

HOKKAIDO UNIVERSITY

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

H. koreana

Shigeyuki Sekimoto

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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 |
|------|--|
| РР | 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 from 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 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

| Desien | Duefestar | Number of | Heterodera | Spe | ecies |
|----------|------------|--------------|------------|-------------|--------------|
| Region | Prefecture | soil samples | positive | 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 1. Numbers of soil samples of the rhizosphere of white clover in eastern Japan

with *Heterodera* detection status.

Table 2. Numbers of soil samples of the rhizosphere of greenhouse carnation inHokkaido and Nagano Prefectures with *Heterodera* detection status.

| Dogion | Drafaatura | Number of soil | Heterodera | Species |
|----------|------------|----------------|------------|-------------|
| Region | samples | | positive | 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 μ l of 0.1% SDS lysis buffer consisting of 10 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 500 μ g 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 μ l 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 | n | Sp | ecies |
|------|----------|------------|-----------------|---------|---|-------------|--------------|
| 110. | Region | Trefecture | Locality | code | п | 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.

| No | Dagian | Drofooturo | Locality | Sample | 12 | Sp | ecies |
|------|--------|------------|-------------------|--------|------|-------------|--------------|
| INO. | Region | Plelectule | Locality | code | 11 - | 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 | Т36 | 6 | 6 | 0 |
| 35 | | Iwate | Tono City | T4 | 6 | 6 | 0 |
| 36 | | | Tono City | T6 | 4 | 4 | 0 |
| 37 | | | Takizawa City | Т8 | 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 |

Table 3. (Continued.)

n: numbers of J2 used for molecular identification.

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

| No | Docion | Drafaatura | Locality | Sample | | Species | |
|------|----------|--------------------------|-----------------------------|--------|-----|-------------|--------------|
| INO. | Region | Prefecture | Locality | code | п – | H. trifolii | H. elachista |
| 1 | Hokkaido | Hokkaido | Hokkaido Nanae Town NAN 6 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 μ l 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 μ l reaction mixture consisting of 10 μ l of 2 × Quick Taq[®] HS DyeMix (Toyobo, Osaka, Japan), 0.4 μ l (10 μ M) of each primer, 5 μ l of DNA template, and 4.2 μ l 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 GelRedTM (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 μ l of the PCR products were digested with 2–3 U of restriction enzyme in a total volume of 10 μ l 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, *AluI*, *MseI*, and *RsaI*. 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. Heter | odera species used in the present study | γ. | | | | | |
|----------------|---|--|--------|-----------------------|----------------------|---------------------|-------------|
| Species | Locality | Host plant | Sample | GenBa | unk accession n | number | Source |
| | | | code | ITS | D2–D3 of 28S rRNA | <i>COI</i> mtDNA | |
| H. trifolii | Sapporo City, Hokkaido Prefecture, Japan | Trifolium repens | HU2 | I | 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 | I | 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 | I | LC208666 | LC208702 | S. Sekimoto |
| H. trifolii | Inawashiro Town, Fukushima Prefecture, Japan | T. repens | FU2 | I | 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 | I | 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 |

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| Table 5. (Contin | nued.) | | | | | | |
|------------------|--|--|--------|-----------------------|-----------------------|--------------|------------|
| Species | Locality | Host plant | Sample | GenBa | unk accession n | number | Source |
| | | | code | 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 | I | I | 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 | I | LC208675, LC208676 | LC208711 | A. Kushida |
| H. glycines | Ninohe City, Iwate Prefecture, Japan | G. max | HG5 | I | 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 | I | LC208679, LC208680 | LC208714 | S. Aiba |
| H. glycines | Fukushima City, Fukushima Prefecture, Japan | G. max | HG7 | I | LC208681, LC208682 | LC208715 | S. Aiba |
| H. glycines | Satte City, Saitama Prefecture, Japan | G. max | HG8 | I | I | 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 μ l 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 μ l reaction mixture containing 2 μ l $10 \times \text{Ex Tag buffer}$ (20 mM Mg²⁺ plus) (Takara Bio, Shiga, Japan), 1.6 μ l dNTP mixture (2.5 mM), 0.4 µl (10 µM) of each primer, 0.1 µl TaKaRa Ex Tag[®] Hot Start Version (5 U μ l⁻¹) (Takara Bio, Shiga, Japan), 3 μ l DNA template, and 12.5 μ l 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 μ l reaction mixture containing 4 μ l 5 × PrimeSTAR Buffer (5 mM Mg²⁺ plus) (Takara Bio, Shiga, Japan), 1.6 μ l dNTP mixture (2.5 mM), 0.4 μ l (10 μ M) of each primer, 0.1 μ l PrimeSTAR[®] HS DNA Polymerase (2.5 U μ l⁻¹) (Takara Bio, Shiga, Japan), 1 μ l DNA template, and 12.5 μ l 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 $0.5 \times \text{TBE}$ buffer, stained by GelRedTM (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). 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 μ m). Fenestral length and semifenestral width 35.1–77.9 μ m and 25.2–54.2 μ m, 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 μ m. 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 = 100μ m; C–F = 20μ m.)



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 = $20 \ \mu$ m.)

| mean ± S.I |). (range). | | | | | | |
|------------|---------------------|---------------------------------|----------------------------------|----------------------------------|-----------------------------------|-----------------------------------|----------------------------------|
| Stage | Character | | White clover | · populations | | Carnation p | opulations |
| | | 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 | $42.0 \pm 4.4 \\ (34.9 - 51.2)$ | 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. Morphometrics of cysts and J2 of six *Heterodera trifolii* populations from Japan. All measurements are in μ m and in the form:

| Stage | Character | | White clover | · populations | | Carnation p | opulations |
|-------|----------------------|-------------------------------|---------------------------------|-------------------------------|-----------------------------------|-------------------------------|--------------------------------|
| | | 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 |
| | Γ | 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) |
| | а | 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) |
| | Э | 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) |
| | ,°J | 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) |

Chapter 2: Characterization of Heterodera trifolii from Japan

Table 6. (Continued.)

| Stage | Character | | White clover | populations | | Carnation p | opulations |
|-------|--------------------------------------|-------------------------------|---------------------------------|-------------------------------|-----------------------------------|-------------------------------|-------------------------------|
| | | 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) |

Table 6. (Continued.)

body. Stylet robust with anteriorly concave knobs. Stylet length 24.1–30.8 μ m. 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 μ m 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% (1-13bp) 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*, 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 *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 μ m), shorter average J2 stylet length (26.9–27.8 vs 29.6–31.0 μ m), shorter average J2 tail length (54–65 vs 71–74 μ m), and shorter average length of hyaline region of J2 tail (30–36 vs 39–42 μ m) (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 μ m), longer average J2 stylet length (26.9–27.8 vs 21.4–24.5 μ m), 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 μ m) (Subbotin *et al.*, 2010b); and from *H. schachtii* by the absence of males, longer average J2 body length (489–528 vs 436–468 μ m), longer average J2 stylet length (26.9–27.8 vs 25.3–26.3 μ m), longer average J2 tail length (54–65 vs 45– 49 μ m), and longer average length of hyaline region of J2 tail (30–36 vs 24–27 μ m)

(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. glvcines*, *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 $5 \times 5 \times 8$ 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 <i>Heterodera trifolii</i> | per pot 63 or 65 days | after in | oculation 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 | q | 0 | 0 | 0 |
| Spinach (<i>Spinacia oleracea</i>) ⁴ | Wasesaradaakari | bcd | 10.0 ± 5.5 | 4.0 ± 2.0 | 59.3 ± 21.1 |
| Brasicaeae | | | | | |
| Broccoli (Brassica oleracea var. italica) | Verde | q | 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 | а | 25.3 ± 13.8 | 114.0 ± 87.9 | 192.0 ± 30.9 |
| Fabaceae | | | | | |
| White clover (<i>Trifolium repens</i>) ⁵ | Ladino | | 500< | 500< | 500< ⁶ |

Chapter 3: Reproductive ability of Heterodera trifolii

Table 7. (Continued.)

- ¹ Mean \pm 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 \pm 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 plantcultivars 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 cy. 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 cys. 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 Siddigi (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 μ l 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 μ l reaction mixture containing 2 μ l $10 \times \text{Ex Tag buffer}$ (20 mM Mg²⁺ plus) (Takara Bio, Shiga, Japan), 1.6 μ l dNTP mixture (2.5 mM), 0.4 µl (10 µM) of each primer, 0.1 µl TaKaRa Ex Tag[®] Hot Start Version (5 U μ l⁻¹) (Takara Bio, Shiga, Japan), 1 μ l DNA template, and 14.5 μ l 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 μ l reaction mixture containing 4 μ l 5 × PrimeSTAR Buffer (5 mM Mg²⁺ plus) (Takara Bio, Shiga, Japan), 1.6 μ l dNTP mixture (2.5 mM), 0.4 μ l (10 μ M) of each primer, 0.1 μ l PrimeSTAR[®] HS DNA Polymerase (2.5 U μ l⁻¹) (Takara Bio, Shiga, Japan), 1 μ l DNA template, and 12.5 μ l 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

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| No. | Locality | Host plant | Sample | GenBa | ank accession nu | umber | Collector |
|-----|--------------------------------------|-------------------------------------|--------|-----------------------|----------------------|-----------|-------------|
| | | | code | STI | D2–D3 of 28S rRNA | COI mtDNA | |
| 1 | Oshu City, Iwate Prefecture | Phyllostachys nigra var. henonis | Miz | LC202133 | LC202092 | LC202153 | M. Sasaki |
| 7 | Tanakura Town, Fukushima Prefecture | P. edulis | Tnk | Ι | LC202093 | LC202154 | S. Sekimoto |
| б | Yamatsuri Town, Fukushima Prefecture | P. aurea | Yam | LC202134, LC202135 | LC202094 | LC202155 | S. Sekimoto |
| 4 | Sakuragawa City, Ibaraki Prefecture | P. bambusoides | Skg | Ι | LC202095 | LC202156 | S. Sekimoto |
| S | Tsukuba City, Ibaraki Prefecture | P. edulis | Tsk | LC202136, LC202137 | LC202096 | LC202157 | S. Sekimoto |
| 9 | Ishioka City, Ibaraki Prefecture | P. edulis | Isk | Ι | LC202097 | LC202158 | S. Sekimoto |
| ٢ | Utsunomiya City, Tochigi Prefecture | P. edulis | Uts | Ι | LC202098 | LC202159 | S. Sekimoto |
| 8 | Shimotsuke City, Tochigi Prefecture | P. bambusoides | Smt | Ι | LC202099 | LC202160 | S. Sekimoto |
| 6 | Moka City, Tochigi Prefecture | P. edulis | Moo | Ι | LC202100 | LC202161 | S. Sekimoto |
| 10 | Ichihara City, Chiba Prefecture | P. bambusoides | Ich | Ι | LC202101 | LC202162 | Z. Nakai |
| 11 | Kamakura City, Kanagawa Prefecture | P. edulis | Kam | Ι | LC202102 | LC202163 | S. Sekimoto |
| 12 | Shibata City, Niigata Prefecture | P. edulis | Shb | Ι | LC202103 | LC202164 | S. Sekimoto |
| 13 | Imizu City, Toyama Prefecture | P. edulis | Imz | I | LC202104 | LC202165 | S. Sekimoto |

| No. | Locality | Host plant | Sample | GenB | ank accession nu | umber | Collector |
|-----|------------------------------------|--------------------------|--------|-----------------------|----------------------|-----------|-------------|
| | | | code | ITS | D2–D3 of 28S rRNA | COI mtDNA | |
| 14 | Tonami City, Toyama Prefecture | Phyllostachys edulis | Tnm | I | LC202105 | LC202166 | S. Sekimoto |
| 15 | Kanazawa City, Ishikawa Prefecture | P. edulis | KnzA | Ι | 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 | I | 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 | I | LC202110 | LC202171 | T. Sengo |
| 20 | Kuwana City, Mie Prefecture | P. edulis | Kuw | I | 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 | I | LC202113 | LC202174 | S. Sekimoto |
| 23 | Owase City, Mie Prefecture | P. edulis | Owa | I | LC202114 | LC202175 | S. Sekimoto |
| 24 | Wakayama City, Wakayama Prefecture | P. bambusoides | Wky | LC202143, LC202144 | LC202115 | LC202176 | T. Sengo |

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Table 9. (Continued.)

| Table | 9. (Continued.) | | | | | | |
|-------|---|----------------------|--------|-----------------------|----------------------|-----------|-------------|
| No. | Locality | Host plant | Sample | GenB: | ank accession nu | umber | Collector |
| | | | code | STI | 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 | I | LC202117 | LC202178 | S. Sekimoto |
| 27 | Mitoyo City, Kagawa Prefecture | P. bambusoides | MtyA | Ι | LC202118 | LC202179 | S. Sekimoto |
| 28 | Mitoyo City, Kagawa Prefecture | P. edulis | MtyB | Ι | 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 | Ι | LC202121 | LC202182 | S. Sekimoto |
| 31 | Shimanto Town, Kochi Prefecture | P. edulis | Sim | Ι | LC202122 | LC202183 | S. Sekimoto |
| 32 | Imari City, Saga Prefecture | P. edulis | Ima | Ι | LC202123 | LC202184 | S. Sekimoto |
| 33 | Takeo City, Saga Prefecture | P. edulis | Tko | Ι | LC202124 | LC202185 | S. Sekimoto |
| 34 | Ureshino City, Saga Prefecture | P. edulis | Ure | Ι | 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 | I | LC202127 | LC202188 | S. Sekimoto |
| 37 | Koshi City, Kumamoto Prefecture | P. edulis | Kos | I | LC202128 | LC202189 | H. Iwahori |

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

Table 9. (Continued.)

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 μ m. Lip region hemispherical and not offset. Stylet robust with rounded knobs. Stylet length 17.5–18.7 μ m. 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 μ m wide, and 176–207 μ m anterior to tail tip. Tail conoid, tapering uniformly to finely rounded terminus. Hyaline region of tail 37–47 μ m 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 μ m; B, C = 20 μ m; E = 50 μ m.)

| and in the form: mean ± S.D. (range). e Character n L (including neck) Body diameter L (including neck)/body diameter ratio | Tsukuba, Japan Tsukuba, Japan Tsk 10 - 10 - 641 ± 65.8 - 641 ± 65.8 - 642 ± 770 - 642 ± 770 - 10 - 11 ± 0.7 - 1.1 ± 0.1 - 1.1 ± 0.1 - 1.1 ± 0.1 - | South Korea Vovlas <i>et al.</i> (1992) 20 20 840 ± 149.0 (630-1174) 520 ± 90.0 (400-653) 1.5 ± 0.4 (1.0-2.9) | China Wang <i>et al.</i> (2012) 5 748 ± 75.0 (700-860) (700-860) (700-860) (620-760) (620-760) (1.0-1.2) | Iran Tanha Maafi <i>et</i> <i>al.</i> (2015) 21 (Vulval cone 7) 502 ± 70.0 (420-640) (420-640) (420-520) (320-520) (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. Morphometric comparison of Heterodera koreana populations from Japan, South Korea, China, and Iran. All measurements are

| Table 10. | (Continued.) | | | | | |
|-----------|----------------------|-------------------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------------|
| Stage | Character | Tsukuba | , Japan | South Korea | China | Iran |
| | | Ts | X | Vovlas <i>et al.</i> (1992) | Wang <i>et al.</i> (2012) | Tanha Maafi <i>et</i> 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 ($437-472$) |
| | 8 | 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) |
| | q | 3.5 ± 0.1 (3.4-3.7) | 3.6 ± 0.2 (3.3-4.1) | I | I | I |
| | 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) |
| | S | 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) |
| | ъ. | 5.9 ± 0.3 (5.1-6.5) | 6.2 ± 0.3 (5.5-6.6) | I | 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) | I | I | I |
| | Stylet knob diameter | 3.9 ± 0.1 (3.8-4.2) | 3.6 ± 0.2 (3.2-4.0) | I | I | I |
| | Lip region height | 3.6 ± 0.2 (3.3-3.9) | 3.6 ± 0.2 (3.3-3.9) | I | I | 3.0 (3.0) |

Chapter 4: Characterization of Heterodera koreana from Japan

| Table 10. | . (Continued.) | | | | | |
|-----------|--|-------------------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------------|
| Stage | Character | Tsukuba | a, Japan | South Korea | China | Iran |
| | | T | sk | Vovlas <i>et al.</i> (1992) | Wang <i>et al.</i> (2012) | Tanha Maafi <i>et</i> 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) | I | I | 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) | I | I | I |
| | 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) | I | I | I |
| | Anterior end to end of pharyngeal glands | 220 ± 21.4 (178–254) | 231 ± 10.7 (205–249) | I | I | I |
| | 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) | I | I | 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) |

Chapter 4: Characterization of Heterodera koreana from Japan

| Stage | Character | Tsukuba | , Japan | South Korea | China | Iran |
|-------|--|----------------------------|-------------------------------|--------------------------------|------------------------------|-------------------------------------|
| | | Tsl | Х | Vovlas <i>et al.</i> (1992) | Wang <i>et al.</i> (2012) | Tanha Maafi <i>et</i> 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) | I | I | I |
| | Tail length/hyaline region | 1.8 ± 0.1 (1.7-1.9) | 1.7 ± 0.1 (1.5-2.0) | I | I | I |
| | Genital primordium length | I | 13.4 ± 0.9 (12.1–15.4) | I | I | I |
| | Genital primordium diameter | I | 8.5 ± 0.7 (7.4-9.7) | I | I | I |
| | Genital primordium to posterior end | I | 193 ± 9.1 (176-207) | I | I | I |

Table 10. (Continued.)

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% (0-28bp). 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).

| | | 393 | С | C |
|---|------------------------|-----|----|----|
| | | 327 | G | IJ |
| | | 288 | Υ | A |
| | | 257 | G | IJ |
| | | 228 | С | Н |
| | | 213 | A | Τ |
| | | 199 | IJ | IJ |
| - | (dq | 192 | Τ | Н |
| • | sition (| 189 | Υ | A |
| | able po | 186 | Α | A |
| | Varia | 171 | Т | Τ |
| | | 159 | IJ | IJ |
| • | | 132 | G | Α |
| - | | 81 | Υ | A |
| | | 75 | Г | IJ |
| | | 72 | С | Н |
| | | 57 | Α | A |
| | | 45 | IJ | IJ |
| • | Number of sequences | | 40 | 40 |
| | Haplotype | | A | В |

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

H

K

G

V

Ε

Ε

A

C

H

Ċ

V

A

A

G

F

F

F

Þ

C



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) μ m) and the shorter length of the hyaline region of the J2 tail (42 (37–47) *vs* 49 (39–56) μ m); and from the Iranian population by the longer J2 tail length (72 (63–80) *vs* 62 (51–75) μ m). 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) μ m) (Kaushal and Swarup, 1988); from *H. fengi* by the larger cyst size (641 (524–770) vs 482 (370–588) μ m), 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) μ m) (Wang *et al.*, 2013); from *H. guangdongensis* by the larger cyst size (641 (524–770) vs 346 (267–415) μ m), absence of fenestrae, longer vulval slit length (50 (41–60 vs 36 (31–41) μ m), 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

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

General Discussion

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

General Discussion

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 *AluI*, *MseI*, and *RsaI*. 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

General Discussion

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

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 *AluI*, *MseI*, and *RsaI*. 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

Summary

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

Summary

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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