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IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF NINE CYST NEMATODE POPULATIONS FROM MAIN FIELD CROPS IN CHINA

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ABSTRACT

Cyst-forming nematodes, a major category of plant parasitic nematodes, cause severe yield losses in many crop plants and are a serious threat to food production in China. However, the reports focused on identification of species abundance and distribution of cyst nematodes on main crops in China are fewer. During 2012-2014, nine populations of cyst nematodes collected from various locations in major crop cultivation areas of China were identified as four species (*Heterodera glycines, H. avenae, H. filipjevi, H. elachista*) based on morphology and molecular analysis. *H. filipjevi* and *H. elachista* were first discovered in Anhui and Jiangxi Provinces, respectively. In addition, a PCR-ITS-RFLP method was applied to distinguish all the studied populations, which revealed molecular characteristics of cyst nematode species on major field crops in China. The present study broadens the knowledge regarding distributions and molecular features of vital *Heterodera* species and populations, and will also provide useful information for early protection of cystnematodes in China.

Key words: cyst nematode, distribution, identification, rDNA-ITS, RFLP, China.

INTRODUCTION

Cyst nematode is an important group of plant pathogenic nematodes and some species among them cause serious economic losses worldwide (Subbotin *et al*., 2010). At present, *Heterodera glycines*, *H. avenae*, *H. filipjevi* and *H. elachista* have been continuously reported to threaten productions of soybean, wheat, rice and other crops in China (Liu *et al*., 1997; Chen *et al*., 1992; Peng *et al*., 2010; Li *et al*., 2010; Ding *et al*., 2012; Zhuo *et al*., 2014).

H. glycines was first discovered by Jaczewski in China in Heilongjiang Province (Dai *et al*., 1958). *H. glycines* hasbeen spreading into 23 provinces in China including Heilongjiang, Jilin, Liaoning, Inner Mongolia, Beijing, Hebei, Shanxi, Shandong, Henan, Anhui, Jiangsu, Zhejiang, Shanghai, Shaanxi, Hubei, Jiangxi, Gansu, Ningxia, Xinjiang, Sichuan, Guizhou, Yunnan and Guangxi Provinces (Liu *et al*., 1994; Liu *et al*., 1997; Zheng*et al*., 2009; Li *et al*., 2014; Wang *et al*., 2015). In 1989, *H. avenae* was first reported in Tianmen County, Hubei Province and its distribution has been confirmed in Beijing, Shandong, Henan, Hebei, Anhui, Shanxi, Hubei, Xinjiang, Qinghai, Inner Mongolia, Jiangsu, Tianjin, Shaanxi, Gansu, Ningxia, and Tibetintotal 16 provinces (Chen *et al*., 1989; Wang *et al*., 1991; Qi *et al*., 1994; Zheng*et al*., 1996; Liu *et al*., 2005; Hou *et al*., 2007; Yang *et al*., 2008; Chen *et al*., 2009; Li *et al*., 2010; Yang *et al*., 2010; Li *et al*., 2016). It causes a major disease on wheat in China. *H. filipjevi* was merely reported in Henan Province. *H. elachista* has been reported in Hunan, Hubei and Guangxi Provinces. It is necessaryto investigate and identify cyst nematode species and distribution in main field crops to prevent and control cyst nematode diseases.

Recently, nematologists, plant pathologists, and state plant regulatory officials carried out surveys to update the map of the known distribution of *H. glycines* in the soybean-producing areas of the China. At various times since these initial discoveries, maps were created of the provinces in China that were known to be infested with the nematode. This recently updated map and those from selected previous years are shown in Figure. 1.

The morphology of cyst nematodes is rendered uniquely complex by their sexual dimorphism, parasitic habits, ontogeny and dormant stages (Subbotin *et al*., 2010). Nevertheless, some features are consistent for juvenile and adult stages. Morphological diagnostics of *Heterodera* species is mainly based on structure and morphometrics of vulval cones and morphometrics of the J2 (Subbotin *et al*., 2010). J2 has a number of features that distinguish genera and species, but cysts may be the stage richest in diagnostic characters. Furthermore, cysts are the stage often most readily available. The shape and prominence of the vulval cone differs among *Heterodera* species (Hesling *et al*., 1978). The width and length of the vulval bridge are used in diagnostics among *Heterodera* species. Also, the length of the vulval slit is a key character for identification of *Heterodera*. Within *Heterodera*, two types of fenestration are further distinguished: ambifenestrate, in which each fenestra is semicircular and bifenestrate, in which each fenestra is circular. Fenestral length and width are used for species identification. Presence, absence, length, shape and

position of the underbridge are all important diagnostic features for species of *Heterodera* (Mulvey *et al.*, 1974).

Nowadays, technological advances also allow for molecular identification of different species. The main DNA regions targeted for diagnostics in eukaryotic cyst nematodes are nuclear ribosomal RNA genes. These include 18S, 28S and especially the internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), which are situated between the 18S and 5.8S, and 5.8S and 28S rRNA genes, respectively (Figure. 2). The flanking 18S and 28S rRNA gene regions contain a rather conservative nucleotide sequences and thus universal primers can be designed and used for amplification of ITS regions. The ITS regions, being under a higher mutation rate than other rRNA regions, usually have sufficient sequence variability to distinguish among nematodes in species level (Subbotin *et al.*, 2010).

A comprehensive analysis of PCR-RFLP of the ITS1-5.8-ITS2-rRNA gene of cyst nematodes has been conducted by Subbotin and the co-workers. In these studies, the universal TW81 and AB28 primers generated amplicons of approximately 1005-1060 bp in length (depending on species) and were used for diagnostic surveys. Twenty-six restriction enzymes were tested to analyze ITS polymorphism among cyst nematode species. Application of the RFLP technique to separate cyst nematode species revealed several restriction enzymes (e.g., *Alu*I, *Bsu*RI, *Bsh*1236I, *Cfo*I and *Scr*FI) that had greater species discriminatory power and generated more polymorphic profiles than other enzymes tested. This enabled more species of cyst nematode to be characterized. Thus, digestion of PCR product by one of seven restriction enzymes (*Alu*I, *Ava*I, *Bsu*RI, *Bsh*1236I, *Cfo*I, *Mva*I and *Rsa*I) distinguishes most cyst nematode species from each other (Subbotin *et al.*, 1997; Subbotin *et al.*, 1999a; Subbotin *et al.*, 1999b; Subbotin *et al.*, 2000; Subbotin *et al.*, 2003; Eroshenko *et al.*, 2001; Amiri *et al.*, 2002; Tanha Maafi *et al.*, 2003; Madani *et al.*, 2004).

The molecular method based on rDNA-ITS region and PCR-RFLP has been an efficient approach to reveal phylogenetic relationship and molecular characteristics amongcyst nematode species. It has been widely applied in the molecular diagnostics of cyst nematodes in recent years (Subbotin *et al.*, 2010; Zhuo *et al.*, 2014). In this study, 9 populations of cyst nematodes were identified by morphologicaland molecular methods. Their molecular features were specifically compared and analyzed using both rDNA-ITS and PCR-RFLP techniques.

MATERIALS AND METHODS

Cyst nematodes collection: The cyst nematodes were collected from the rhizosphere soil of wheat, soybean, and rice plants growing in China during 2012-2014. The detailed information of samples including the nematode species, location, and cultivated cropsis listed in Table 1 and shown in Figure. 3.

Cyst nematodes extraction: The cysts were isolated from the rhizosphere soil using a sieving method (Madani *et al.*, 2004). Each soil sample was mixed thoroughly with waterin individual pots. The supernatant was poured sequentially through 20 to 80 mesh sieve sets (pore size 850-180 μm) after stirring, and the whole procedure was repeated three times. Residual soil on the 80 mesh sieve was rinsed through with water into a beaker, and was then filtered with screen cloth. Cyst samples were picked under a dissecting microscope and then surface-sterilized using 0.5% NaClO solution for 2-3 min, and finally washed with sterile water.

Morphological identification: Morphological identification was based on the morphological characteristics and morphometrics of cysts and second-stage juveniles (J2s). After cystswere soaked in sterile water for 24 h, the vulval conesof each cyst was dissected under a microscope (Liu, 1995). Within each cyst, both the J2s and eggs were collected. Some of them were fixed with 5% formalin for the morphological identification, and the others were put into a 1.5 mL centrifuge tube for DNA extraction. The morphological characteristics of vulval cones and J2s was observed under a microscope and morphometrics was determined. Morphological identification followed the method described by Subbotin *et al.* (2010).

DNA extraction: DNA was extracted using the protocol described by Subbotin *et al.* (2000) with some modifications. Single cyst containing eggs and J2s was transferredinto a 1.5 mL centrifuge tube containing 50 μL of sterilized, double-distilled water and frozen in liquid N_2. The samples were crushed using a tissue grinder for 1 min with 40 μL lysis buffer (125 mM KCl, 25 mM Tris-HCl [pH 8.3], 3.75 mM $MgCl_2$, 2.5 mM DDT, 1.125% Tween 20, and 0.025% gelatin), together with 3 μL of proteinase K (20 mg/ mL) which was added after centrifugation. The tubes were then frozen at -80°Cfor 2 min, incubated at 65°C for 1 h and finally at 95°C for 10 min to inactivate the proteinase K. The nematode lysis mix was centrifuged at 12,000 rpm for 1 min and the supernatant was transferred to a clean tube and stored at –20°C for PCR amplification.

PCR amplification and sequencing: The forward primer TW81 (5′- GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′), as described by Curran *et al.* (1994), were used to amplify the internal transcribed spacer (ITS1-5.8S-ITS2) regions of the rRNA gene (Subbotin *et al.*, 2000). 1 μL of genomic DNA as a template, 12.5μL 2×Taq PCR MasterMix (Tiangen Bio

Tech), 0.5μL each of 10uM primers TW81 and AB28, and 10.5μL sterilized, deionized-distilled water were added (total 25 μL) into a sterile Eppendorf tube. The PCR reaction was performed on a BioRad S1000TM Thermal Cycler (Hercules, CA, USA) using the following run parameters: 94℃ (4 min); 25 cycles of 94℃ (30 sec), 55℃ (30 sec), 72℃ (1 min); 72℃ (7 min); 4℃(hold). PCR products were purified and sequenced directly on an ABI-PRISM 3730 Genetic Analyzer by Sangon Bio Tech (http://www.sangon.com/).

Phylogenetic analysis: ITS rDNA in this study and sequences of several *Heterodera* species from GenBankwere used for phylogenetic reconstruction. *Cryphodera brinkmani* (AF274418)was used as an outgroup taxa. The newly obtained and published sequences were edited with Chromas 2.3 and then aligned using ClustalX 1.83 with default parameters. Phylogenetic analysis of the sequences was performed with a Neighbor-joining method using MEGA 5.0 software.

PCR-RFLP analysis: 6 μL of each PCR product of the rDNA-ITS was digested with one of the following 8 restriction enzymes: *Alu*I, *Bsu*RI, *Cfo*I, *Eco*RI, *Hinf*I, *Pst*I, *Rsa*I and *Taq*I (Takara bioinc.) in the buffer stipulated by the manufacture. The digested DNA was loaded onto a 1.5% agarose gel, stained with ethidium bromide, visualized on a UV transilluminator, and photographed.

RESULTS

Morphological identification: Nine populations of cyst nematodes collected were identified based on morphological characteristics and morphometrics of cysts and J2s (Table 2, 3). Each was further classified into 4 species: *Heterodera avenae* (Figure 4A, 5A, 6A and 6E), *H. elachista* (Figure. 4B, 5B, 6B and 6F), *H. filipjevi* (Figure. 5C, 5C, 6C and 6G) and *H. glycines* (Figure. 4D, 5D, 6D and 6H). Among them, we assigned 3 populations (HaBJ, HaKF and HaLH) to *H. avenae*, 2 populations (HgTG and HgJN) as *H. glycines*, 2 populations (HfAH and HfHN) as *H. filipjevi*, and 2 populations (HeHuN and HeJX) as *H. elachista*.

Molecular identification and phylogenetic analysis: rDNA-ITS region was used to analyze the molecular characters of cyst nematode populations. The amplicon of ITS region from all the population DNAs yielded a single band with an approximately 1000 bp in size (Figure 7), which was then sequenced. Final sequences were Blasted in GenBank and highly conserved sequences were selected for further phylogenetic analysis with *Cryphodera brinkmani* (AF274418) as an outgroup (Figure 8).

Phylogenetic analysis showed that 3 populations (HaBJ, HaKF and HaLH) and *H. avenae* (KJ921716, AF274395 and AF274397) were located within the same species. This cluster shared more than 99% sequence similarity with previously identified *H. avenae* population from China (KJ921716), as well as 98.2% to 98.6% sequence similarity with *H. avenae* population from India (AF274397), and 98.4% to 98.8% sequence similarity with *H. avenae* population from France (AF274395). Populations HgTG and HgJN matched well with *H. glycines* sequences in GenBank with the sequence similarities of 99.3% to 99.6%. Two populations (HfAH and HfHN) and *H. filipjevi* (AY347922, AF274399, GU079654, GU083595 and AY148397) are located in the same subgroup and share 99.7% to 100% sequence similarity. The sequences of two populations (HeHuN and HeJX) were 98.6% to 99.7% similar to *H. elachista* (JN202916 and KC618466). Together with morphological identification, 3 populations (HaBJ, HaKF and HaLH) were recognized as *H. avenae*, 2 populations (HgTG and HgJN) as *H. glycines*, 2 populations (HfAH and HfHN) as *H. filipjevi*, and 2 populations (HeHuN and HeJX) as *H. elachista*.

Molecular characterization among cyst nematode populations: A comparative analysis of ITS-rDNA among species and populations of cyst nematodes on major field crops was established based on PCR-RFLP method. The PCR products of rDNA-ITS regions from 9 populations were digested with 8 different the restriction enzymes (Table 4, Figure. 9). The results indicated that *Cfo*I and *Taq*I are able to distinguish 4 different species (Figure. 9C and 9H). The whole sequence of *H. glycines* populations (HgTG and HgJN) was cut into two fragments (582 and 369 bp, respectively) by *Eco*RI, while no recognition site was found in ITS sequences of other cyst nematode populations (Figure. 9D). The restriction enzymes *Alu*I, *Bsu*RI, *Cfo*I, *Rsa*I and *Taq*I were able to distinguish *H. glycines* and *H. elachista* (Figure. 9A, 9B, 9C, 9G and 9H). *Cfo*I, *Pst*I and *Taq*I distinguished *H. avenae* and *H. filipjevi* (Figure. 9C, 9F and 9H). Two different patterns were produced by *Hinf* I for 4 *Heterodera* species. Specifically, a 500 bp PCR fragment from *H. glycines* and *H. elachista*, while a 770 bp PCR fragment from *H. avenae* and *H. filipjevi* (Figure. 9E).

Table 1.Information on cyst-forming nematodes used in this study.

Code	Sample time	Species	Source	Host
HaBJ	2012	*H. avenae*	Beijing	Wheat
HaKF	2012	*H. avenae*	Kaifeng, Henan	Wheat
HgTG	2013	*H. glycines*	Taigu, Shanxi	Soybean
HgJN	2014	*H. glycines*	Jining, Shandong	Soybean
HaLH	2014	*H. avenae*	Luohe, Henan	Wheat
HfAH	2014	*H. filipjevi*	Suzhou, Anhui	Wheat
HfHN	2013	*H. filipjevi*	Zhengzhou, Henan	Wheat
HeHuN	2013	*H. elachista*	Changsha, Hunan	Rice
HeJX	2014	*H. elachista*	Nanchang, Jiangxi	Rice

Table 2.Morphological characteristics and morphometrics of cysts(all measurements in µm).

Species	***H. avenae***	***H. elachista***	***H. filipjevi***	***H. glycines***
n	20	20	20	20
L	643.1±25.6 (551.7-703.8)	447.1±25.1 (327.6-556.9)	767.3±13.9 (625.8-933.6)	617.6±66.1 (445.7-721.5)
W	493.2±25.4 (447.4-561.3)	322.9±25.8 (227.9-448.3)	531.6±14.3 (369.0-677.9)	450.1±55.8 (343.6-546.9)
L/W	1.3±0.1 (1.1-1.9)	1.4±0.2 (1.1-1.8)	1.4±0.1 (1.3-1.6)	1.4±0.1 (1.1-1.6)
Fenestral length	48.6±10.1 (45.1-54.9)	29.3±2.6 (22.9-34.7)	50.6±6.9 (45.3-57.8)	48.6±8.9 (37.1-62.5)
Fenestral width	24.3±3.5 (20.7-29.3)	32.2±2.9 (24.9-40.2)	27.9±5.6 (24.1-32.6)	38.5±7.8 (22.7-53.5)
Underbridge	Absent	Slender	Present	Strong
Vulval slit length	8.3±2.8 (7.8-8.6)	37.7±2.4 (26.9-41.3)	10.1±1.5 (9.0-12.4)	42.3±10.6 (30.5-59.5)
Bullae	Prominent	Few	Prominent	Prominent
Fenestration type	Bifenestrate	Ambifenestrate	Bifenestrate	Ambifenestrate
Group	*Avenae*	*Cyperi*	*Avenae*	*Schachtii*

Table 3. Morphological characteristics and morphometrics of J2s (all measurements in µm).

Species	***H. avenae***	***H. elachista***	***H. filipjevi***	***H. glycines***
n	20	20	20	20
L	455.6±17.9 (413.7-486.5)	403.4±9.8 (378.6-455.1)	526.9±10.6 (486.0-571.1)	430.8±28.9 (371.1-466.8)
SP	26.8±1.1 (23.9-27.8)	19.3±0.5 (16.8-22.0)	24.8±1.1 (23.5-26.3)	23.5±1.1 (21.3-24.8)
Stylet knob shape	Anteriorly flattened to projected	Rounded or anteriorly projected	Moderately anteriorly projected	Anteriorly projected
Tail	55.8±4.6 (45.5-67.2)	56.9±3.2 (48.1-70.2)	59.4±3.7 (57.6-67.1)	46.6±4.6 (38.5-54.5)
h.	32.0±3.1 (24.1-47.8)	31.8±2.9 (27.6-36.7)	36.5±2.5 (30.1-40.8)	23.0±3.1 (17.7-29.9)
Incisures number	4	3	4	4
Group	*Avenae*	*Cyperi*	*Avenae*	*Schachtii*

Table 4. Length (bp) of restriction fragments of rDNA-ITS regions based on restriction fragment length polymorphisms (RFLPs) and sequence data.

Restriction enzymes	*Alu*I	*Bsu*RI	*Cfo*I	*Eco*RI	*Hinf*I	*Pst*I	*Rsa*I	*Taq* I
HaBJ	(529,437)	(383,353,130,52,24,24)	(708,108,106,44)	(966)	(771,187,8)	(671,295)	(683,283)	(384,273,148,96,65)
HaKF	(527,438)	(381,353,131,52,24,24)	(706,108,107,44)	(965)	(772,185,8)	(669,296)	(684,281)	(384,248,148,94,65,26)
HaLH	(528,438)	(382,353,131,52,24,24)	(704,108,107,44)	(966)	(772,186,8)	(670,296)	(684,282)	(384,274,148,95,65)
HfAH	(538,437)	(391,378,130,52,24)	(717,108,60,46,44)	(975)	(775,192,8)	(680,130,165)	(682,293)	(339,273,118,101,79,65)
HfHN	(538,437)	(391,378,130,52,24)	(717,108,106,44)	(975)	(775,192,8)	(680,130,165)	(682,293)	(339,273,118,101,79,65)
HgJN	(341,284,134,133,44,15)	(467,252,197,24,11)	(422,278,146,59,46)	(582,369)	(494,449,8)	(663,288)	(786,159,6)	(526,266,94,65)
HgTG	(341,284,135,134,44,15)	(468,253,197,24,11)	(422,279,146,60,46)	(583,370)	(495,450,8)	(664,289)	(787,160,6)	(526,267,95,65)
HeHuN	(338,324,146,142,28,17)	(506,203,183,79,24)	(251,248,215,96,94,64,27)	(995)	(505,482,8)	(680,315)	(572,423)	(637,293,65)
HeJX	(338,323,146,142,28,17)	(505,203,183,79,24)	(342,250,215,96,64,27)	(994)	(505,481,8)	(680,314)	(571,423)	(637,292,65)

Figure. 1. A-E: Known distribution of the soybean cyst nematode, *H. glycines*, in provinces of China in selected years from 1899 to 2016. Known infested provinces are indicated in red. F: Known distribution of the *H. glycines*, *H. avenae*, *H. elachista*, *H. filipjevi* in Provinces of China.

Figure. 2. Structure of rRNA genes and positions of universal primers.

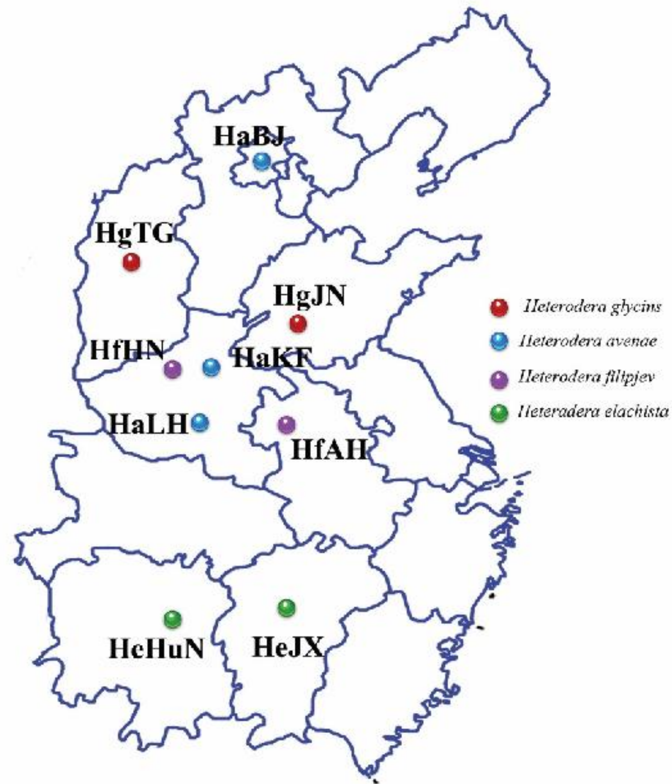

Figure. 3. Locations of 9 cyst nematode populations collected from the Middle and Eastern parts of China

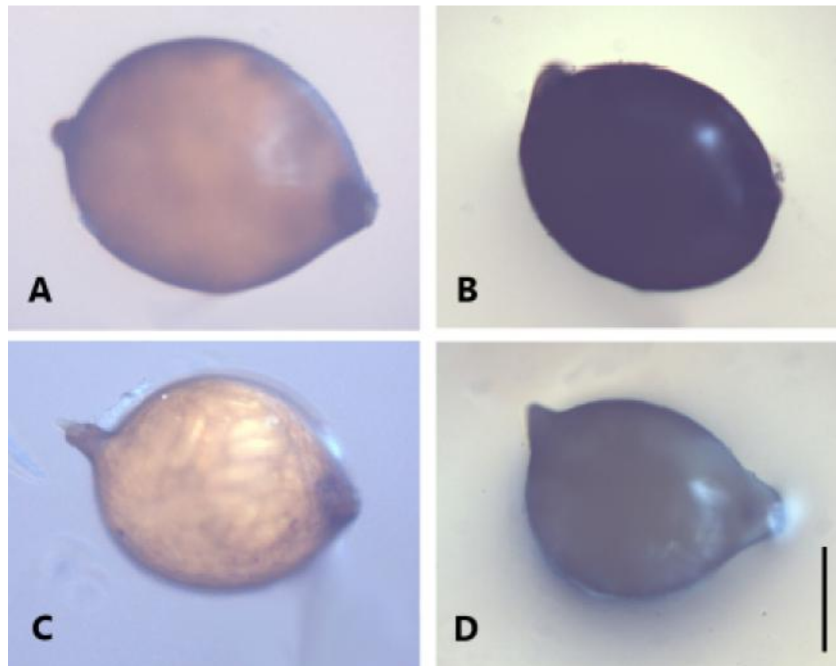

Figure. 4. Light micrographs of the cysts.（scale bar = 200 μm）A: *H. avenae*; B: *H. elachista*; C: *H. filipjevi*; D: *H. glycines.*

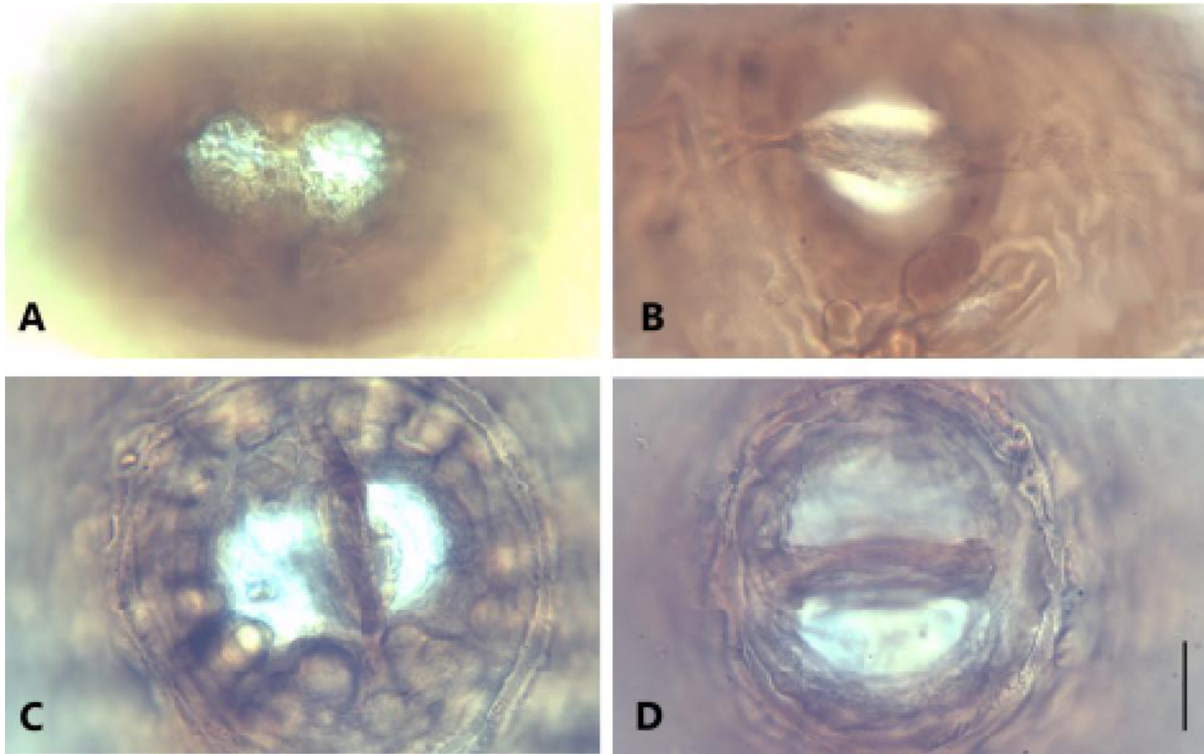

Figure 5. Light micrographs for the terminal region of the cysts. （**scale bar = 20 µm**）**A:** ***H. avenae*; (Bifenestrate; Underbridge: Absent), B: *H. elachista*; (Ambifenestrate; Underbridge: Slender), C: *H. filipjevi*; (Bifenestrate Underbridge: Present), D: *H. glycines*. (Ambifenestrate; Underbridge: Strong)**

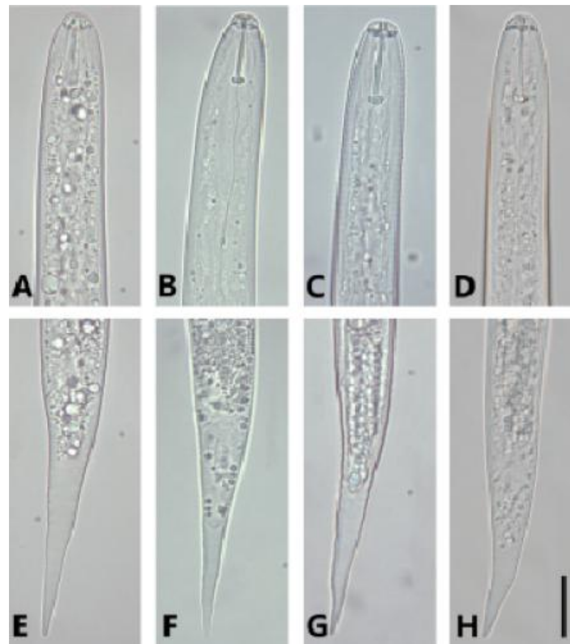

Figure. 6. Light micrographs of the second-stage juveniles (J2s).（**scale bar = 20 µm**）**A & E: *H. avenae*; B &F: *H. elachista*; C & G: *H. filipjevi*; D & H: *H. glycines.***

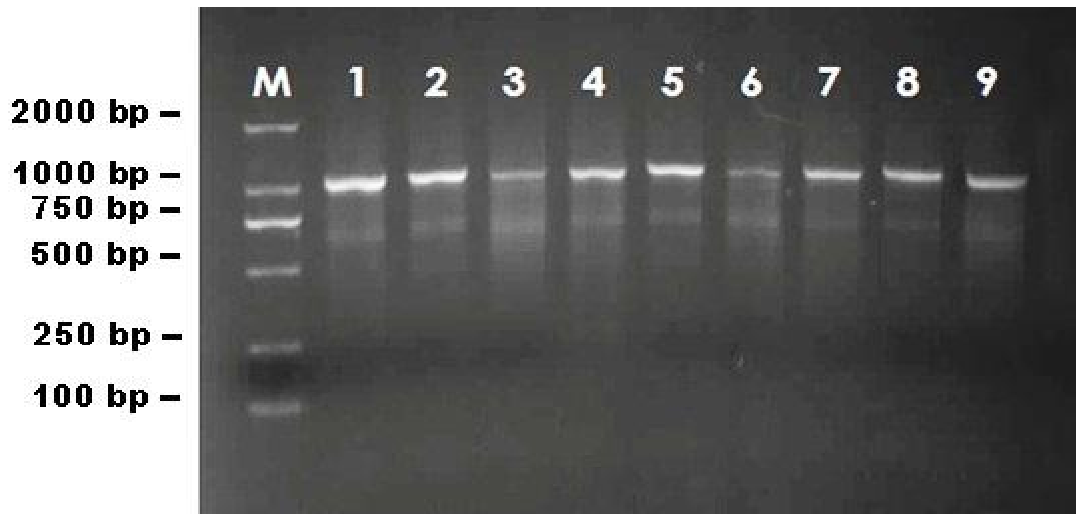

Figure. 7. The amplicon of rDNA-ITS regions for 9 cyst-forming nematode populations of major crops.(M: 2000 bp marker, 1: HaBJ, 2: HaKF, 3: HgTG, 4: HgJN, 5: HaLH, 6: HfAH, 7: HfHN, 8: HeHuN, 9: HeJX).The lane labeled 'M' indicates the 2000 bp DNA ladder.

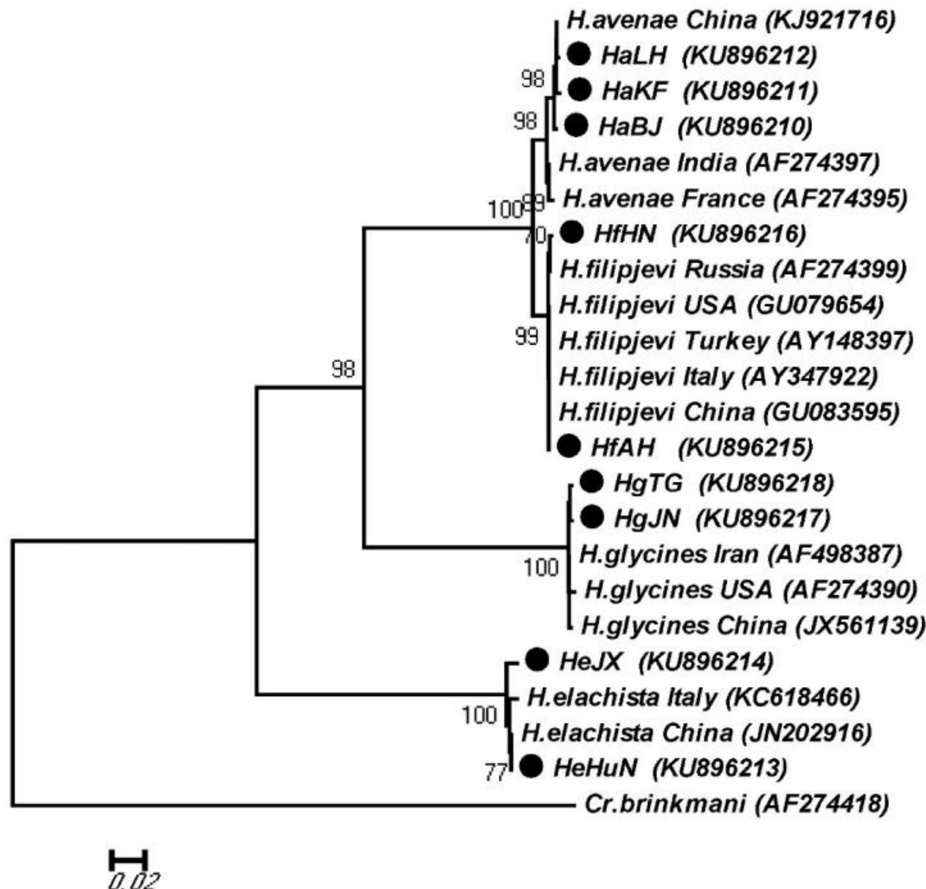

Figure. 8. Phylogenetic tree for the 9 cyst-forming nematode populations characterized in this study and 13 populations previously reported. The tree was constructed based on the rDNA-ITS sequences using the neighbor-joining method (MEGA 5.0). *Cryphodera brinkmani* was used as the outgroup. Nodal values were supported by 1000 bootstrap replications. The bar represents 0.02 substitutions per site. Only bootstrap values>70% were shown.

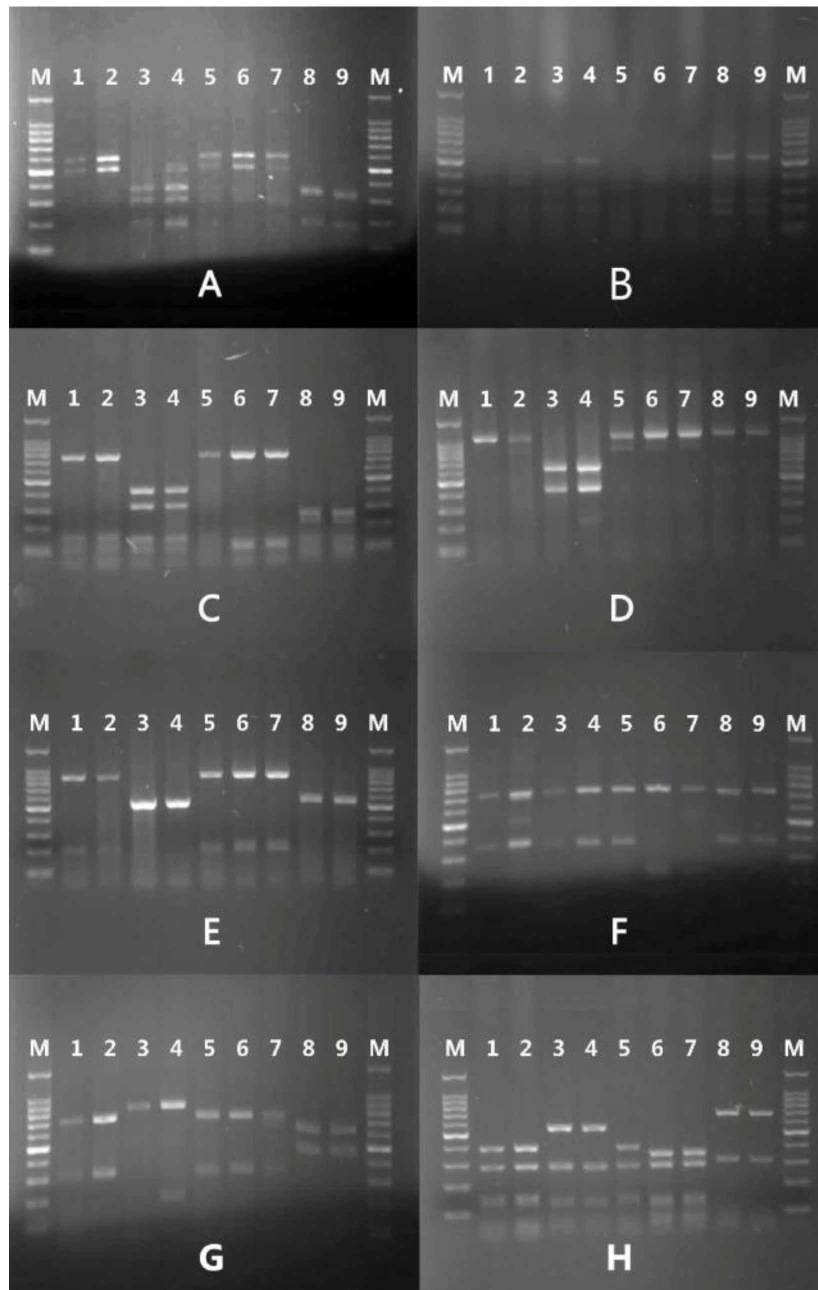

Figure. 9. Restriction fragments of amplified rDNA-ITS regions for 9 cyst-forming nematode populations of major crops. The PCR products were generated using 8 different enzymes (A: *Alu*I; B: *Bsu*RI; C: *Cfo*I; D:*Eco*RI; E:*Hinf*I; F:*Pst*I; G: *Rsa*I;H:*Taq*I). The lanes correspond to DNA samples from each of the 9 populations (1: HaBJ; 2: HaKF; 3: HgTG; 4: HgJN; 5: HaLH; 6: HfAH; 7: HfHN; 8: HeHuN; 9: HeJX). The lane labeled 'M' indicates the 100 bp DNA ladder.

DISCUSSION

Cyst nematodes cause severe damages on crop productions in China. Investigation and identificationof cyst nematode diseases and cyst nematode populationson crops in the main cultivating areas of China will provide important information for early control. In this study, 9 populations of cyst nematodes were collected from the main field crop growing regions in middle and eastern parts of China, and they were divided into 4 species including *H. avenae*, *H. glycines*, *H. filipjevi*and *H. elachista* based on morphological and molecular characters.

The 4 *Heterodera* species are the most important for main field crops in China. Among them, *H. filipjevi* mainly parasitizes wheat and was first found in Henan

Province (Peng *et al*., 2010; Li *et al*., 2010). In our study, *H. filipjevi* occurred in Anhui province, which is the first recorded incidence in any other Chinese province except the Henan Province. *H. elachista* was first observed in rice fields in Hunan province in 2011 (Ding *et al*., 2012), and has since been identified in severalother regions, including the Guangxi and Ningxia Provinces (Zhuo *et al*., 2014). Our results demonstrate that *H. elachista*also resides in Jiangxi Province, suggesting that the migration of cyst nematodes may occur in China or other reasons such as lack of system investigation. So there are still a lot of basic work needs to be completed. Cyst nematode species share similar morphological characters and they often co-occur, even in the same field. Therefore, it is often difficult to identify them to the species-level using morphological characteristics alone. PCR-RFLP is a suitable method for wide application and now PCR-RFLP maps are available to identify 49 different cyst nematode species (Subbotin *et al.*, 2010). In this study, we utilized 8 different restriction enzymes to analyze molecular character of 9 *Heterodera* populations parasitizing main field crops.

Heterogeneity exists in rDNA-ITS region among different species of cyst nematodes (Subbotin *et al.*, 1997; Subbotin *et al.*, 1999a; Subbotin *et al.*, 1999b; Subbotin *et al.*, 2000; Subbotin *et al.*, 2003; Eroshenko *et al.*, 2001; Amiri *et al.*, 2002; Tanha Maafi *et al.*, 2003; Madani *et al.*, 2004). Subbotin and co-workers think that heterogeneity occurs in the ITS region of *H. avenae*, and thus divided them further into types"A" and "B" (Subbotin *et al.*, 2000). Furthermore, Zheng and his colleagues identified a new type "C" of *H. avenae* from Taigu in Shanxi Province, which was different from previously identified *H. avenae*populations (Zheng *et al.*, 2010). PCR-RFLP analysis with *Hinf* I showed that all of *H. Avenae* populations in our study can be distinguished astype "C". They are also close to previously identified type "C" populations of *H. Avenae* in the phylogenetic tree (Figure 8).

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REFERENCES

Amiri, S., S.A. Subbotin, and M. Moens (2002). Identification of the beet cyst nematode *Heterodera schachtii*by PCR. Eur. J. Plant. Pathol. 108(6): 497-506.

Chen, P.S., M.Z. Wang, and D.L. Peng (1989). Preliminary report of identification on cereal cyst nematode of wheat in China. Scientia Agricultura Sinica. 24(5):89-91.

Chen, P.S., M.Z. Wang, and D.L. Peng (1992). A study of cereal cyst-nematode (*Heterodera avenae* Wollenweber) in China. Acta. Agron. Sin. 22(4): 339-343.

Chen, X., H.Y. Zhou, and X. Ma (2009). Distribution of *Heterodera avenae* in the middle and west regions of Inner Mongolia, China. Plant Protection. 35(5):114-117.

Curran, J., F. Driver, J. W. O. Ballard, and R.J. Milner (1994). Phylogeny of Metarhizium: analysis of ribosomal DNA sequence data. Mycol. Res. 98(5): 547-552.

Dai, F.L., W.N. Xiang and R.Y. Zheng (1958). Chinese Plant Disease Pathogens. Science Press; Peking (China).

Ding, Z., J. Namphueng, X.F. He, D.L. Peng, and W.K. Huang (2012). First report of the cyst nematode (*Heterodera elachista*) on rice in Hunan Province, China. Plant. Dis. 96(1):151.

Eroshenko, A.S., S.A. Subbotin, and I.P. Kazachenko (2001). *Heterodera vallicola* sp. n. (Tylenchida: Heteroderidae) from elm trees, *Ulmus japonica* (Rehd.)Sarg. in the Primorsky territory, the Russian Far East, with rDNA identification of closely related species. Nematology. 9(1):9-17.

Hesling, J.J. (1978). Cyst nematodes: morphology and identification of *Heterodera, Globodera* and *Punctodera*. Plant nematology; London (UK). p125-155.

Hou, S.Y., D.L. Peng, A.L Wang, W.K. Huang, G.G. Geng, and G. Zhang (2007). Preliminary report on the investigation of Cereal cyst nematode in Qinghai. J. Qinghai University. 26(5): 84-86.

Li, H.L., H.X. Yuan, J.W. Sun, B. Fu, G.L. Nian, X.S. Hou, X.P. Xing, and B.J. Sun (2010). First record of the cereal cyst nematode *Heterodera filipjevi* in China. Plant. Dis. 94(12): 1505.

Li, H.M., X. Wang, S.A. Pei, X.H. Le, and Z.W. Liang (2010). A preliminary survey of the cereal cyst nematode *Heterodera avenae* on wheat in Jiangsu Province of China. Plant Protection. 36(6): 172-175.

Li, J.R., P. Zhao, P.G. Xu, J.Z. Jian, D.L. Peng, and H.X. Li (2016). Investigation and identification of cyst nematode in agricultural and pastoral oats fields in Tianzhu of Gansu Province. J. Triticeae Crops. 36(7): 961-968.

Li, K.M., Riziwangguli, and Y.Q. Dong (2014). A preliminary study on the soybean cyst nematode *Heterodera glycines* in Xinjiang uygurautonomous region. Plant Protection. 40(2): 172-174.

Liu, W.Z., X.Y. Liu, and Z.J. Luan (2005). The Detection of *Heterodera avenae* Wollenweber 1924 From the Wheat field in Heze City of Shandong Province. J. Laiyang Agricultural College. 22(4):266-269.

Liu, W.Z., Y. Liu, and Y.X. Duan (1994). Morphological Observation of Soybean Cyst Nematode from China. J. Shenyang Agri. University. 25(2): 164-167.

Liu, W. Z. (1995). Technology for Nematological Research. Liaoning Science and Technology Publishing House; Shenyang (China). p86-87.

Liu, X.H., Li, J.Q., and Zhang, D.S. (1997). History and status of soybean cyst nematode in China. International J. Nematology 7(1): 18-25.

Madani, M., N. Vovlas, P. Castillo, S.A. Subbotin, and M. Moens (2004). Molecular characterization of cyst nematode species (*Heterodera* spp.) from the Mediterranean basin using RFLPs and sequences of ITS-rDNA. J. Phytopathol. 152(4):229-234.

Mulvey, R.H. (1974). Cone-top morphology of the white females and cysts of the genus *Heterodera* (subgenus *Heterodera*), a cyst-forming nematode. Can. J. Zool. 52(1): 77-81.

Peng, D.L., W.X. Ye, H. Peng, and X.C. Gu (2010). First report of the cyst nematode (*Heterodera filipjevi*) on wheat in Henan Province, China. Plant. Dis. 94(10): 1262.

Qi, S.H., D.L. Peng, D.S. Zhang, and P.S. Chen (1994). Preliminary report on the new host and new distribution area of cereal cyst nematode in China. Plant Protection. 20(4):52.

Subbotin, S.A., P.D. Halford, and R.N. Perry (1999a). Identification of populations of potato cyst nematodes from Russia using protein electrophoresis, rDNA-RFLPs and RAPDs. Russ. J. Nematol. 7(1): 57-64.

Subbotin, S.A., M. Mundo-Ocampo, and J.G. Baldwin (2010). Systematics of cyst nematodes (Nematodes: Heteroderinae), Volume 8, Part B. Brill Academic Publishers; The Netherlands.

Subbotin, S.A., D. Sturhan, H.J. Rumpenhorst, and M. Moens (2003). Molecular and morphological characterisation of the *Heterodera avenae* complex species (Tylenchida: Heteroderidae). Nematology. 5(4): 515-538.

Subbotin, S.A., D. Sturhan, L. Waeyenberge, and M. Moens(1997). *Heterodera riparia* sp. n. (Tylenchida: Heteroderidae) from common nettle, *Urticadioica* L., and rDNA-RFLP separation of species from the *H. humuli* group. Russ. J. Nematol. 5(2): 143-157.

Subbotin, S. A., L. Waeyenberge, and M. Moens (2000). Identification of cyst-forming nematodes of the genus *Heterodera* (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLPs. Nematology. 2(2):153-164.

Subbotin, S.A., L. Waeyenberge, I.A. Molokanova, and M. Moens (1999b). Identification of *Heterodera avenae* group species by morphometrics and rDNA-RFLPs. Nematology. 1(2):195-207.

Tanha, M. Z., S. A. Subbotin, and M. Moens (2003). Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on ITS-rDNA sequences. Nematology. 5(1):99-111.

Wang, D., Y.X. Duan, Y.Y. Wang, X.F. Zhu, L.J. Chen, X.Y. Liu, and J.S. Chen (2015). First Report of Soybean Cyst Nematode, *Heterodera glycines*, on Soybean from Guangxi, Guizhou, and Jiangxi Provinces, China. Plant. Dis. 99(6): 893.

Wang, M.Z., D.L. Peng, and X.Q. Wu (1991). Study on wheat cyst nematode disease: I. Pathogen identification. J. Huazhong Agricultural University. 10(4):352-356.

Yang, C.G., H.P. Wu, G.J. Tan, and Y.Y. Wang (2008). Investigations on the distribution of and damages caused by cereal cyst nematode in Anhui. Plant Protection. 34(2):107-110.

Yang, Y.Y., H.H. Zhao, and D.L. Peng (2010). New Distribution Report of Cereal Cyst Nematode on Wheat in Shandong Province. J. Qingdao Agricultural University. 27(1):17-20.

Zheng, J., S.A. Subbotin, L. Waeyenberge, and M. Moens (2000). Molecular characterization of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. Russ. J. Nematol.8(2): 109-113.

Zheng, J.W., M.S. Lin, H.R. Cheng, and Z.D. Fang (1996). Identification of two cyst nematode populations parasitizing wheat in Anhui, China. Jiangsu J. Agricultural Sciences. 12(1): 31-35.

Zheng, J., Y. Zhang, X. Li, L. Zhao, and S. Chen (2009). First report of the soybean cyst nematode, *Heterodera glycines*, on soybean in Zhejiang, eastern China. Plant. Dis. 93(3):319.

Zhuo, K., H.D. Song, H.H. Wang, Y. Tao, H.L. Zhang, X.H. Lu, J.L. Huang, Z.M. Liu, and J.L. Liao (2014). Occurrence of *Heterodera elachista* in Guangxi Region and Its Intra-species Heterogeneity in rDNA-ITS Region. Chinese science of Rice Science. 28(1): 78-84.