological measurements were made and photomicrographs were taken from four *H. trifolii* populations of white clover (HU2, H61, T20, and FU2) and two populations of carnation (NAN and SAK). Live J2 specimens were temporarily mounted onto a glass slide in a drop of tap water, killed with gentle heat (65°C) on a hot plate, examined, and measured immediately. Cyst vulval cones were prepared from cysts and mounted in glycerin jelly. Specimens were examined using a compound Olympus BX51 microscope equipped with DIC. Photomicrographs were taken with a digital camera Olympus DP21 attached to Olympus BX51.

DNA extraction, PCR and sequencing

DNA was extracted from several J2 individuals of each population. The protocol used for DNA extraction with proteinase K was described in Chapter 1, which yielded 200 μl of lysate for each specimen. The ITS1-5.8S-ITS2 region was amplified using the forward primer TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and reverse primer

AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) (Joyce *et al.*, 1994). The D2–D3 expansion segments of the 28S rRNA gene was amplified using the forward primer D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and reverse primer D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (De Ley *et al.*, 1999). PCR amplifications for ITS and D2–D3 were performed in a final volume of 20 μl reaction mixture containing 2 μl 10 × Ex Taq buffer (20 mM Mg^{2+} plus) (Takara Bio, Shiga, Japan), 1.6 μl dNTP mixture (2.5 mM), 0.4 μl (10 μM) of each primer, 0.1 μl TaKaRa Ex Taq® Hot Start Version (5 U μl^{-1}) (Takara Bio, Shiga, Japan), 3 μl DNA template, and 12.5 μl distilled water. The amplification conditions for ITS and D2–D3 were as follows: a single step of pre-denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C (ITS) or 60°C (D2–D3) for 30 sec, and extension at 72°C for 1 min. The partial mtDNA *COI* gene was amplified using the forward primer JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and reverse primer JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′) (Derycke *et al.*, 2005) in a final volume of 20 μl reaction mixture containing 4 μl 5 × PrimeSTAR Buffer (5 mM Mg^{2+} plus) (Takara Bio, Shiga, Japan), 1.6 μl dNTP mixture (2.5 mM), 0.4 μl (10 μM) of each primer, 0.1 μl PrimeSTAR® HS DNA Polymerase (2.5 U μl^{-1}) (Takara Bio, Shiga, Japan), 1 μl DNA template, and 12.5 μl distilled water. The amplification conditions for *COI* were as follows: a single pre-denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 46°C for 15 sec, and extension at 72°C for 30 sec.

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 visualised under UV light. PCR

products were purified with ExoSAP-IT® (Affymetrix, USB products, Santa Clara, CA, USA) and used for direct sequencing or for cloning. *COI* and D2–D3 PCR products were directly sequenced in both directions using the primers described above. ITS and some D2–D3 PCR products were cloned into pCR4-TOPO using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Two positive clones from each PCR product were isolated using blue/white selection and sequenced in both directions using T3 and T7 universal primers. For the ITS sequencing, the additional internal forward primer 5.8SM2 (5′-CTT ATC GGT GGA TCA CTC GG-3′) and the reverse primer 5.8SM5 (5′-GGC GCA ATG TGC ATT CGA-3′) (Zheng *et al.*, 2000) were also used. The resulting products were purified with Agencourt CleanSEQ® (Beckman Coulter, Brea, CA, USA) and analysed in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The newly obtained sequences were submitted to the GenBank database under the accession numbers indicated in Table 5.

Phylogenetic analysis

The newly obtained sequences of the ITS, D2–D3 of 28S rRNA gene, and *COI* mtDNA gene were edited with DNADynamo (Blue Tractor Software, UK) and were aligned with their corresponding published gene sequences using MUSCLE (Edgar, 2004), as implemented in MEGA 5.2.2 (Tamura *et al.*, 2011), with default parameters. Out group taxa for each data set were selected based on previously published data (Mundo-Ocampo *et al.*, 2008; Zheng *et al.*, 2008). Sequence datasets were analysed with BI using MrBayes 3.2.2 (Ronquist *et al.*, 2011). The best-fit model of DNA

evolution was selected using the AIC as implemented in MrModeltest 2.3 (Nylander, 2004) in conjunction with PAUP* 4.0b10 (Swofford, 2003). The GTR + I + G model for ITS and *COI* and GTR + I model for D2–D3 were selected. BI analysis was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. Tracer v.1.6 (Rambaut and Drummond, 2013) was used to test for ESS (> 100) and convergence of parameters. The topologies were used to generate a 50% majority rule consensus tree. PP were given on appropriate clades. The tree was visualized using FigTree v1.3.1 (Rambaut, 2009). Further sequence analyses of alignments were performed with PAUP* 4.0b10 (Swofford, 2003). Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data.

2.3 Results

Morphological characterization of Japanese populations (Figs 7, 8; Table 6)

Cyst variable in size, light to dark brown in colour, and lemon-shaped with prominent neck and vulval cone. Cuticle showing an irregular zigzag pattern. Vulval cone ambifenestrate with long vulval slit (34.9–62.1 μm). Fenestral length and semifenestral width 35.1–77.9 μm and 25.2–54.2 μm, respectively. Underbridge well developed with bifurcate ends. Prominent brown bullae scattered at or near underbridge.

J2 cylindrical in shape, tapering posteriorly, and curved ventrally after heat-killing. Body length 424–587 μm. Lip region hemispherical and slightly offset from rest of

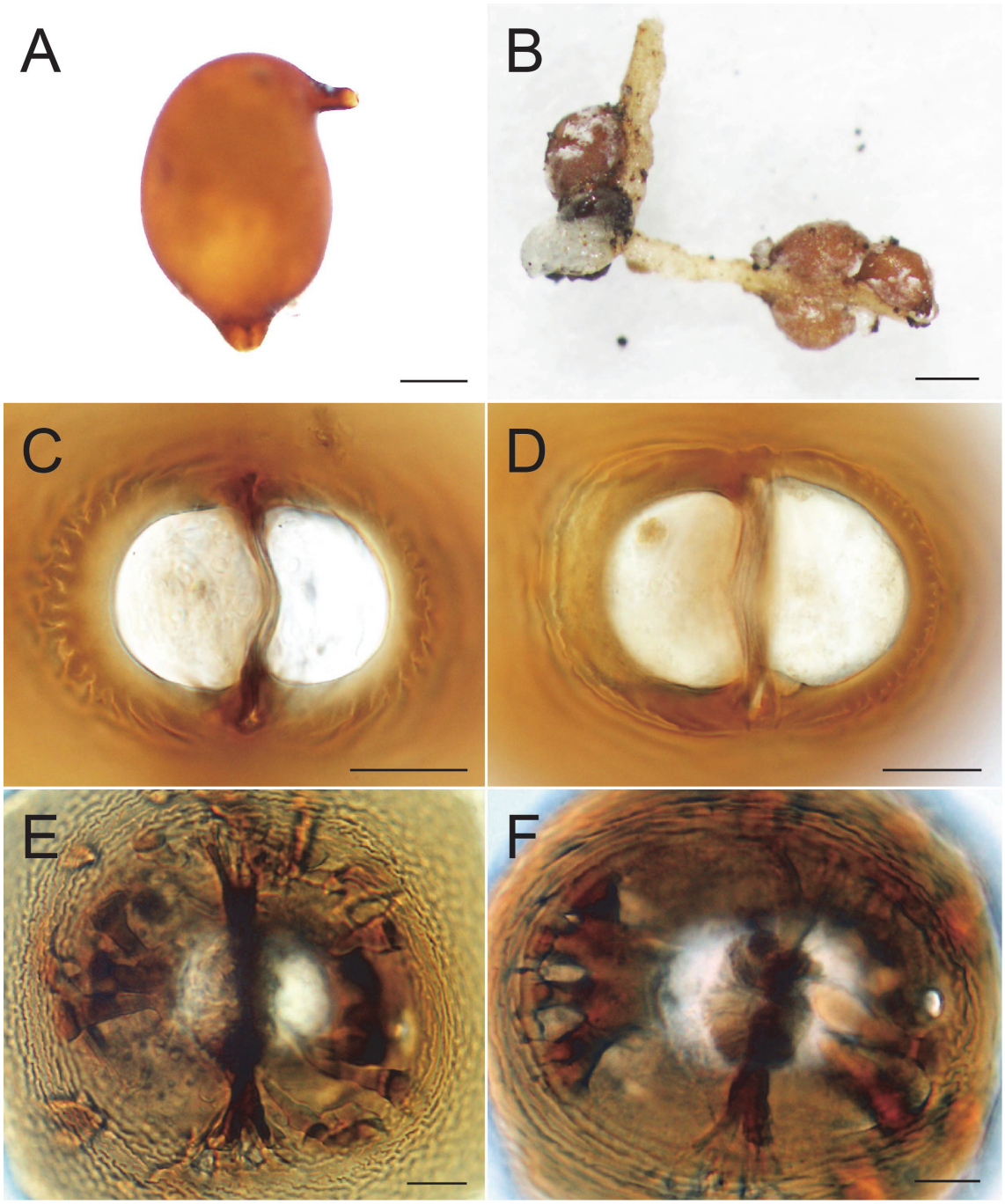

Fig. 7. Light micrographs of *Heterodera trifolii*. A: Entire cyst (H61); B: Cysts on a root (HU2); C, D: Vulval plate (HU2 and NAN, respectively); E, F: Underbridge and burae (HU2 and NAN, respectively). (Scale bars: A, B = 100 μm; C–F = 20 μm.)

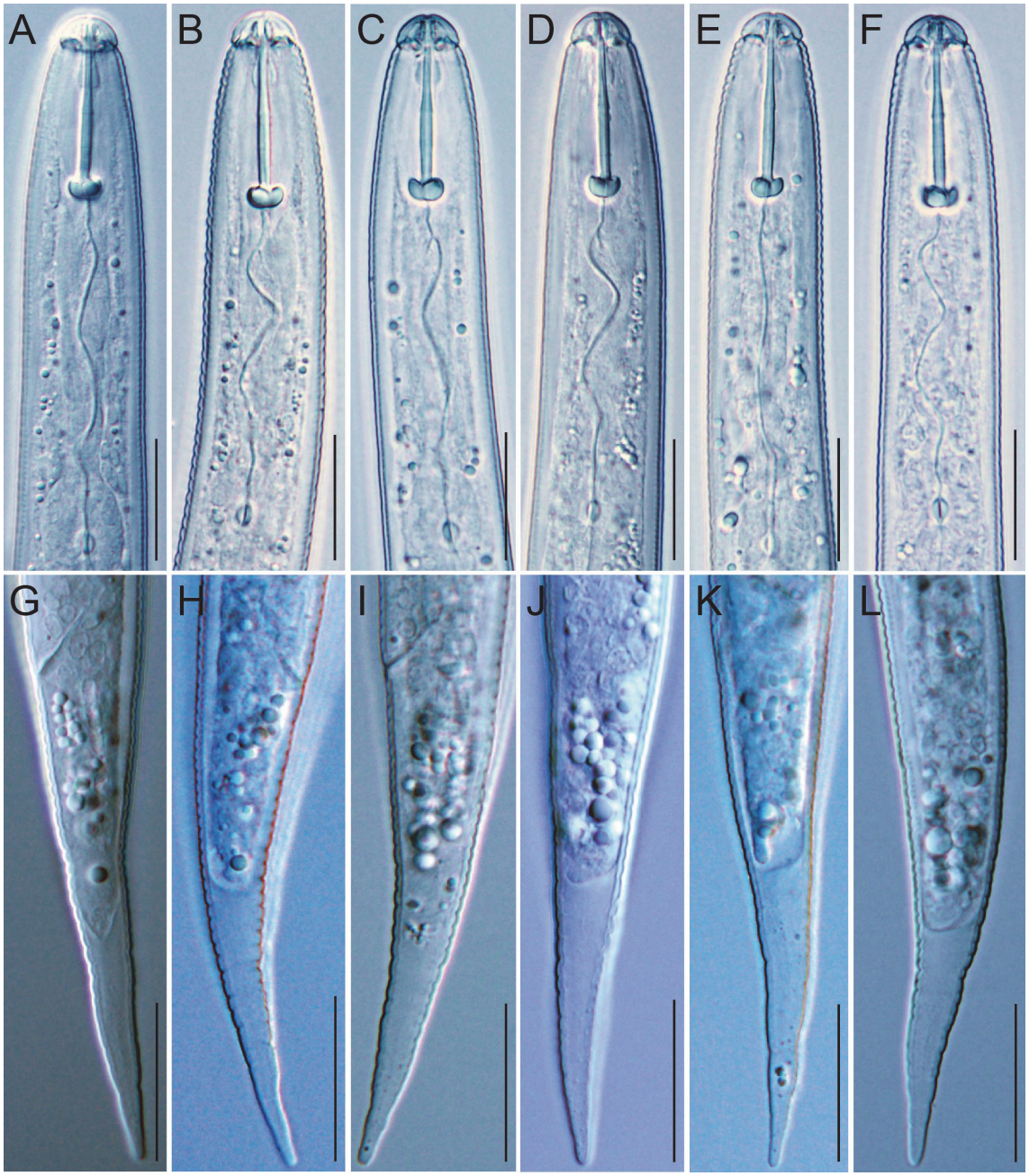

Fig. 8. Light micrographs of *Heterodera trifolii*. A–F: Anterior region of J2 (HU2, H61, NAN, T20, FU2, and SAK, respectively); G–L: Tail of J2 (HU2, H61, NAN, T20, FU2, and SAK, respectively). (Scale bars = 20 μm.)

Table 6. Morphometrics of cysts and J2 of six *Heterodera trifolii* populations from Japan. All measurements are in μm and in the form: mean ± S.D. (range).

Stage	Character	White clover populations				Carnation populations	
		Sapporo, Hokkaido (HU2)	Teshikaga, Hokkaido (H61)	Kuroishi, Aomori (T20)	Inawashiro, Fukushima (FU2)	Nanae, Hokkaido (NAN)	Sakuho, Nagano (SAK)
Cyst	n	10	10	10	10	10	10
	L (excluding neck)	561 ± 70.0 (466–697)	685 ± 90.9 (592–906)	599 ± 111.9 (444–763)	608 ± 83.8 (486–726)	786 ± 83.3 (669–924)	701 ± 131.2 (532–980)
	Body diameter	350 ± 77.5 (260–532)	439 ± 63.9 (332–583)	382 ± 68.7 (271–482)	375 ± 44.6 (315–440)	472 ± 79.7 (400–636)	430 ± 67.1 (348–563)
	Fenestral length	41.2 ± 3.5 (35.9–47.6)	46.7 ± 12.0 (36.6–77.9)	42.0 ± 3.6 (35.7–47.5)	49.6 ± 6.6 (38.9–60.6)	59.1 ± 6.3 (52.0–71.8)	46.1 ± 7.1 (35.1–58.1)
	Semifenestral width	30.8 ± 1.9 (27.9–33.0)	34.5 ± 7.4 (28.5–54.2)	31.3 ± 1.7 (28.9–34.5)	31.4 ± 4.3 (25.2–41.3)	42.7 ± 5.0 (34.5–53.3)	40.2 ± 5.8 (27.3–49.5)
	Vulval bridge width	5.4 ± 1.4 (3.3–7.3)	5.7 ± 1.5 (3.4–8.6)	5.3 ± 0.8 (4.2–6.6)	5.3 ± 0.9 (3.6–6.6)	6.9 ± 2.3 (4.4–12.1)	6.9 ± 1.6 (4.4–9.6)
	Vulval slit length	42.0 ± 4.4 (34.9–51.2)	46.2 ± 4.5 (41.0–57.2)	42.7 ± 3.5 (37.6–46.9)	44.4 ± 5.2 (38.6–54.9)	50.7 ± 4.8 (46.6–62.1)	48.4 ± 5.1 (39.2–56.7)
	Underbridge length	98.0 ± 10.5 (78.1–111.1)	103.2 ± 11.8 (79.9–119.2)	100.2 ± 15.1 (68.2–118.4)	95.3 ± 13.0 (73.9–112.6)	135.0 ± 18.1 (108.5–161.3)	135.8 ± 20.1 (91.1–160.8)
	Underbridge width	29.5 ± 1.9 (26.2–32.9)	31.0 ± 6.6 (20.3–42.6)	32.5 ± 4.7 (26.0–39.8)	29.1 ± 5.1 (17.4–36.2)	37.3 ± 6.3 (25.7–46.5)	34.0 ± 4.0 (27.5–39.6)

Table 6. (Continued.)

Stage	Character	White clover populations				Carnation populations	
		Sapporo, Hokkaido (HU2)	Teshikaga, Hokkaido (H61)	Kuroishi, Aomori (T20)	Inawashiro, Fukushima (FU2)	Nanae, Hokkaido (NAN)	Sakuho, Nagano (SAK)
J2	n	20	20	20	20	14	20
	L	520 ± 30.6 (451–587)	501 ± 26.3 (424–544)	521 ± 33.1 (441–565)	512 ± 26.5 (467–549)	528 ± 20.7 (499–562)	489 ± 17.3 (458–533)
	a	24.2 ± 1.8 (20.1–27.3)	23.8 ± 1.0 (21.5–26.0)	24.2 ± 1.4 (20.5–26.7)	24.6 ± 0.9 (22.9–26.4)	24.1 ± 0.7 (22.8–25.6)	23.7 ± 0.6 (22.7–24.8)
	c	8.3 ± 0.4 (7.7–9.0)	8.0 ± 0.4 (7.5–8.7)	8.1 ± 0.4 (7.2–8.9)	8.3 ± 0.3 (7.6–9.0)	8.6 ± 0.4 (7.7–9.3)	9.1 ± 0.4 (8.4–10.4)
	c′	4.4 ± 0.3 (3.8–4.7)	4.4 ± 0.2 (3.9–4.7)	4.6 ± 0.2 (4.3–5.0)	4.5 ± 0.2 (4.2–4.8)	4.2 ± 0.3 (3.7–4.8)	3.8 ± 0.2 (3.4–4.3)
	Stylet length	27.4 ± 1.6 (24.1–30.8)	26.9 ± 1.2 (24.8–28.9)	27.8 ± 0.8 (26.1–29.4)	27.4 ± 0.8 (25.7–29.2)	27.2 ± 1.0 (25.3–28.6)	26.9 ± 0.9 (24.4–28.2)
	Stylet knob height	3.1 ± 0.2 (2.7–3.5)	3.2 ± 0.2 (2.8–3.6)	3.4 ± 0.3 (2.9–4.2)	3.3 ± 0.3 (2.6–3.8)	3.4 ± 0.3 (2.9–4.1)	3.2 ± 0.2 (2.9–3.8)
	Stylet knob diameter	5.3 ± 0.3 (4.7–6.1)	5.5 ± 0.3 (4.7–6.0)	5.3 ± 0.2 (4.9–5.6)	5.2 ± 0.6 (3.0–5.9)	5.6 ± 0.4 (4.6–6.1)	5.4 ± 0.2 (5.0–5.9)
	Lip region height	4.7 ± 0.2 (4.3–5.0)	4.6 ± 0.2 (4.1–5.0)	4.7 ± 0.3 (4.0–5.3)	4.5 ± 0.2 (4.1–5.2)	4.9 ± 0.3 (4.4–5.5)	4.5 ± 0.3 (3.8–4.9)
	Lip region diameter	9.9 ± 0.3 (9.6–10.4)	9.9 ± 0.4 (9.3–10.6)	9.8 ± 0.3 (9.3–10.4)	9.9 ± 0.4 (9.2–10.5)	10.4 ± 0.2 (10.0–10.7)	9.8 ± 0.3 (9.3–10.4)

Table 6. (Continued.)

Stage	Character	White clover populations				Carnation populations	
		Sapporo, Hokkaido (HU2)	Teshikaga, Hokkaido (H61)	Kuroishi, Aomori (T20)	Inawashiro, Fukushima (FU2)	Nanae, Hokkaido (NAN)	Sakuho, Nagano (SAK)
J2	n	20	20	20	20	14	20
	DGO	6.3 ± 0.6 (5.2–8.2)	6.1 ± 0.5 (4.9–6.7)	6.6 ± 0.7 (4.8–7.8)	6.4 ± 0.6 (5.3–7.8)	6.2 ± 0.6 (5.4–7.1)	5.3 ± 0.6 (4.0–6.7)
	Anterior end to median bulb valve	84 ± 3.9 (74–93)	79 ± 3.9 (68–84)	79 ± 7.8 (64–92)	80 ± 6.0 (71–91)	82 ± 2.2 (79–85)	77 ± 2.2 (73–81)
	Anterior end to excretory pore	124 ± 5.3 (114–137)	119 ± 5.4 (105–129)	120 ± 10.1 (89–133)	118 ± 8.8 (104–132)	124 ± 3.5 (117–129)	114 ± 3.0 (108–121)
	Body diameter at mid-body	21.5 ± 0.8 (20.5–23.3)	21.0 ± 0.7 (19.7–22.6)	21.6 ± 1.2 (20.1–24.4)	20.8 ± 0.6 (19.8–22.2)	21.9 ± 0.7 (20.3–23.0)	20.6 ± 0.4 (19.8–21.5)
	Body diameter at anus	14.3 ± 0.4 (13.5–14.9)	14.4 ± 0.7 (13.4–15.8)	14.2 ± 0.5 (13.0–14.8)	13.9 ± 0.5 (12.9–14.6)	14.8 ± 0.5 (14.0–15.5)	14.0 ± 0.6 (13.2–15.0)
	Tail length	62 ± 4.2 (53–70)	63 ± 4.4 (54–70)	65 ± 2.7 (60–69)	62 ± 2.9 (58–69)	62 ± 4.7 (54–73)	54 ± 3.3 (44–58)
	Length of hyaline region	33 ± 3.7 (26–41)	34 ± 2.3 (29–38)	36 ± 1.8 (33–40)	34 ± 2.3 (31–39)	32 ± 2.9 (30–41)	30 ± 2.3 (22–34)
	Hyaline region/stylet length	1.2 ± 0.1 (1.0–1.5)	1.3 ± 0.1 (1.1–1.5)	1.3 ± 0.1 (1.2–1.5)	1.2 ± 0.1 (1.1–1.4)	1.2 ± 0.1 (1.1–1.5)	1.1 ± 0.1 (0.8–1.3)
	Tail length/hyaline region	1.9 ± 0.1 (1.6–2.2)	1.9 ± 0.1 (1.7–2.1)	1.8 ± 0.1 (1.6–2.0)	1.8 ± 0.1 (1.7–2.1)	1.9 ± 0.1 (1.8–2.0)	1.8 ± 0.1 (1.7–2.0)

body. Stylet robust with anteriorly concave knobs. Stylet length 24.1–30.8 μm. Dorsal pharyngeal gland orifice distinct. Median bulb oval and prominent. Pharyngeal glands well developed, extending well posterior to pharyngo-intestinal valve. Lateral field with four incisures. Tail conoid, tapering uniformly to finely rounded terminus. Hyaline region 22–41 μm long.

Molecular characterization and phylogeny

The amplification of ITS, D2–D3 expansion segments of 28S rRNA gene, and partial *COI* gene yielded a single fragment of *ca* 1000, 700, and 400 bp, respectively, as visualized by gel electrophoresis.

ITS

The alignment of ITS contained 67 sequences of *Heterodera* species including seven *H. trifolii* sequences and two sequences of *Cryphodera brinkmani* Karssen & van Aelst, 1999 and *Meloidodera alni* Turkina & Chizhov, 1986 as outgroups and was 1423 bp in length. Six new sequences of *H. trifolii*, three new sequences of *H. betae*, two new sequences of *H. schachtii*, and four new sequences of *H. glycines* were included in this study. Intraspecific ITS sequence variation was 0.1–1.4% (1–13 bp) for *H. trifolii*, 0.1–1.3% (1–12 bp) for *H. betae*, 0.2–1.7% (2–16 bp) for *H. schachtii*, and 0.1–1.4% (1–13 bp) for *H. glycines*. Phylogenetic relationships between *Heterodera* species, as inferred from BI, are presented in Figure 9. Phylogenetic relationships between the seven species groups were resolved. *Heterodera trifolii* clustered with species of the *schachtii* group, including *H. betae*, *H. cajani*, *H. ciceri*, *H. daverti*, *H. glycines*, *H. medicaginis*, *H. mediterranea*, and *H. schachtii*, with high support value (PP = 100). Phylogenetic

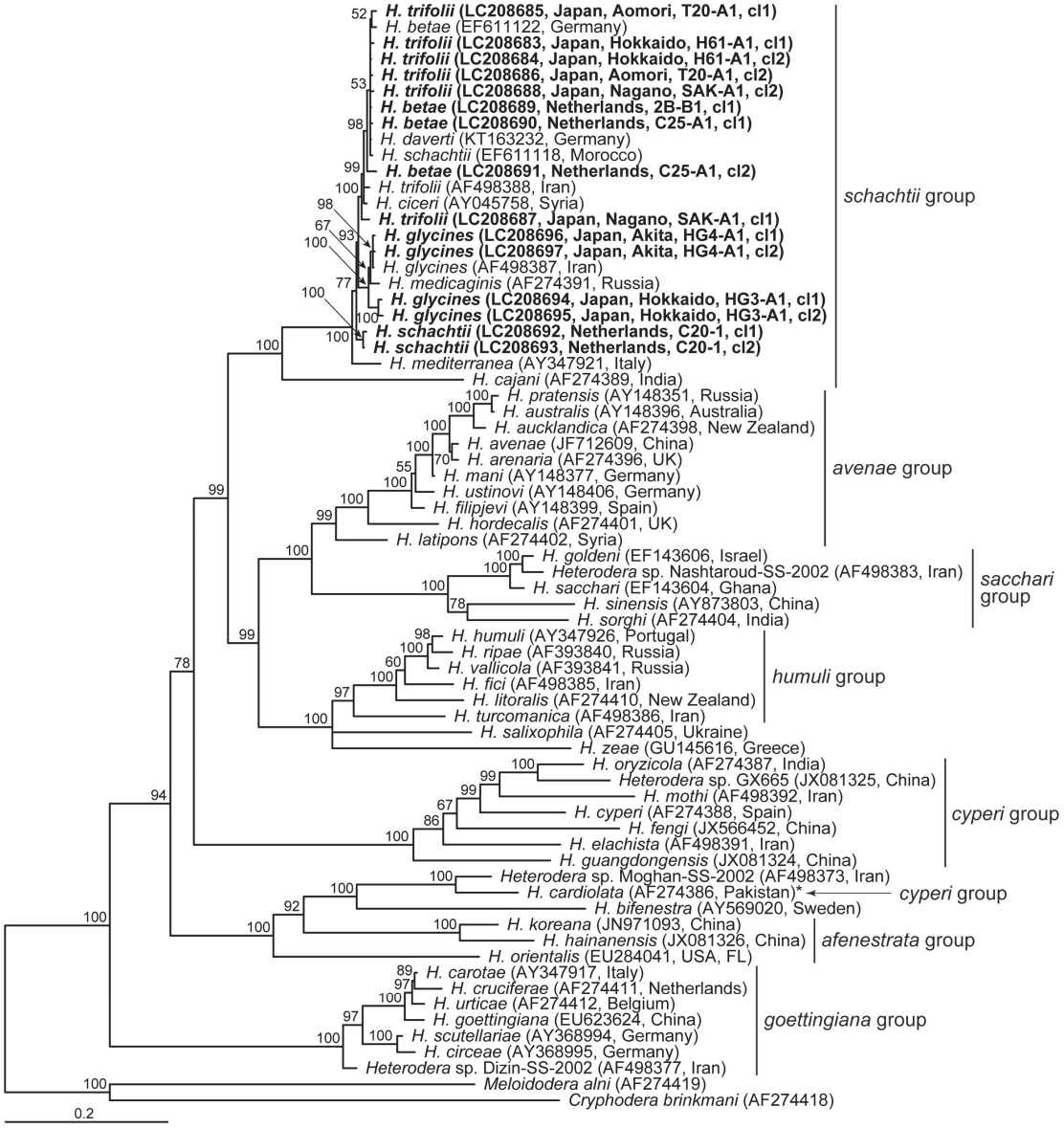

Fig. 9. Phylogenetic relationships between *Heterodera* species: Bayesian 50% majority rule consensus tree as inferred from the analysis of the ITS sequence alignment under the GTR + I + G model. PP over 50% are given for appropriate clades. Newly obtained sequences are indicated by bold letters. * Identified as *H. cynodontis* by Subbotin *et al.* (2001), the species is considered as a synonym of *H. cardiolata* by Subbotin *et al.* (2010b).

relationships between species of the *schachtii* group were not well resolved.

D2–D3 of 28S rRNA gene

The alignment of the D2–D3 of 28S rRNA gene was 739 bp in length and contained 32 sequences of species of the *schachtii* group and two sequences of *H. avenae* and *H. filipjevi* (Madzhidov, 1981) Stelter, 1984 as outgroups. Eight new sequences of *H. trifolii*, two new sequences of *H. betae*, one new sequence of *H. schachtii*, and ten new sequences of *H. glycines* were obtained in this study. Intraspecific D2–D3 sequence variation was 0–0.2% (0–1 bp) for *H. trifolii*, 0% (0 bp) for *H. betae*, 0–0.3% (0–2 bp) for *H. schachtii*, and 0–1.4% (0–10 bp) for *H. glycines*. Phylogenetic relationships between species of the *schachtii* group, as inferred from BI, are presented in Figure 10. Phylogenetic relationships between species of the *schachtii* group were resolved, except for relationship between *H. trifolii* and *H. betae*, with almost identical D2–D3 sequences (different in only 0–1 bp). Interspecific differences were 0–0.2% (0–1 bp) between *H. trifolii* and *H. betae*, 0.1–0.4% (1–3 bp) between *H. trifolii* and *H. schachtii*, 6.4–6.6% (42–49 bp) between *H. trifolii* and *H. cajani*, 0.4–1.4% (3–10 bp) between *H. trifolii* and *H. glycines*, and 0.3–1.3% (2–9 bp) between *H. glycines* and *H. schachtii*.

***COI* of the mtDNA gene**

The alignment of *COI* gene included 31 sequences of *Heterodera* species and two sequences of *Rotylenchus eximius* Siddiqi, 1964 and *R. urmiaensis* Noruzi, Asghari, Atighi, Eskandari, Cantalapiedra-Navarrete, Archidona-Yuste, Liébanas, Castillo & Palomares-Rius, 2015 as outgroups, and was 393 bp in length. Eight new sequences of *H. trifolii*, two new sequences of *H. betae*, one new sequence of *H. schachtii*, and eight

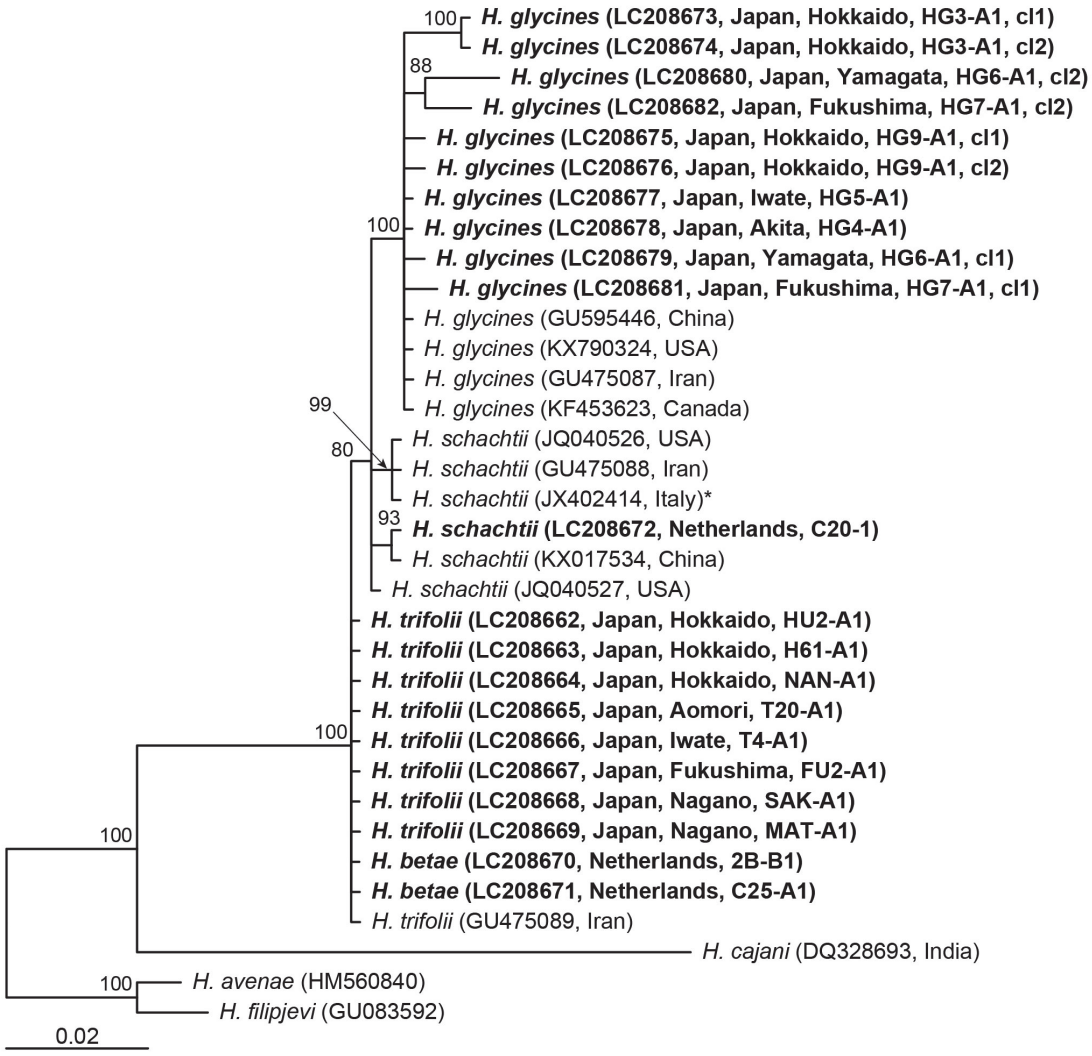

Fig. 10. Phylogenetic relationships between species of the *schachtii* group: Bayesian 50% majority rule consensus tree as inferred from the analysis of the D2–D3 of 28S rRNA gene sequence alignment under the GTR + I model. PP over 50% are given for appropriate clades. Newly obtained sequences are indicated by bold letters. * Identified as *H. cruciferae* by Sasanelli *et al.* (2013).

new sequences of *H. glycines* were obtained in the present study. No insertions or deletions occurred between the sequences. Intraspecific *COI* sequence variation was 0–0.3% (0–1 bp) for *H. trifolii*, 0% (0 bp) for *H. betae*, 0–0.5% (0–2 bp) for *H. daverti*, 0% (0 bp) for *H. schachtii*, and 0–2.3% (0–9 bp) for *H. glycines*. Two *COI* haplotypes were found for *H. trifolii* populations. Phylogenetic relationships between species of the *schachtii* group, as inferred from BI, are presented in Figure 11. Phylogenetic relationships between species of the *schachtii* group were well resolved, except for relationship between *H. trifolii* and *H. betae*, with almost identical *COI* sequences (different in only 0–1 bp). Interspecific differences were 0–0.3% (0–1 bp) between *H. trifolii* and *H. betae*, 4.6–4.9% (18–19 bp) between *H. trifolii* and *H. daverti*, 4.0–4.4% (16–17 bp) between *H. trifolii* and *H. ciceri*, 9.0–10.1% (35–40 bp) between *H. trifolii* and *H. schachtii*, 9.0–9.9% (35–39 bp) between *H. trifolii* and *H. glycines*, and 6.4–7.7% (25–30 bp) between *H. glycines* and *H. schachtii*.

2.4 Discussion

The morphology and morphometrics of the cysts and J2 of the six Japanese populations of *H. trifolii* were within the ranges reported for *H. trifolii* populations from the USA (Hirschmann, 1956; Hirschmann and Triantaphyllou, 1979), Japan (Yuhara *et al.*, 1961; Momota *et al.*, 1990; Momota and Mizukoshi, 2000), The Netherlands (Wouts and Weischer, 1977; Maas *et al.*, 1982), New Zealand (Wouts and Weischer, 1977), the UK (Hirschmann and Triantaphyllou, 1979), Germany (Maas *et al.*, 1982), Russia (Subbotin, 1984), and Italy (Ambrogioni and Irdani, 2001). The morphometric characters of the six Japanese populations were also in congruence with those of the redescription of *H. trifolii* from Germany by Wouts and Sturhan (1978), who designated

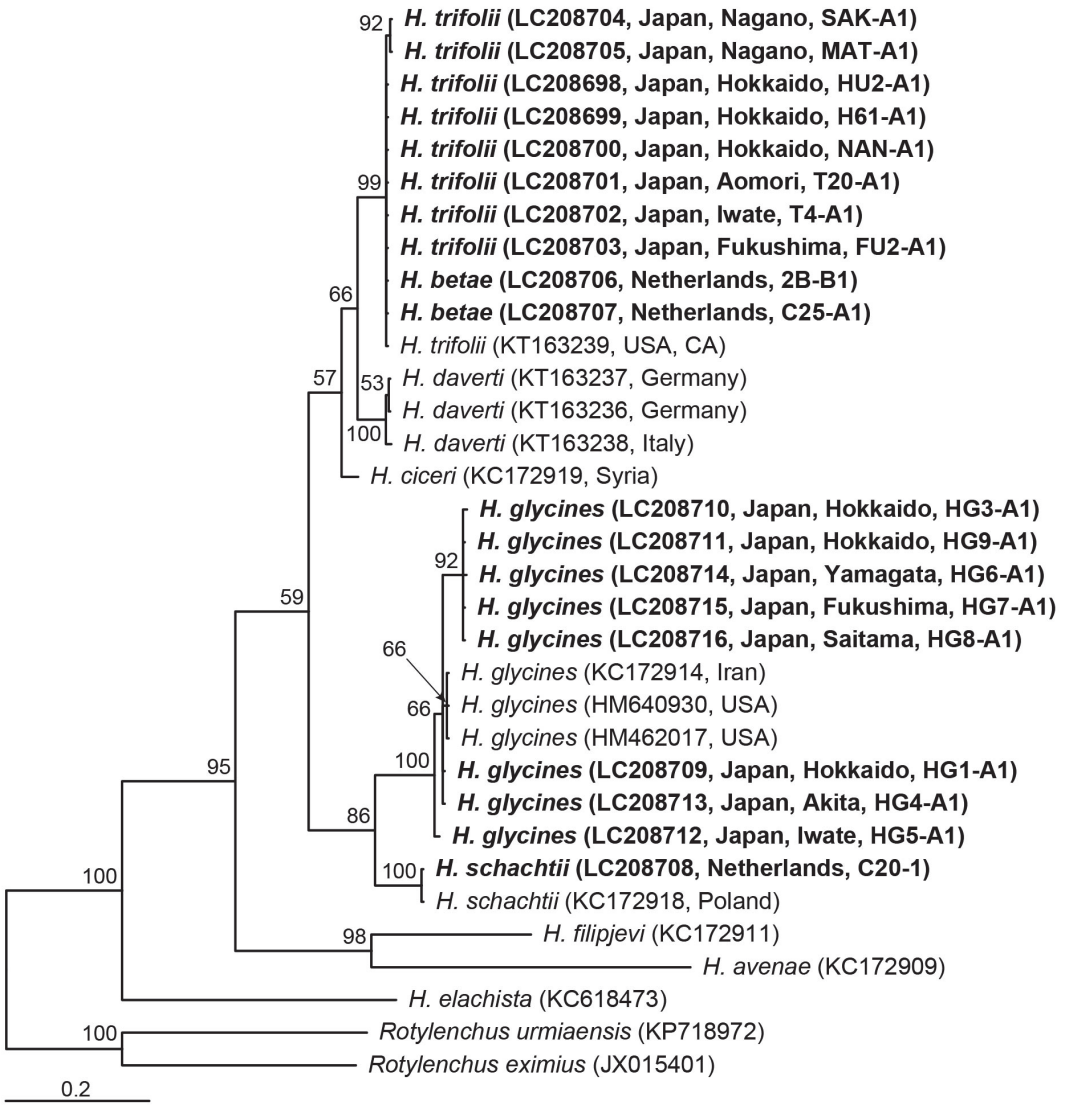

Fig. 11. Phylogenetic relationships between species of the *schachtii* group: Bayesian 50% majority rule consensus tree as inferred from the analysis of the *COI* gene sequence alignment under the GTR + I + G model. PP over 50% are given for appropriate clades. Newly obtained sequences are indicated by bold letters.

a neotype of this species. Although minor variations were observed in some morphometric characters between the examined populations, *e.g.*, J2 body length, J2 tail length, and length of the hyaline region of the J2 tail, these might have been due to a genetic variation arising from an interaction between environmental and genetic factors (Dawabah *et al.*, 2012).

Heterodera trifolii belongs to the *schachtii* group. All the species can be distinguished from each other by minor morphological and morphometric differences. Recent morphological and molecular studies have revealed that *H. trifolii* is closely related to *H. betae*, *H. daverti*, *H. glycines*, and *H. schachtii* (Ambrogioni and Irdani, 2001; Amiri *et al.*, 2002, 2003; Madani *et al.*, 2007; Vovlas *et al.*, 2015). The Japanese populations of *H. trifolii* can be morphologically distinguished from *H. betae* by the shorter average J2 body length (489–528 *vs* 584–607 μm), shorter average J2 stylet length (26.9–27.8 *vs* 29.6–31.0 μm), shorter average J2 tail length (54–65 *vs* 71–74 μm), and shorter average length of hyaline region of J2 tail (30–36 *vs* 39–42 μm) (Subbotin *et al.*, 2010b); from *H. daverti* by the absence of males, longer average J2 body length (489–528 *vs* 457–477 μm), longer average J2 stylet length (26.9–27.8 *vs* 25.0–26.0 μm), and longer average J2 tail length (54–65 *vs* 54–57 μm) (Subbotin *et al.*, 2010b); from *H. glycines* by the absence of males, longer average J2 body length (489–528 *vs* 385–471 μm), longer average J2 stylet length (26.9–27.8 *vs* 21.4–24.5 μm), longer average J2 tail length (54–65 *vs* 39–51 μm), and longer average length of hyaline region of J2 tail (30–36 *vs* 22–30 μm) (Subbotin *et al.*, 2010b); and from *H. schachtii* by the absence of males, longer average J2 body length (489–528 *vs* 436–468 μm), longer average J2 stylet length (26.9–27.8 *vs* 25.3–26.3 μm), longer average J2 tail length (54–65 *vs* 45–49 μm), and longer average length of hyaline region of J2 tail (30–36 *vs* 24–27 μm)

(Subbotin *et al.*, 2010b).

The results of the analysis of the ITS sequences support the separation of *Heterodera* species into seven species groups, *i.e.*, *afenestrata*, *avenae*, *cyperi*, *goettingiana*, *humuli*, *sacchari*, and *schachtii*, as previously described by Mundo-Ocampo *et al.* (2008), Zheng *et al.* (2008), Skantar *et al.* (2012), De Luca *et al.* (2013), Wang *et al.* (2013), and Zhuo *et al.* (2013, 2014). However, this molecular marker could not distinguish among some closely related species of the *schachtii* group, such as *H. betae*, *H. daverti*, *H. glycines*, *H. schachtii*, and *H. trifolii*, with high sequence similarity. The present study demonstrated that the D2–D3 of 28S rRNA gene and the *COI* mtDNA gene sequences clearly discriminated *H. trifolii* from other closely related species of the *schachtii* group, except for *H. betae*, with almost identical sequences. In addition, intraspecific sequence differences in the *schachtii* group were much higher in *COI* than in D2–D3, *e.g.*, differences between *H. trifolii* and *H. schachtii* were 9.0–10.1% (35–40 bp) in *COI* and 0.1–0.4% (1–3 bp) in D2–D3. This result was also in congruence with the results of a previous study by Vovlas *et al.* (2015), who noticed that the *COI* gene sequences appears to be a useful and promising molecular marker for deciphering the diversity of some *Heterodera* group complexes, such as the *schachtii* group. Although the molecular results showed it is impossible to discriminate molecularly between *H. trifolii* and *H. betae* using the ITS, D2–D3 of 28S rRNA gene, and the *COI* mtDNA gene sequences, they can be distinguished from each other by the above-mentioned morphometric characters and host plant range. Sugar beet, cabbage (*Brassica oleracea* L. var. *capitata*), and broad bean (*Vicia faba* L.) are non-hosts of *H. trifolii*, whereas these plants are good hosts of *H. betae* (Wouts *et al.*, 2001; Subbotin *et al.*, 2010b). In contrast, white clover is a good host of *H. trifolii* but

poor host of *H. betae* (Subbotin *et al.*, 2010b).

Only two *COI* haplotypes were found in eight Japanese *H. trifolii* populations, with 0.3% intraspecific sequence variation between these haplotypes. The *COI* sequence of *H. trifolii* from California, USA (KT163239) was entirely identical to those of Japanese *H. trifolii* populations except for the sequences from two populations of carnation in Nagano Prefecture (SAK and MAT), which differed by 1 bp only. In addition, four *COI* haplotypes were found in eight Japanese *H. glycines* populations with 0.3–2.3% intraspecific sequence variation among these haplotypes. Subbotin (2015) recently reported similar low intraspecific variations of 0.7% and 0.7% each for *H. sturhani* Subbotin, 2015 and *H. pratensis* Gäbler, Sturhan, Subbotin & Rumpenhorst, 2000. On the other hand, relatively high intraspecific variation was reported for *H. koreana* with 1.3–4.3% in Chapter 4.

Chapter 3

Reproductive ability of three Japanese populations of *Heterodera trifolii* on five crops and three carnation cultivars

3.1 Introduction

Variation in reproductive ability across different host plants has been previously observed among different populations of *H. trifolii* (Singh and Norton, 1970; Hirschmann and Triantaphyllou, 1979; Maas *et al.*, 1982; Wang and Riggs, 1999; Wang *et al.*, 2001). In Japan, only Momota *et al.* (1990) reported that a Japanese population of *H. trifolii* collected from carnation in Iida City, Nagano Prefecture, Japan reproduced well on red clover and azuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi), but not on soybean, sugar beet, or cabbage. However, no additional research has been conducted on other Japanese *H. trifolii* populations. For an effective pest control strategy, it is important to clarify the reproductive ability of these pests on different host plants. In this study, I examined the reproductive ability of three Japanese populations of *H. trifolii* on five crops and three carnation cultivars.

3.2 Materials and Methods

Three Japanese populations of *H. trifolii* collected in Chapter 1 were used in this study. The Hokkaido population (HU2) and Aomori population (T20) were originally collected from white clover in Sapporo City, Hokkaido Prefecture, Japan and Kuroishi City, Aomori Prefecture, Japan, respectively, and propagated on white clover cv. Ladino (Kaneko Seeds, Gunma, Japan) pot cultures in a greenhouse. The Nagano population

(SAK) was originally collected from carnation in Sakuho Town, Nagano Prefecture, Japan and propagated on carnation cv. Enfant de Nice mixed (Takii, Kyoto, Japan) pot cultures in a greenhouse. Species identification of the nematodes was confirmed by molecular methods using PCR-RFLP analysis of the ITS1-5.8S-ITS2 region (Orui, 1997). To obtain cyst nematode eggs and J2, cysts were extracted from soils of pot cultures using the dry flotation-sieving method (Aiba, 2014) and crushed to release eggs and J2. An aqueous suspension of eggs and J2 was used as inoculum.

A mixture (1:1) of sterilized andosol and commercial nursery soil (Kureha Engei Baido; Kureha, Tokyo, Japan) was used in the experiment. The five crops and three carnation cultivars are shown in Table 7. White clover was grown from seeds in 9 cm diameter polyethylene pots. Azuki bean cv. Tamba-dainagon (Takii, Kyoto, Japan), broccoli (*Brassica oleracea* L. var. *italica*) cv. Verde (Takii, Kyoto, Japan), spinach (*Spinacia oleracea* L.) cv. Wasesaradaakari (Takii, Kyoto, Japan), and sugar beet cv. Amahomare were grown from seeds in 5 × 5 × 8 cm plug seedling trays and then transplanted into 9 cm diameter polyethylene pots. Seedlings of carnation cvs. Barbara, Cherry Tessino, and Peachy Mambo (Hilverda Kooij, De Kwakel, The Netherlands) were purchased from M & H Bloemen, Yamanashi, Japan and transplanted into 9 cm diameter polyethylene pots. Plants were inoculated with approximately 2,000 eggs + J2 per pot at 42 days (spinach), 46 days (azuki bean, broccoli, and sugar beet), or 78 days (white clover) after sowing, or 42 days (carnation) after transplanting seedlings. The experiment was conducted in a greenhouse with an average temperature of approximately 25°C. Each pot was filled with approximately 300 ml soil and contained a single seedling, except for white clover that included more than two plants. Three replicates of each plant were tested. Plants were maintained for 63 days (spinach) or 65

Table 7. Number of cysts of *Heterodera trifolii* per pot 63 or 65 days after inoculation with 2,000 eggs + J2 per pot[1].

Plant (scientific name)	Cultivar		White clover populations		Carnation population
			Hokkaido (HU2) B[2]	Aomori (T20) B	Nagano (SAK) A
Fabaceae					
Azuki bean (*Vigna angularis*)	Tamba-dainagon	abc[3]	70.0 ± 27.3	77.3 ± 9.3	95.0 ± 12.1
Amaranthaceae					
Sugar beet (*Beta vulgaris* ssp. *vulgaris*)	Amahomare	d	0	0	0
Spinach (*Spinacia oleracea*)[4]	Wasesaradaakari	bcd	10.0 ± 5.5	4.0 ± 2.0	59.3 ± 21.1
Brasicaeae					
Broccoli (*Brassica oleracea* var. *italica*)	Verde	d	0	0	0
Caryophyllaceae					
Carnation (*Dianthus caryophyllus*)	Barbara	cd	16.7 ± 10.8	16.7 ± 2.4	19.7 ± 3.8
Carnation (*D. caryophyllus*)	Cherry Tessino	ab	59.0 ± 23.5	58.0 ± 15.5	151.0 ± 73.2
Carnation (*D. caryophyllus*)	Peachy Mambo	a	25.3 ± 13.8	114.0 ± 87.9	192.0 ± 30.9
Fabaceae					
White clover (*Trifolium repens*)[5]	Ladino		500<	500<	500<[6]

Table 7. (Continued.)

[1] Mean ± S.E. of three replicates except for as mentioned below.

[2] Same capital letter indicates no significant difference between populations (Tukey-Kramer HSD test, $P > 0.05$).

[3] Same small letter indicates no significant difference between cultivars (Tukey-Kramer HSD test, $P > 0.05$).

[4] Plants died approximately 35 days after nematode inoculation.

[5] Data were excluded from stastical analyses because number of cysts was counted up to 500 cysts per pot.

[6] Mean ± S.E. of two replicates.

days (azuki bean, broccoli, carnation, sugar beet, and white clover) after nematode inoculation. At the end of each culture period, the newly formed cysts in each pot were collected from the soil using the dry flotation-sieving method (Aiba, 2014) and counted. The number of cysts on white clover was counted up to 500 cysts per pot.

Cyst number data were analyzed using a two-way ANOVA, with nematode population and plant cultivar as factors. Significant differences between nematode populations or plant cultivars were further analyzed using the Tukey-Kramer HSD test for multiple comparisons at 5% significant level. Statistical analyses were performed using JMP 9.0.2 (SAS Institute, Cary, NC, USA.).

3.3 Results and Discussion

Data from one replicate of white clover hosting the Nagano population were omitted because of poor growth of the test plant and poor reproduction of cysts. Although the terrestrial part of all spinach plants hosting all three populations died approximately 35 days after nematode inoculation because of bolting, we included data from these plants as cysts were found in all pots (all pots were maintained in a greenhouse for 63 days after nematode inoculation). In addition, data from white clover hosting all three populations were excluded from statistical analyses as the number of cysts on white clover was counted up to 500 cysts per pot.

The number of cysts was significantly affected by nematode population and plant cultivar, but there were no significant interactions between nematode populations and plant cultivars (Table 8). The number of cysts from all populations was significantly higher on carnation cvs. Cherry Tessino and Peachy Mambo than that on sugar beet, broccoli, and carnation cv. Barbara (Tukey-Kramer HSD test, $P < 0.05$; Table 8). The

Table 8. Results of two-way ANOVA on the effcts of nematode populations and plant cultivars on the number of cysts of *Heterodera trifolii*.

Factor	*df*	MS	*F* value	*P* value
Nematode population	2	14,086.30	5.9342	0.0054
Plant cultivar	6	19,722.74	8.3086	< 0.0001
Nematode population × plant cultivar	12	3,531.95	1.4879	0.1672
Residual	62	4,654.68		

number of cysts from the Nagano population was significantly higher than that of the Hokkaido and Aomori populations on all plants, except for white clover (Tukey-Kramer HSD test, $P < 0.05$; Table 8).

In this study, although all three populations produced cysts on white clover, azuki bean, spinach, and the three carnation cultivars, none produced cysts on sugar beet or broccoli. These results are in agreement with previous studies (Mulvey, 1959; Holtzmann and Aragaki, 1963; Singh and Norton, 1970; Maas *et al.*, 1982; Riggs, 1982; Momota *et al.*, 1990; Wang and Riggs, 1999; Wang *et al.*, 2001). White clover was the most susceptible host of all plants examined. Azuki bean was also moderately susceptible to all populations. Although only *H. glycines* has been reported as a pest of azuki bean in Japan (Momota, 2004), these results indicated that *H. trifolii* could also be a potential pest of azuki bean. The Nagano population reproduced moderately well on spinach, but the Hokkaido and Aomori populations reproduced poorly. However, Momota (2004) noted that *H. trifolii* reproduced well on spinach after inoculation. Carnation cv. Cherry Tessino was susceptible, whereas carnation cv. Barbara was less susceptible to reproduction by all nematode populations. Carnation cv. Peachy Mambo was susceptible to the Aomori and Nagano populations, but less susceptible to the Hokkaido population. The number of cysts from all populations was much lower on the three carnation cultivars than on white clover. Similar results were reported by Wang and Riggs (1999), who compared the reproductive abilities of seven *H. trifolii* populations from the USA and Australia, and by Singh and Norton (1970), who studied variability of host-parasitic relationships in six *H. trifolii* populations from the USA and Canada. Consequently, carnation might not be as good a host for *H. trifolii* as white clover. Sugar beet and broccoli were apparently non-preferred hosts for *H. trifolii*.

Carnation is one of the common hosts of *H. trifolii* (Subbotin *et al.*, 2010b). In Japan, severe damage to carnation caused by *H. trifolii* has been reported from Hokkaido (Momota and Mizukoshi, 2000) and Nagano Prefectures (Momota *et al.*, 1990; Toyoshima *et al.*, 1992). The damage caused by *H. trifolii* to carnation includes stunted plant growth, wilting, curling of leaves, and delays in flower bud appearance (Momota *et al.*, 1990; Momota and Mizukoshi, 2000). Momota and Mizukoshi (2000) suggested that carnation cultivars can be divided into three types, *i.e.*, susceptible type (significant growth inhibition and high or low nematode population density), tolerant type (little growth inhibition and high nematode population density), and resistant type (little growth inhibition and low nematode population density), based on the relationship between plant growth inhibition and population densities of J2 in soil. They noticed that carnation cv. Cherry Tessino was the tolerant type. In this study, the number of cysts from all populations was significantly lower on carnation cv. Barbara than on cvs. Cherry Tessino or Peachy Mambo (Tukey-Kramer HSD test, $P < 0.05$; Table 8). These results suggested that carnation cv. Barbara may be the resistant type. Conversely, the number of cysts from all populations on carnation cvs. Cherry Tessino and Peachy Mambo varied from approximately 25 to 190 and 60 to 150, respectively. Momota and Mizukoshi (2000) pointed out that carnation cultivars that were resistant to Dutch populations of *H. trifolii* were susceptible to Hokkaido populations because of the difference in host preference between Dutch and Japanese populations. In addition, previous studies indicated that geographically distinct populations of *H. trifolii* had different reproductive abilities on carnation (Singh and Norton, 1970; Hirschmann and Triantaphyllou, 1979; Wang and Riggs, 1999; Wang *et al.*, 2001). The variation in the number of cysts from the three populations on spinach and carnation cvs. Cherry

Tessino and Peachy Mambo suggested variation in reproductive ability on different host plants in Japanese *H. trifolii* populations.

Chapter 4

Morphological and molecular characterization of *Heterodera koreana* from bamboo in Japan

4.1 Introduction

To date, five *Heterodera* species belonging to the *afenestrata* or *cyperi* groups have been recorded from bamboo: *H. bamboosi* (Kaushal & Swarup, 1988) Wouts & Baldwin, 1988, *H. fengi* Wang, Zhou, Ye, Zhang, Peng & Liao, 2013, *H. guangdongensis* Zhou, Wang, Zhang, Peng & Liao, 2014, *H. hainanensis* Zhuo, Wang, Ye, Peng & Liao, 2013, and *H. koreana*. The bamboo cyst nematode, *H. bamboosi*, has only been found from bamboo (*Bambusa* sp.) in India (Kaushal and Swarup, 1988). This species was originally described as a new species of *Brevicephalodera* Kaushal & Swarup, 1988 and was subsequently transferred to *Afenestrata* Baldwin & Bell, 1985 by Siddiqi (2000) and to *Heterodera* by Mundo-Ocampo *et al.* (2008). Three Chinese species, *H. fengi*, *H. guangdongensis*, and *H. hainanensis*, were recently described from moso bamboo (*Phyllostachys edulis* (Carrière) J.Houz.) in China (Wang *et al.*, 2013; Zhuo *et al.*, 2013, 2014). The Korean cyst nematode, *H. koreana*, was isolated from moso bamboo in South Korea and originally described as a new species of *Afenestrata* (Vovlas *et al.*, 1992). Subsequently, this species was transferred to *Heterodera* by Mundo-Ocampo *et al.* (2008). This species was recorded from fish pole bamboo (*P. aurea* Riviere & C. Riviere) in the USA (Florida) (Inserra *et al.*, 1999), unidentified bamboo in Thailand (Sturhan, 2010), moso bamboo in China (Wang *et al.*, 2012), and black bamboo (*P. nigra* (Lodd. ex Lindl.) Munro) in Iran (Tanha Maafi *et al.*, 2015).

Momota (2004) pointed out that an unidentified *Afenestrata* sp. (= *Heterodera* sp.) on bamboo in Japan is likely to be *A. koreana* (= *H. koreana*) or a related species. However, the correct identification of this nematode species using molecular and morphological analyses has not yet been achieved.

During nematological surveys conducted during 2012–2014 in Japan, cyst nematode populations were recovered from soil samples collected from the rhizosphere of bamboo. The aims of the present study were: *i*) to identify the cyst nematode species from bamboo in Japan; *ii*) to provide molecular characterization of the cyst nematode populations using the ITS, the D2–D3 expansion segments of 28S rRNA gene, and the partial of *COI* mtDNA gene sequences; and *iii*) to reveal phylogenetic relationships within *Heterodera* using rRNA and *COI* gene sequences.

4.2 Materials and Methods

Nematode populations

During 2012–2014, 88 soil samples were collected from the rhizosphere of four bamboo species in Japan: 70 samples from moso bamboo, 11 samples from madake (*P. bambusoides* Siebold & Zucc.), five samples from henon bamboo (*P. nigra* var. *henonis* (Mitford) Stapf ex Rendle), and two samples from fish pole bamboo. Soil samples were collected at a depth of approximately 5–15 cm of soil around roots of bamboo using a trowel. Then, approximately 1 kg of soil was mixed well in a plastic bag, labelled, and kept in a refrigerator at 10°C for use in further studies. J2 were extracted from 2 × 20 g soil samples by the Baermann funnel method for 3 days at approximately 25°C. Cysts were extracted from soil samples by the dry flotation-sieving method (Aiba, 2014). For one soil sample collected from moso bamboo in Tsukuba City, Ibaraki Prefecture, Japan

(Tsk), additional samples were collected in 2015–2016 from the same locality in order to obtain the necessary specimens for morphological identification. One population from moso bamboo in Tsukuba City, Ibaraki Prefecture (Tsk) was used for morphological examination by LM and molecular analyses. The other populations were used only for molecular analyses.

Morphological analysis

Morphological measurements were made and photomicrographs were taken from one population from moso bamboo in Tsukuba City, Ibaraki Prefecture (Tsk). J2 specimens were killed in gently heated water (65°C for 1 min), fixed in a solution of 4% formaldehyde, processed in a glycerin-ethanol series using modified Seinhorst's method (Minagawa and Mizukubo, 1994), and mounted on permanent slides. Additional measurements were obtained from live J2 specimens temporarily mounted on to a glass slide in a drop of tap water, killed with gentle heat (65°C for 10 sec) on a hot plate, examined, and measured immediately. Cyst vulval cones were prepared from cysts and mounted in glycerin jelly. Specimens were examined using a compound Olympus BX51 microscope equipped with DIC. Photomicrographs were taken with a digital camera Olympus DP21 attached to Olympus BX51.

DNA extraction, PCR and sequencing

DNA was extracted from three J2 individuals of each population. The protocol used for DNA extraction with proteinase K was described in Chapter 1, which yielded 200 μl of lysate for each specimen. The ITS1-5.8S-ITS2 region was amplified using the forward primer TW81 (5′ -GTT TCC GTA GGT GAA CCT GC-3′) and reverse primer

AB28 (5′ -ATA TGC TTA AGT TCA GCG GGT-3′) (Joyce *et al.*, 1994). The D2–D3 expansion segments of the 28S rRNA gene was amplified using the forward primer D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and reverse primer D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (De Ley *et al.*, 1999). PCR amplifications for ITS and D2–D3 were performed in a final volume of 20 μl reaction mixture containing 2 μl 10 × Ex Taq buffer (20 mM Mg^{2+} plus) (Takara Bio, Shiga, Japan), 1.6 μl dNTP mixture (2.5 mM), 0.4 μl (10 μM) of each primer, 0.1 μl TaKaRa Ex Taq® Hot Start Version (5 U μl^{-1}) (Takara Bio, Shiga, Japan), 1 μl DNA template, and 14.5 μl distilled water. The amplification conditions for ITS and D2–D3 were as follows: a single step of pre-denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C (ITS) or 60°C (D2–D3) for 30 sec, and extension at 72°C for 30 sec (D2–D3) or 1 min (ITS and D2–D3). For ITS and D2–D3, two J2 individuals of each population were analysed. The partial *COI* of the mtDNA gene was amplified using the forward primer JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and reverse primer JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′) (Derycke *et al.*, 2005). PCR amplification for *COI* was performed in a final volume of 20 μl reaction mixture containing 4 μl 5 × PrimeSTAR Buffer (5 mM Mg^{2+} plus) (Takara Bio, Shiga, Japan), 1.6 μl dNTP mixture (2.5 mM), 0.4 μl (10 μM) of each primer, 0.1 μl PrimeSTAR® HS DNA Polymerase (2.5 U μl^{-1}) (Takara Bio, Shiga, Japan), 1 μl DNA template, and 12.5 μl distilled water. The amplification conditions for *COI* were as follows: a single pre-denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 46°C for 15 sec, and extension at 72°C for 30 sec. For *COI*, three J2 individuals of each population were analysed. The PCR products were purified and sequenced following the methods described in Chapter

2. The newly obtained sequences were submitted to the GenBank database under the accession numbers indicated in Table 9.

Phylogenetic analysis

The newly obtained sequences of the ITS, D2–D3 of 28S rRNA gene, and *COI* mtDNA gene were edited with DNADynamo (Blue Tractor Software, UK) and aligned using MUSCLE (Edgar, 2004) as implemented in MEGA 5.2.2 (Tamura *et al.*, 2011) with default parameters with their corresponding published gene sequences. Outgroup taxa for each data set were selected based on previously published data (Mundo-Ocampo *et al.*, 2008; Zheng *et al.*, 2008; Wang *et al.*, 2013; Zhuo *et al.*, 2013, 2014). Sequence datasets were analysed with BI using MrBayes 3.2.2 (Ronquist *et al.*, 2011). The best-fit model of DNA evolution was selected using the AIC as implemented in MrModeltest 2.3 (Nylander, 2004) in conjunction with PAUP* 4.0b10 (Swofford, 2003). The GTR + I + G model for ITS and GTR + G model for D2–D3 and *COI* were selected. BI analysis was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. Tracer v.1.6 (Rambaut and Drummond, 2013) was used to test for ESS (> 100) and convergence of parameters. The topologies were used to generate a 50% majority rule consensus tree. PP were given on appropriate clades. The tree was visualised using FigTree v1.3.1 (Rambaut, 2009). Further sequence analyses of alignments were performed with PAUP* 4.0b10 (Swofford, 2003). Pairwise divergences between taxa were computed as absolute distance values and as percentage mean

Table 9. Japanese populations of *Heterodera koreana* used in the present study.

No.	Locality	Host plant	Sample code	GenBank accession number			Collector
				ITS	D2–D3 of 28S rRNA	*COI* mtDNA	
1	Oshu City, Iwate Prefecture	*Phyllostachys nigra* var. *henonis*	Miz	LC202133	LC202092	LC202153	M. Sasaki
2	Tanakura Town, Fukushima Prefecture	*P. edulis*	Tnk	–	LC202093	LC202154	S. Sekimoto
3	Yamatsuri Town, Fukushima Prefecture	*P. aurea*	Yam	LC202134, LC202135	LC202094	LC202155	S. Sekimoto
4	Sakuragawa City, Ibaraki Prefecture	*P. bambusoides*	Skg	–	LC202095	LC202156	S. Sekimoto
5	Tsukuba City, Ibaraki Prefecture	*P. edulis*	Tsk	LC202136, LC202137	LC202096	LC202157	S. Sekimoto
6	Ishioka City, Ibaraki Prefecture	*P. edulis*	Isk	–	LC202097	LC202158	S. Sekimoto
7	Utsunomiya City, Tochigi Prefecture	*P. edulis*	Uts	–	LC202098	LC202159	S. Sekimoto
8	Shimotsuke City, Tochigi Prefecture	*P. bambusoides*	Smt	–	LC202099	LC202160	S. Sekimoto
9	Moka City, Tochigi Prefecture	*P. edulis*	Moo	–	LC202100	LC202161	S. Sekimoto
10	Ichihara City, Chiba Prefecture	*P. bambusoides*	Ich	–	LC202101	LC202162	Z. Nakai
11	Kamakura City, Kanagawa Prefecture	*P. edulis*	Kam	–	LC202102	LC202163	S. Sekimoto
12	Shibata City, Niigata Prefecture	*P. edulis*	Shb	–	LC202103	LC202164	S. Sekimoto
13	Imizu City, Toyama Prefecture	*P. edulis*	Imz	–	LC202104	LC202165	S. Sekimoto

Table 9. (Continued.)

No.	Locality	Host plant	Sample code	GenBank accession number			Collector
				ITS	D2–D3 of 28S rRNA	*COI* mtDNA	
14	Tonami City, Toyama Prefecture	*Phyllostachys edulis*	Tnm	–	LC202105	LC202166	S. Sekimoto
15	Kanazawa City, Ishikawa Prefecture	*P. edulis*	KnzA	–	LC202106	LC202167	S. Sekimoto
16	Kanazawa City, Ishikawa Prefecture	*P. nigra var. henonis*	KnzB	LC202138	LC202107	LC202168	S. Sekimoto
17	Komatsu City, Ishikawa Prefecture	*P. edulis*	Kmt	–	LC202108	LC202169	S. Sekimoto
18	Fuji City, Shizuoka Prefecture	*P. edulis*	Fuj	LC202139, LC202140	LC202109	LC202170	S. Sekimoto
19	Tokoname City, Aichi Prefecture	*P. edulis*	Tok	–	LC202110	LC202171	T. Sengo
20	Kuwana City, Mie Prefecture	*P. edulis*	Kuw	–	LC202111	LC202172	T. Sengo
21	Inabe City, Mie Prefecture	*P. edulis*	Ina	LC202141, LC202142	LC202112	LC202173	T. Sengo
22	Watarai Town, Mie Prefecture	*P. edulis*	Wat	–	LC202113	LC202174	S. Sekimoto
23	Owase City, Mie Prefecture	*P. edulis*	Owa	–	LC202114	LC202175	S. Sekimoto
24	Wakayama City, Wakayama Prefecture	*P. bambusoides*	Wky	LC202143, LC202144	LC202115	LC202176	T. Sengo

Table 9. (Continued.)

No.	Locality	Host plant	Sample code	GenBank accession number			Collector
				ITS	D2–D3 of 28S rRNA	*COI* mtDNA	
25	Yonago City, Tottori Prefecture	*Phyllostachys edulis*	Yng	LC202145, LC202146	LC202116	LC202177	S. Sekimoto
26	Kanonji City, Kagawa Prefecture	*P. edulis*	Koj	–	LC202117	LC202178	S. Sekimoto
27	Mitoyo City, Kagawa Prefecture	*P. bambusoides*	MtyA	–	LC202118	LC202179	S. Sekimoto
28	Mitoyo City, Kagawa Prefecture	*P. edulis*	MtyB	–	LC202119	LC202180	S. Sekimoto
29	Ino Town, Kochi Prefecture	*P. edulis*	Ino	LC202147, LC201248	LC202120	LC202181	S. Sekimoto
30	Nakatosa Town, Kochi Prefecture	*P. edulis*	Kre	–	LC202121	LC202182	S. Sekimoto
31	Shimanto Town, Kochi Prefecture	*P. edulis*	Sim	–	LC202122	LC202183	S. Sekimoto
32	Imari City, Saga Prefecture	*P. edulis*	Ima	–	LC202123	LC202184	S. Sekimoto
33	Takeo City, Saga Prefecture	*P. edulis*	Tko	–	LC202124	LC202185	S. Sekimoto
34	Ureshino City, Saga Prefecture	*P. edulis*	Ure	–	LC202125	LC202186	S. Sekimoto
35	Higashisonogi Town, Nagasaki Prefecture	*P. bambusoides*	Hsg	LC202149, LC201250	LC202126	LC202187	S. Sekimoto
36	Sasebo City, Nagasaki Prefecture	*P. edulis*	Ssb	–	LC202127	LC202188	S. Sekimoto
37	Koshi City, Kumamoto Prefecture	*P. edulis*	Kos	–	LC202128	LC202189	H. Iwahori

Table 9. (Continued.)

No.	Locality	Host plant	Sample code	GenBank accession number			Collector
				ITS	D2–D3 of 28S rRNA	*COI* mtDNA	
38	Satsuma Town, Kagoshima Prefecture	*Phyllostachys edulis*	Sat	–	LC202129	LC202190	H. Morioka
39	Kagoshima City, Kagoshima Prefecture	*P. bambusoides*	KagA	–	LC202130	LC202191	H. Morioka, A. Mukoshimizu
40	Kagoshima City, Kagoshima Prefecture	*P. edulis*	KagB	–	LC202131	LC202192	H. Morioka, A. Mukoshimizu
41	Minamikyushu City, Kagoshima Prefecture	*P. edulis*	Mei	LC202151, LC201252	LC202132	LC202193	H. Morioka

distance values based on whole alignment, with adjustment for missing data.

4.3 Results

Nematode populations from bamboo

In total, 88 soil samples were collected in 2012–2014 from the rhizosphere of four bamboo species in Japan. Cyst nematode J2 were detected in 46% of the soil samples (41 of 88 samples) (Table 9). The detection rate of cyst nematodes was 44% (31 of 70 samples) from moso bamboo, 63% (seven of 11 samples) from madake, 40% (two of five samples) from henon bamboo, and 50% (one of two samples) from fish pole bamboo.

Morphological characterization of Japanese population (Fig. 12; Table 10)

Cyst variable in size, light to dark brown in colour, and lemon-shaped or ovoid with distinct neck and vulval cone. Cuticle showing an irregular zigzag pattern. Vulval cone lacking fenestration and covered with tuberculate pattern. Bullae and underbridge absent. Egg-sac absent. Vulval lips enclosing a sunken vulval slit.

J2 cylindrical in shape, tapering posteriorly and curved ventrally after heat-killing. Body length 417–465 μm. Lip region hemispherical and not offset. Stylet robust with rounded knobs. Stylet length 17.5–18.7 μm. Dorsal pharyngeal gland orifice distinct. Median bulb oval and prominent. Pharyngeal glands well developed, extending well posterior to pharyngo-intestinal valve. Lateral field with three incisures. Genital primordium oval, 12.1–15.4 long × 7.4–9.7 μm wide, and 176–207 μm anterior to tail tip. Tail conoid, tapering uniformly to finely rounded terminus. Hyaline region of tail 37–47 μm long, occupying 50–68% of tail length.

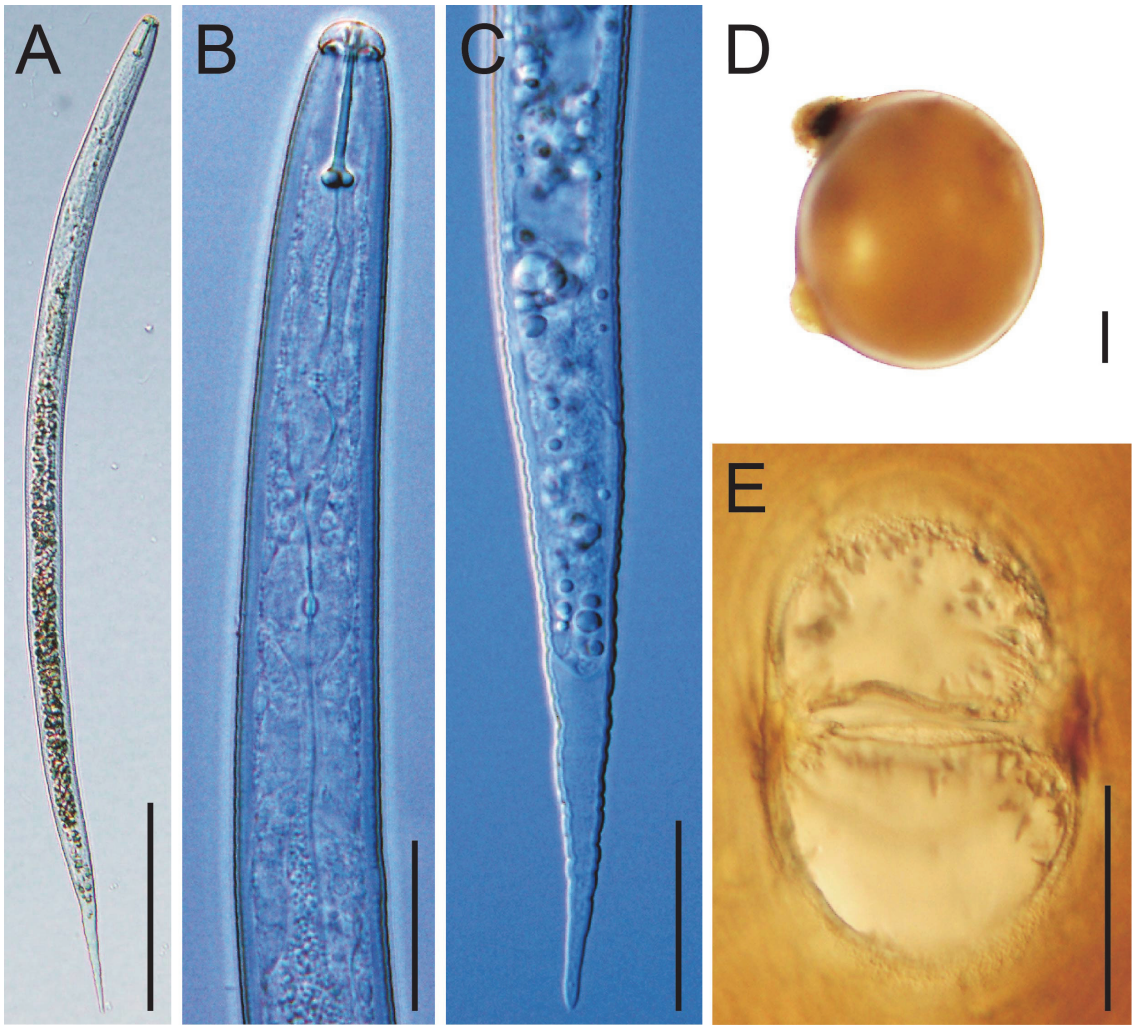

Fig. 12. Light micrographs of *Heterodera koreana* from Tsukuba City, Ibaraki Prefecture (Tsk). A: Entire body of J2; B: Anterior region of J2; C: Tail of J2; D: Entire cyst; E: Vulval plate. (Scale bars: A, D = 100 μm; B, C = 20 μm; E = 50 μm.)

Table 10. Morphometric comparison of *Heterodera koreana* populations from Japan, South Korea, China, and Iran. All measurements are in μm and in the form: mean ± S.D. (range).

Stage	Character	Tsukuba, Japan		South Korea	China	Iran
		Tsk		Vovlas *et al.* (1992)	Wang *et al.* (2012)	Tanha Maafi *et al.* (2015)
Cyst	n	10	–	20	5	21 (Vulval cone 7)
	L (including neck)	641 ± 65.8 (524–770)	–	840 ± 149.0 (630–1174)	748 ± 75.0 (700–860)	502 ± 70.0 (420–640)
	Body diameter	574 ± 60.7 (482–706)	–	520 ± 90.0 (400–653)	680 ± 63.0 (620–760)	408 ± 60.0 (320–520)
	L (including neck)/body diameter ratio	1.1 ± 0.1 (1.0–1.2)	–	1.5 ± 0.4 (1.0–2.9)	1.1 ± 0.1 (1.0–1.2)	1.2 ± 0.1 (1.1–1.5)
	Vulval slit length	49.9 ± 4.9 (40.5–59.8)	–	49.0 ± 4.3 (42.0–56.0)	48.8 ± 5.1 (41.3–55.0)	51.9 ± 4.3 (46.0–59.0)

Table 10. (Continued.)

Stage	Character	Tsukuba, Japan		South Korea	China	Iran
		Tsk		Vovlas *et al.* (1992)	Wang *et al.* (2012)	Tanha Maafi *et al.* (2015)
J2	n	20 (live)	20 (fixed)	20	20	14
	L	458 ± 17.3 (424–483)	442 ± 13.4 (417–465)	446 ± 28.0 (390–509)	513 ± 29.2 (448–553)	455 ± 11.3 (437–472)
	a	26.3 ± 0.8 (25.3–27.8)	27.7 ± 0.7 (26.3–29.1)	28.0 ± 1.7 (25.0–32.0)	29.9 ± 1.6 (26.0–32.2)	29.9 ± 0.9 (28.3–31.5)
	b	3.5 ± 0.1 (3.4–3.7)	3.6 ± 0.2 (3.3–4.1)	–	–	–
	b′	2.1 ± 0.2 (1.9–2.5)	1.9 ± 0.1 (1.8–2.2)	2.5 ± 0.2 (2.3–3.4)	3.3 ± 0.3 (2.7–3.8)	2.7 ± 0.4 (2.2–3.5)
	c	6.3 ± 0.2 (6.0–7.0)	6.2 ± 0.4 (5.6–7.2)	6.7 ± 0.3 (6.1–7.3)	6.7 ± 0.7 (6.2–8.1)	7.4 ± 0.9 (6.0–8.9)
	c′	5.9 ± 0.3 (5.1–6.5)	6.2 ± 0.3 (5.5–6.6)	–	6.6 ± 0.7 (5.2–7.3)	6.1 ± 0.4 (5.1–6.7)
	Stylet length	17.5 ± 0.6 (16.4–18.9)	18.1 ± 0.3 (17.5–18.7)	18.0 ± 1.5 (16.0–20.0)	19.0 ± 0.9 (17.5–20.5)	18.1 ± 0.5 (17.0–19.0)
	Stylet knob height	2.3 ± 0.1 (2.1–2.6)	2.3 ± 0.2 (2.0–2.6)	–	–	–
	Stylet knob diameter	3.9 ± 0.1 (3.8–4.2)	3.6 ± 0.2 (3.2–4.0)	–	–	–
	Lip region height	3.6 ± 0.2 (3.3–3.9)	3.6 ± 0.2 (3.3–3.9)	–	–	3.0 (3.0)

Table 10. (Continued.)

Stage	Character	Tsukuba, Japan		South Korea	China	Iran
		Tsk		Vovlas *et al.* (1992)	Wang *et al.* (2012)	Tanha Maafi *et al.* (2015)
J2	n	20 (live)	20 (fixed)	20	20	14
	Lip region diameter	8.1 ± 0.2 (7.7–8.4)	7.9 ± 0.2 (7.5–8.2)	–	–	7.5 ± 0.5 (7.0–8.0)
	DGO	5.2 ± 0.4 (4.4–6.2)	5.3 ± 0.5 (4.5–6.0)	–	–	–
	Anterior end to median bulb valve	70 ± 3.0 (65–77)	70 ± 2.4 (67–76)	72 ± 7.2 (65–98)	72 ± 6.8 (60–80)	72 ± 1.7 (70–75)
	Anterior end to excretory pore	105 ± 3.0 (98–111)	101 ± 3.2 (95–107)	95 ± 4.3 (88–103)	111 ± 6.7 (97–118)	100 ± 2.5 (96–103)
	Anterior end to pharyngo-intestinal valve	130 ± 5.8 (121–141)	123 ± 7.3 (109–135)	–	–	–
	Anterior end to end of pharyngeal glands	220 ± 21.4 (178–254)	231 ± 10.7 (205–249)	–	–	–
	Body diameter at mid-body	17.4 ± 0.4 (16.5–18.1)	16.0 ± 0.3 (15.3–16.7)	16.0 ± 1.0 (14.0–17.0)	17.2 ± 0.5 (16.0–18.0)	15.2 ± 0.4 (15.0–16.0)
	Body diameter at anus	12.2 ± 0.4 (11.5–12.7)	11.7 ± 0.5 (10.7–12.8)	–	–	10.1 ± 1.0 (8.0–11.0)
	Tail length	72 ± 4.4 (63–79)	72 ± 4.8 (63–80)	66 ± 4.4 (59–74)	77 ± 8.9 (62–88)	62 ± 6.9 (51–74)
	Length of hyaline region	41 ± 3.2 (33–45)	42 ± 2.9 (37–47)	40 ± 2.5 (35–46)	49 ± 5.2 (39–56)	44 ± 1.8 (40–47)

Table 10. (Continued.)

Stage	Character	Tsukuba, Japan		South Korea	China	Iran
		Tsk		Vovlas *et al.* (1992)	Wang *et al.* (2012)	Tanha Maafi *et al.* (2015)
J2	n	20 (live)	20 (fixed)	20	20	14
	Hyaline region/stylet length	2.3 ± 0.2 (2.0–2.6)	2.3 ± 0.2 (2.1–2.6)	–	–	–
	Tail length/hyaline region	1.8 ± 0.1 (1.7–1.9)	1.7 ± 0.1 (1.5–2.0)	–	–	–
	Genital primordium length	–	13.4 ± 0.9 (12.1–15.4)	–	–	–
	Genital primordium diameter	–	8.5 ± 0.7 (7.4–9.7)	–	–	–
	Genital primordium to posterior end	–	193 ± 9.1 (176–207)	–	–	–

Molecular characterization and phylogeny

Amplification of ITS, D2–D3 expansion segments of 28S rRNA gene, and the partial *COI* gene yielded a single fragment of *ca* 1000, 700, and 400 bp, respectively, based on the results of gel electrophoresis.

ITS

The ITS sequence alignment contained 76 sequences of *Heterodera* species including 25 *H. koreana* sequences and two sequences of *C. brinkmani* and *M. alni* used as outgroups, and was 1308 bp in length. Twenty new sequences were obtained in the present study. Intraspecific ITS sequence variation for *H. koreana* was 0–3.1% (0–28 bp). Phylogenetic relationships between *Heterodera* species, as inferred from BI, are presented in Figure 13. Phylogenetic relationships between the seven species groups were resolved. *Heterodera koreana* clustered with *H. bifenestra*, *H. cardiolata* Kirjanova & Ivanova, 1969, *H. hainanensis*, *H. orientalis* (Kazachenko, 1989) Mundo-Ocampo, Troccoli, Subbotin, Del Cid, Baldwin & Inserra, 2008, and *Heterodera* sp. Moghan-SS-2002 from Iran, with high support value (PP = 100). Interspecific differences between *H. koreana* and *H. hainanensis* were 9.2–9.8% (86–92 bp) and *H. koreana* and *H. orientalis* were 20.0–22.0% (194–213 bp).

D2–D3 of 28S rRNA gene

The D2–D3 of 28S rRNA gene alignment contained 63 sequences of *Heterodera* species including 44 *H. koreana* sequences and two sequences of *C. brinkmani* and *Atalodera crassicrustata* (Bernard, 1981) de Souza & Huang, 1994 used as outgroups, and was 756 bp in length. Forty-one new sequences were obtained in this study.

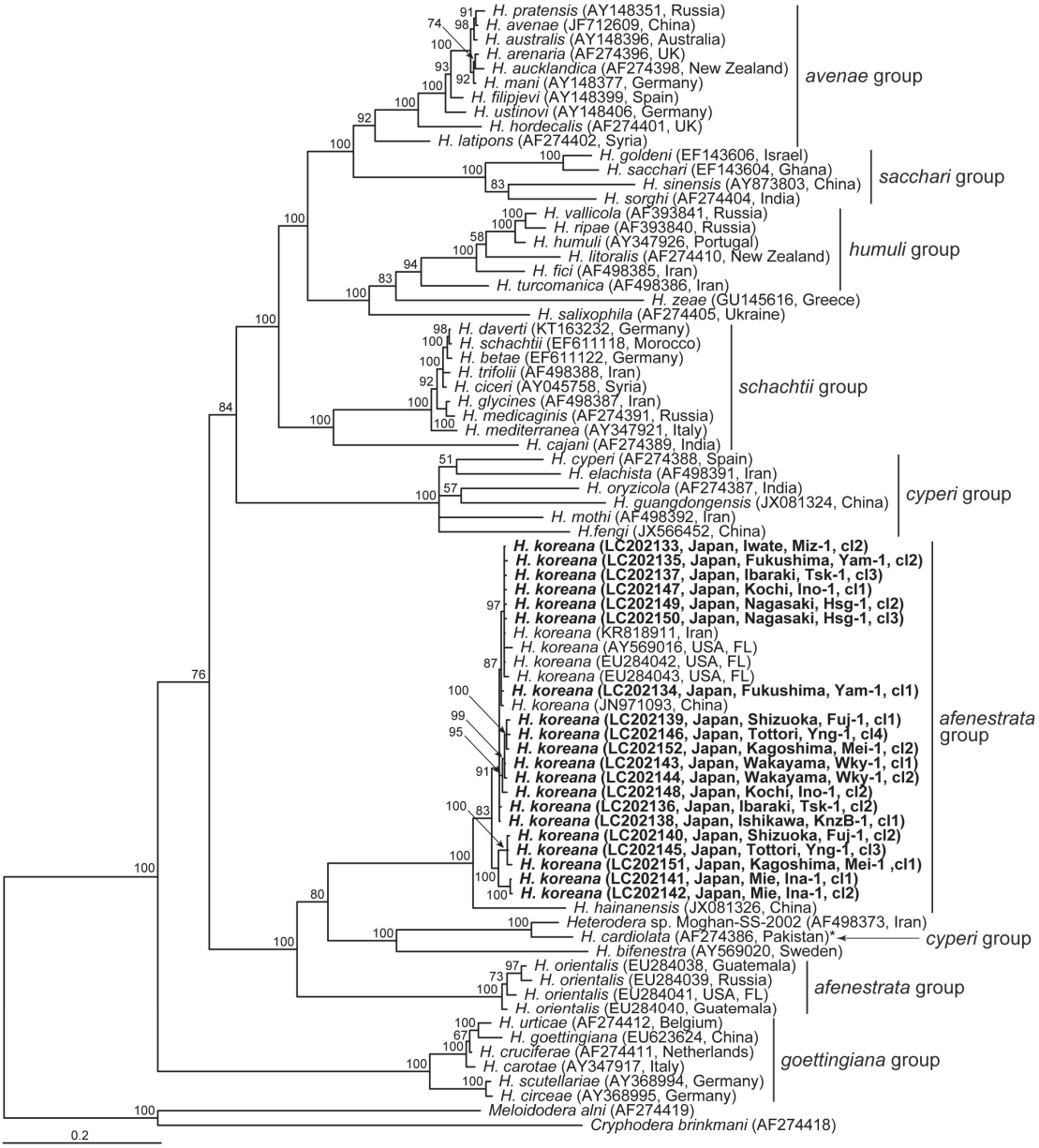

Fig. 13. Phylogenetic relationships between *Heterodera* species: Bayesian 50% majority rule consensus tree as inferred from the analysis of the ITS sequence alignment under the GTR + I + G model. PP over 50% are given for appropriate clades. Newly obtained sequences are indicated by bold letters. * Identified as *H. cynodontis* by Subbotin *et al.* (2001), the species is considered as a synonym of *H. cardiolata* by Subbotin *et al.* (2010b).

Intraspecific D2–D3 sequence variation for *H. koreana* was 0–2.9% (0–22 bp). The phylogenetic relationships of *H. koreana* with related species, as inferred from BI, are presented in Figure 14. *Heterodera koreana* clustered with *H. hainanensis* with high support value (PP = 100). Interspecific differences between *H. koreana* and *H. hainanensis* were 2.6–4.7% (19–35 bp) and *H. koreana* and *H. orientalis* were 7.5–8.3% (52–61 bp).

COI of the mtDNA gene

COI gene alignment included 73 sequences of *Heterodera* species and two sequences of *R. eximius* and *R. urmiaensis* as outgroups, and was 393 bp in length. Forty-one sequences were obtained in the present study. Intraspecific *COI* sequence variation for *H. koreana* was 0–4.3% (0–17 bp). No insertions or deletions occurred between the sequences. Eighteen positions were variable and 16 were third-base substitutions (Table 11). Three *COI* haplotypes were found for Japanese *H. koreana* populations. Nucleotide sequences were also converted into amino-acid sequences, the analysis revealing that intraspecific amino-acid sequence variation for *H. koreana* was only 1.5% (two amino acids). Phylogenetic relationships between *Heterodera* species, as inferred from BI, are presented in Figure 15. *Heterodera koreana* clustered with *H. cardiolata* with high support value (PP = 100). Interspecific differences between *H. koreana* and *H. cardiolata* were 9.7–11.2% (38–44 bp). The distribution of *COI* haplotypes across Japan is given in Figure 16. Haplotypes A and B were found from 20 populations, whereas haplotype C was only detected from one population.

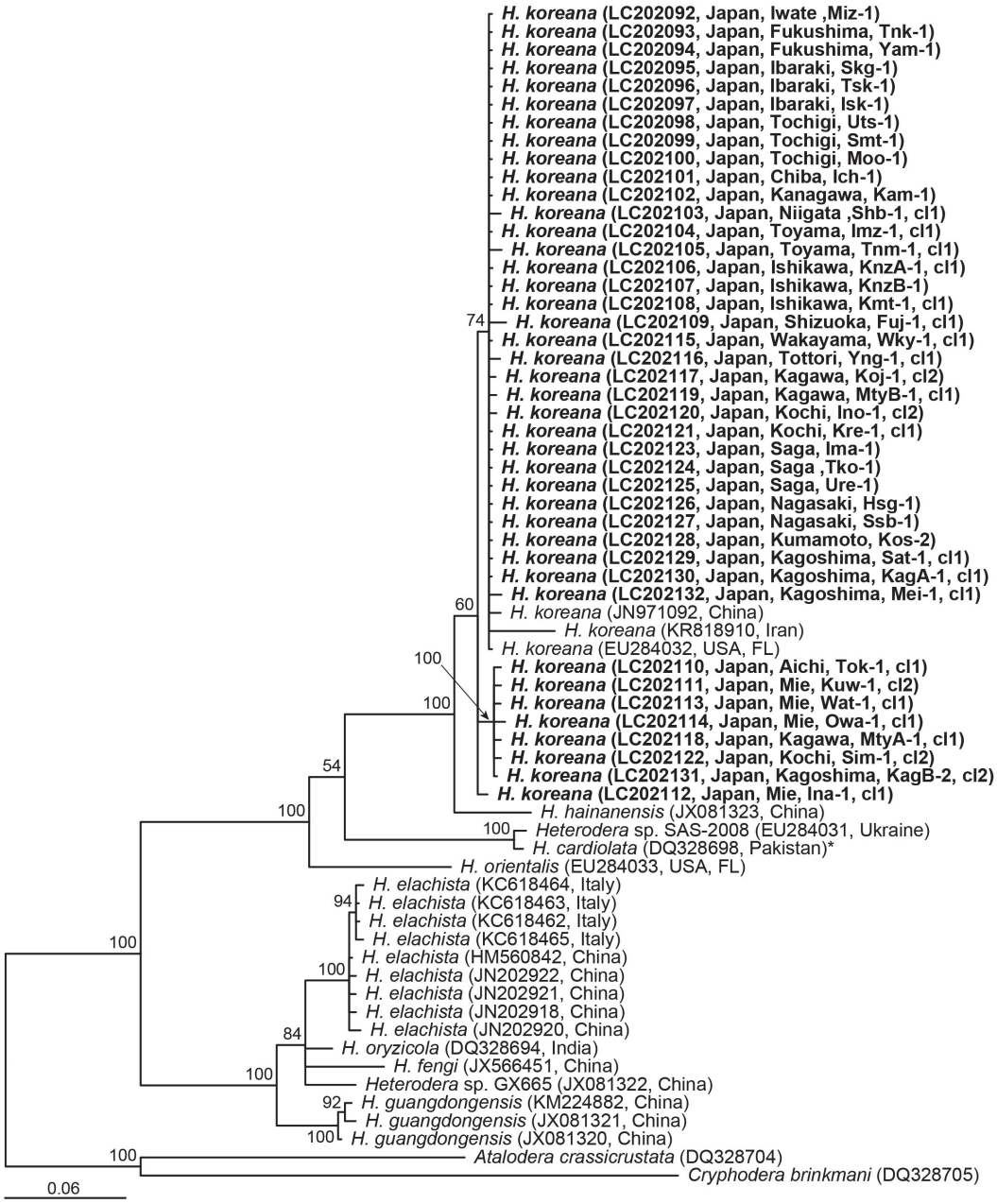

Fig. 14. Phylogenetic relationships between *Heterodera koreana* and other related species: Bayesian 50% majority rule consensus tree as inferred from the D2–D3 of 28S rRNA gene sequence alignment under the GTR + G model. PP over 50% are given for appropriate clades. Newly obtained sequences are indicated by bold letters. * Identified as *H. cynodontis* by Subbotin *et al.* (2006), the species is considered as a synonym of *H. cardiolata* by Subbotin *et al.* (2010b).

Table 11. Variable position in the three *COI* haplotypes of Japanese *Heterodera koreana* populations.

Haplotype	Number of sequences	Variable position (bp)																	
		45	57	72	75	81	132	159	171	186	189	192	199	213	228	257	288	327	393
A	40	G	A	C	T	A	G	G	T	A	A	T	G	A	C	G	A	G	C
B	40	G	A	T	G	A	A	G	T	A	A	T	G	T	T	G	A	G	C
C	1	A	T	T	T	G	A	A	A	G	T	C	A	T	T	A	G	A	T

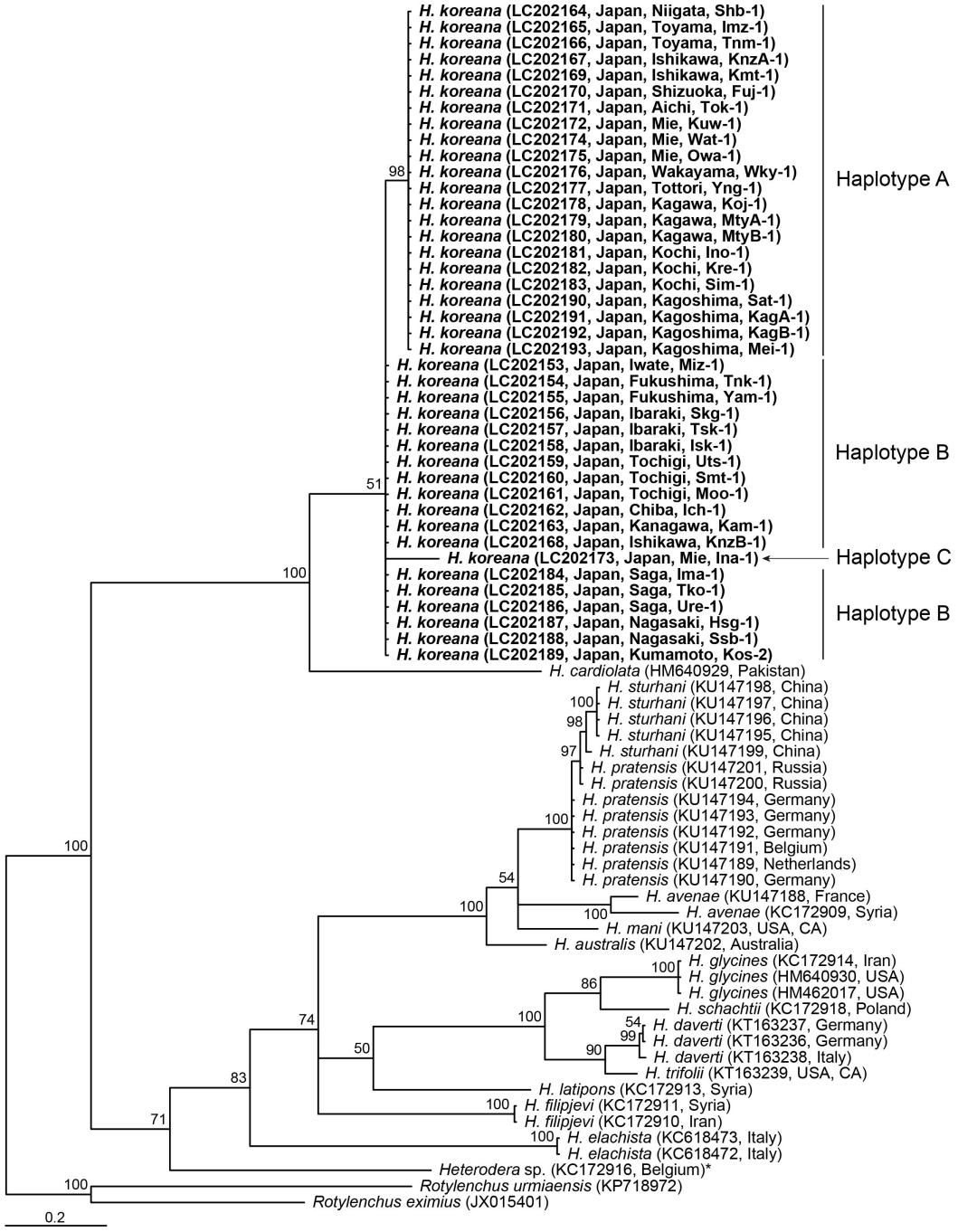

Fig. 15. Phylogenetic relationships between *Heterodera* species: Bayesian 50% majority rule consensus tree as inferred from the analysis of the *COI* gene sequence alignment under the GTR + G model. PP over 50% are given for appropriate clades. Newly obtained sequences are indicated by bold letters. * Identified as *H. pratensis* by Toumi *et al.* (2013).

Fig. 16. Geographical distribution of the studied *Heterodera koreana COI* haplotypes in Japan. Closed circles represent the localities where haplotype A was collected. Open circles represent the localities where haplotype B was collected. The circled bullet represents the locality where haplotype C was collected. Numbers correspond to those in Table 9.

4.4 Discussion

This is the first report of *H. koreana* from Japan. This survey revealed that *H. koreana* is widely distributed in Japan throughout Honshu, Shikoku, and Kyushu. In addition, madake and henon bamboo were newly recorded as hosts of *H. koreana*.

The morphology and morphometrics of the cysts and J2 of the Japanese population were in agreement with those of the original description of *H. koreana* from South Korea (Vovlas *et al.*, 1992). The morphometric characters of the Japanese population (Table 10) were also in agreement with those of the subsequent description of *H. koreana* from China (Wang *et al.*, 2012) and Iran (Tanha Maafi *et al.*, 2015), but the Japanese population differs from the Chinese population by the shorter J2 body length (442 (417–465) *vs* 513 (448–553) μm) and the shorter length of the hyaline region of the J2 tail (42 (37–47) *vs* 49 (39–56) μm); and from the Iranian population by the longer J2 tail length (72 (63–80) *vs* 62 (51–75) μm). These differences can be explained by the occurrence of intraspecific variation because similar variability was reported between *H. koreana* populations from South Korea and China (Wang *et al.*, 2012).

Heterodera koreana is similar to four other *Heterodera* species recorded from bamboo: *H. bamboosi*, *H. fengi*, *H. guangdongensis*, and *H. hainanensis*. However, the Japanese population of *H. koreana* differs from *H. bamboosi* by the presence of a vulval cone, the absence of males, and a shorter J2 stylet length (18.1 (17.5–18.7) *vs* 20.0 (19.0–23.0) μm) (Kaushal and Swarup, 1988); from *H. fengi* by the larger cyst size (641 (524–770) *vs* 482 (370–588) μm), absence of fenestrae, absence of males, and a shorter J2 stylet length (18.1 (17.5–18.7) *vs* 23.2 (22.0–24.0) μm) (Wang *et al.*, 2013); from *H. guangdongensis* by the larger cyst size (641 (524–770) *vs* 346 (267–415) μm), absence of fenestrae, longer vulval slit length (50 (41–60 *vs* 36 (31–41) μm), absence of males, a

shorter J2 stylet length (18.1 (17.5–18.7) *vs* 20.2 (19.3–21.3) *μ*m), and the longer hyaline region of the J2 tail (42 (37–47) *vs* 25 (18–34) *μ*m) (Zhuo *et al.*, 2014); and from *H. hainanensis* by the larger cyst size (641 (524–770) *vs* 472 (404–641) *μ*m), absence of an underbridge, the longer vulval slit length (50 (41–60) *vs* 35 (25–40) *μ*m), longer J2 body length (442 (417–465) *vs* 379 (337–411) *μ*m), longer J2 stylet length (18.1 (17.5–18.7) *vs* 16.6 (15.8–17.5) *μ*m), and longer J2 tail length (72 (63–80) *vs* 57 (48–63) *μ*m) (Zhuo *et al.*, 2013).

Heterodera koreana belongs to the *afenestrata* group, which includes the following seven species: *H. africana* (Luc, Germani & Netscher, 1973) Mundo-Ocampo, Troccoli, Subbotin, Del Cid, Baldwin & Inserra, 2008, *H. axonopi* (Souza, 1996) Mundo-Ocampo, Troccoli, Subbotin, Del Cid, Baldwin & Inserra, 2008, *H. bamboosi*, *H. hainanensis*, *H. koreana*, *H. orientalis*, and *H. saccharophila* Mundo-Ocampo, Troccoli, Subbotin, Del Cid, Baldwin & Inserra, 2008 (Subbotin *et al.*, 2010b; Zhuo *et al.*, 2013). These species are characterized by a vulval cone without fenestration and bullae, but with a long vulval slit and a weak or absent underbridge (Subbotin *et al.*, 2010b). On the basis of molecular phylogenetic analyses and the consideration that the key diagnostic characters of this group are convergent, Mundo-Ocampo *et al.* (2008) acknowledged that the *afenestrata* group is a paraphyletic and artificial assemblage. In the present study, analysis of the phylogenetic relationships between *H. koreana* and other species using the ITS and D2–D3 of 28S rRNA gene sequences revealed a close relationship between *H. koreana* and *H. hainanensis*, and the paraphyly of the *afenestrata* group within *Heterodera*, as previously presented by Mundo-Ocampo *et al.* (2008), De Luca *et al.* (2013), Wang *et al.* (2013), Zhuo *et al.* (2013, 2014), and Kang *et al.* (2016). However, sequence data from other species of the *afenestrata* group, *viz.*, *H. africana*, *H. axonopi*,

H. bamboosi, and *H. saccharophila*, are still lacking. Additional molecular phylogenetic analyses including the sequences of these species is needed to clarify the relationships between *afenestrata* group species.

Recently, although several studies have been carried out using the *COI* gene in *Heterodera* species (De Luca *et al.*, 2013; Toumi *et al.*, 2013; Subbotin, 2015; Vovlas *et al.*, 2015), this molecular marker has never been used for *H. koreana*. The *COI* gene sequences, without heterogeneity, of *H. koreana* were obtained for the first time in this study. Only three *COI* haplotypes were found in 41 Japanese *H. koreana* populations with 1.3–4.3% intraspecific sequence variation between these haplotypes. Intraspecific variation of *H. koreana* is higher than that of *H. sturhani* and *H. pratensis* with 0.7% (Subbotin, 2015). Armenteros *et al.* (2014) analysed partial *COI* gene sequences of free-living marine nematodes and noted that all conspecific genetic distances were <3% of the genetic divergence, whereas all interspecific distances (within and among genera) were >14%. Intraspecific variation of *H. koreana* slightly exceeded this standard level. Much higher intraspecific sequence variations were reported for *Xiphinema brevicolle* Lordello & Costa, 1961 with 7.2–7.6% (Sakai *et al.*, 2012), *X. diversicaudatum* (Micoletzky, 1927) Thorne, 1939 with 9.5% (Orlando *et al.*, 2016), and *Longidorus orientalis* Loof, 1982 with 15.5% (Subbotin *et al.*, 2015). However, such high intraspecific sequence variation in the *COI* gene has not been reported for *Heterodera* species.

Each *COI* haplotype of Japanese *H. koreana* population showed a characteristic geographical distribution in Japan regardless of the bamboo species (Fig. 16). Haplotype A is widely distributed in south-west Japan throughout western Honshu, Shikoku, and the southern part of Kyushu. Haplotype B is distributed in the southern

part of the Pacific Ocean side of the Tohoku region, the Kanto region, and the northern part of Kyushu. Haplotype C was solely detected from one population from moso bamboo in Inabe City, Mie Prefecture (Ina). Although haplotypes A and B are distributed almost allopatrically in Japan, these two haplotypes were detected from two geographically close populations (KnzA from moso bamboo and KnzB from henon bamboo, located *ca* 2 km apart) in Kanazawa City, Ishikawa Prefecture, which is the main area of distribution for haplotype A. This result indicates that the distribution of these *COI* haplotypes may be complicated in Japan.

General Discussion

The cyst nematode species of the genus *Heterodera* are economically important pests of many crops. Some species cause considerable yield losses to important crops in the world and are regulated as quarantine pests in international trade. Accurate and rapid identification of *Heterodera* species is essential not only for selection of effective and appropriate control measures against these nematodes, but for effective quarantine inspection and implementation of phytosanitary measures. Although eight *Heterodera* species, *viz.*, *H. avenae*, *H. elachista*, *H. glycines*, *H. humuli*, *H. latipons*, *H. trifolii*, an unidentified species on soybean, and an unidentified species on bamboo, have been recorded in Japan (Momota, 2004), there is little information available on the distribution, morphology and morphometric characters, and the biology for most of these species. The aim of the present study was to provide distributional, morphological, morphometric, molecular, and biological data of *H. trifolii* populations and unidentified cyst nematode species on bamboo in Japan. First, the geographical distribution of *H. trifolii* on white clover and greenhouse carnation was investigated in eastern Japan (Chapter 1). Then, Japanese populations of *H. trifolii* were characterized morphologically, morphometrically, molecularly, and biologically (Chapters 2 and 3). Finally, an unidentified species on bamboo from Japan was identified as *H. koreana*, and the Japanese population of this species was characterized morphologically, morphometrically, and molecularly (Chapter 4).

Geographical distribution of *H. trifolii* and *H. koreana* in Japan

In Japan, *H. trifolii* have been reported only from Hokkaido and Nagano

Prefectures (Inoue, 1961; Sakurai *et al.*, 1961; Yamada *et al.*, 1961; Yuhara *et al.*, 1961; Kureha, 1962; Momota *et al.*, 1990; Toyoshima *et al.*, 1992; Mizukoshi, 2000; Momota and Mizukoshi, 2000). The present study newly revealed that *H. trifolii* is widely distributed in eastern Japan from Hokkaido to the Chubu region (Chapter 1). This species still remains unknown from western Japan. Considering its wide distribution in the world, it is likely that this cosmopolitan species is also distributed in western Japan. *Heterodera koreana* has not been recorded from Japan to date. It was newly revealed that *H. koreana* is widely distributed in Japan throughout Honshu, Shikoku, and Kyushu (Chapter 4).

Morphological identification and characterization

Nematodes are considered as one of the most difficult organisms to identify due to their microscopic size, morphological similarity, limited number of diagnostic characters, and overlapping morphometric measurements (Oliveira *et al.*, 2011). The identification of *Heterodera* species has traditionally been based on morphological and morphometric characters. The structure and morphometrics of vulval cone of the cyst have become widely used for *Heterodera* species delimitation and identification since 1960 (*e.g.*, fenestral length, semifenestral width, and vulval slit length), and importance of morphometric characters of J2 (*e.g.*, body length, stylet length, tail length, and length of hyaline region of the J2 tail) also become evident (Wouts and Baldwin, 1998). In the present study, Japanese populations of *H. trifolii* and *H. koreana* were characterized morphologically and morphometrically (Chapters 2 and 4). The morphology and morphometrics of their cysts and J2 were well in agreement with those of the original description and the redescriptions of each species. Although minor intraspecific

variations and interspecific overlapping were also observed in some important morphometric characters, the present results strongly suggested great value of the cyst and J2 characters for *Heterodera* species delimitation and identification.

Morphological identification, however, is time-consuming and also requires technical skills. It can be unreliable sometimes because of the significant intraspecific variation of morphological and morphometric characters. In addition, the increasing number of nominal species in the genus *Heterodera* makes reliable morphological identification more difficult (Subbotin *et al.*, 2003), although correct species identification of these nematodes is becoming increasingly important for effective nematode control, such as crop rotation and plant resistance, and for implementation of appropriate plant quarantine measures. Unfortunately, skilled nematologists in classical morphology-based taxonomy are decreasing in recent years (Ferris, 1994; Coomans, 2000). Due to such a decline in taxonomical expertise and an increasing demand for extensive diagnostic tolls, DNA-based techniques have been developed to identify *Heterodera* species with high sensitivity and accuracy, *e.g.*, PCR, PCR with specific primers, RFLP, Real-time PCR, and DNA sequencing (Subbotin *et al.*, 2010a). These routine, rapid, and robust techniques make species diagnosis more effective and accessible, even to scientists not skilled in morphological taxonomy (Waeyenberge *et al.*, 2009).

Molecular identification and phylogenetic relationships

The main DNA regions targeted for diagnosing of *Heterodera* species are nuclear rRNA genes, that include conserved and variable regions of the 18S and 28S rRNA genes, and the more variable two ITS regions, ITS1 and ITS2, which are situated

between 18S and 5.8S and 5.8S and 28S rRNA genes, respectively (Subbotin *et al.*, 2010a). PCR-RFLP of the ITS1-5.8S-ITS2 region has frequently been used for the identification of *Heterodera* species. As shown in Chapter 1, *H. trifolii* and *H. elachista* were easily identified by PCR-RFLP analysis of the ITS1-5.8S-ITS2 region by using digestion patterns using *Alu*I, *Mse*I, and *Rsa*I. Several studies have revealed that, in some cases, some species cannot be discriminated from each other, *e.g.*, *H. trifolii*, *H. daverti*, and *H. ciceri*, because of a lack of differences between the restriction patterns obtained (Subbotin *et al.*, 2000; 2010a).

The ITS sequences have also been utilized as useful diagnostic marker for species identification and characterization of phylogenetic relationships within *Heterodera*, especially when morphological characters lead to ambiguous interpretation (Subbotin *et al.*, 2001, 2003, 2006; Tanha Maafi *et al.*, 2003; Madani *et al.*, 2004; Mundo-Ocampo *et al.*, 2008; Zheng *et al.*, 2008; Skantar *et al.*, 2012; De Luca *et al.*, 2013; Toumi *et al.*, 2013; Wang *et al.*, 2013; Zhuo *et al.*, 2013, 2014; Kang *et al.*, 2016). In the present study, the phylogenetic analyses of the ITS sequences strongly supported the identification of *H. koreana* from Japan and the separation of *Heterodera* species into seven species groups, *i.e.*, *afenestrata*, *avenae*, *cyperi*, *goettingiana*, *humuli*, *sacchari*, and *schachtii* (Chapters 2 and 4). Thus, the ITS regions, being under a higher mutation rate than other rRNA regions, seem to have sufficient sequence variability for *Heterodera* species identification and for determination of species group of *Heterodera*. These molecular markers, however, do not often contain enough variation for distinguishing some closely related species, such as species of the *schachtii* group (Chapter 2).

The D2–D3 expansion segments of the 28S rRNA gene have also been used as

useful genetic markers for resolving phylogenetic relationship within *Heterodera* (Subbotin *et al.*, 2006; Mundo-Ocampo *et al.*, 2008; Skantar *et al.*, 2012; De Luca *et al.*, 2013; Wang *et al.*, 2013; Zhuo *et al.*, 2013, 2014; Kang *et al.*, 2016). In this study, most closely related species within the *schachtii* group and the *afesestrata* group were more clearly discriminated by D2–D3 than by ITS (Chapters 2 and 4). De Luca *et al.* (2013) pointed out that the separation of *Heterodera* species into seven species groups are better supported in ITS than in D2–D3.

More recently, the *COI* mtDNA gene sequence has been proposed as a standard genetic marker for accurate nematode species identification, including discriminating closely related species within species group of *Heterodera* (De Luca *et al.*, 2013; Toumi *et al.*, 2013; Subbotin, 2015; Vovlas *et al.*, 2015). Genes of mtDNA, with their higher rate of mutations relative to rRNA genes, have great potential for diagnosing races and populations of cyst nematodes (Subbotin *et al.*, 2010a). By using *COI*, *H. trifolii* was more clearly discriminated from most other species of the *schachtii* group, than by ITS and D2–D3, except for *H. betae* showing almost identical *COI* sequences (Chapter 2). In addition, the present study revealed three *COI* haplotypes in Japanese *H. koreana* populations, with a geographical distributional pattern regardless of the bamboo species (Chapter 4). Thus, the *COI* region is also regarded as a powerful tool for assessing intraspecific genetic structure and phylogeographic patterns of *Heterodera* species. Intraspecific sequence variations were identified also in the ITS and D2–D3 regions, as well as *COI*, in both *H. trifolii* and *H. koreana*. These variations were much higher in *H. koreana* than in *H. trifolii*: 0.0–3.1% (0–28 bp) *vs* 0.1–1.4% (1–3 bp) in ITS, 0.0–2.9% (0–22 bp) *vs* 0.0–0.2% (0–1 bp) in D2–D3, and 0.0–4.3% (0–17 bp) *vs* 0.0–0.3% (0–1 bp) in *COI* (Chapters 2 and 4).

Although molecular techniques provide fast and accurate identification, it should not be regarded as a panacea as proposed by Tautz *et al.* (2003). Morphological and molecular approaches should be applied in complementary manner for species taxonomy and identification of *Heterodera*.

Heterodera trifolii* and *H. betae

The yellow beet cyst nematode, *H. betae*, was previously considered to be a biotype of *H. trifolii*. This nematode is a parasite of sugar beet, a crop not normally affected by *H. trifolii*, and subsequently the species was newly described as *H. betae* (Wouts *et al.*, 2001). This species has been found in Europe and Morocco and causes severe damage to sugar beet (Amiri *et al.*, 2002; Subbotin *et al.*, 2010b; Gracianne *et al.*, 2014). In recent morphological and molecular studies, it has been revealed that *H. trifolii* is closely related to *H. betae* (Ambrogioni and Irdani, 2001; Amiri *et al.*, 2002). The present study demonstrated that *H. trifolii* can be morphologically distinguished from *H. betae* by the J2 body length, J2 stylet length, J2 tail length, and length of hyaline region of J2 tail (Chapter 2). These species also can be clearly distinguished from each other by difference of their host plant range. For example, *H. trifolii* well reproduces on white clover but not on sugar beet and cabbage (Chapter 4). On the other hand, *H. betae* well reproduces on sugar beet and cabbage but not on white clover (Maas *et al.*, 1982; Steele *et al.*, 1983; Ambrogioni *et al.*, 2004). However, *H. trifolii* and *H. betae* could not be molecularly differentiated because of their almost identical ITS, D2–D3, and *COI* sequences (Chapter 2). Further analyses of other genes, *e.g.*, actin gene and Hsp90 gene, are needed to differentiate *H. trifolii* and *H. betae* molecularly.

Summary

The cyst nematode species of the genus *Heterodera* are economically important pests of many crops in the world. Accurate and rapid identification of these species is essential not only for selection of appropriate control measures against these nematodes but also for effective implementation of phytosanitary measures. However, there is little information available on the distributional, morphological, morphometric, molecular, and biological data for most Japanese *Heterodera* species. The present study was conducted to characterize Japanese populations of *H. trifolii* and *H. koreana* morphologically, morphometrically, molecularly, and biologically.

Chapter 1. The geographical distribution of the clover cyst nematode *Heterodera trifolii* in eastern Japan on white clover and greenhouse carnation 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. J2 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 PCR-RFLP analysis of the ITS1-5.8S-ITS2 region 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%). The results showed that *H. trifolii* is widely distributed in eastern Japan and can be potentially a serious threat to carnation production.

Chapter 2. The Japanese populations of *H. trifolii* were characterized morphologically, morphometrically, and molecularly. The morphology and morphometrics of six Japanese populations of *H. trifolii* were in congruence with those

of previous descriptions of this species from Germany, Italy, Japan, New Zealand, Russia, The Netherlands, UK, and USA. The results of the phylogenetic analyses of the D2–D3 expansion segments of 28S rRNA gene and the *COI* mtDNA gene sequences discriminated *H. trifolii* from most of the other closely related species of the *schachtii* group. The intraspecific sequence differences in the *schachtii* group were much higher in *COI* than in D2–D3. The *H. trifolii* populations displayed very low levels of intraspecific variations (up to 0.3%) in the *COI* mtDNA gene sequences. The phylogenetic relationships of *H. trifolii* with other *Heterodera* species, as inferred from the analyses of the ITS, D2–D3 of 28S rRNA gene, and the *COI* mtDNA gene sequences, were determined.

Chapter 3. Three Japanese populations of *H. trifolii* were examined for their ability to reproduce on five crops and three carnation cultivars. All three populations produced cysts on white clover, azuki bean, spinach, and carnation cvs. Barbara, Cherry Tessino, and Peachy Mambo. No cysts were produced on sugar beet or broccoli. The number of cysts from the Nagano population was significantly higher than that from the Hokkaido and Aomori populations on all plants, except for white clover. The number of cysts from all populations was significantly lower on carnation cv. Barbara than on cvs. Cherry Tessino and Peachy Mambo. These results suggested that carnation cv. Barbara may be the resistant type. Variation in cyst number of the three populations on spinach and carnation cvs. Cherry Tessino and Peachy Mambo indicated variation in reproductive ability on different host plants in Japanese *H. trifolii* populations.

Chapter 4. The Korean cyst nematode, *Heterodera koreana*, was recorded for the first time from Japan and characterized morphologically, morphometrically, and molecularly. In total, 41 populations were detected from soil samples collected from the

rhizosphere of four bamboo species in Japan: 31 populations from moso bamboo, seven from madake, two from henon bamboo, and one from fish pole bamboo. The morphology and morphometrics of the Japanese population were in agreement with those of the original description of *H. koreana* from South Korea and other subsequent descriptions from China and Iran, with the exception of some minor differences. The results of the phylogenetic analyses of the D2–D3 expansion segments of 28S rRNA gene and ITS sequences confirmed the species identification and phylogenetic relationship of *H. koreana* with other *Heterodera* species. The *COI* mtDNA gene sequences were obtained for the first time for *H. koreana*. Three *COI* haplotypes found in Japanese *H. koreana* populations showed a characteristic geographical distribution in Japan.

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