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Genetic Variation Analysis of *Heterodera avenae* Wollenweber (Nematoda: Heteroderidae) using ISSR Marker and ITS-rDNA Sequence

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ABSTRACT

Heterodera avenae is distributed in 13 provinces of China and causes considerable yield losses of wheat. Inter-Simple Sequence Repeats (ISSR) fingerprint patterns and sequence analysis of the ITS region were used for the comparative study of genetic variation of *H. avenae*. DNAs of second-stage Juveniles from 16 populations of *H. avenae* and one population of *Heterodera filipjevi* in 4 provinces were analysed with 3 ISSR primers. Results of ISSR revealed a high genetic diversity (the percentage of polymorphic bands was 78.6%) within *H. avenae* on a large geographical scale. The analysis of molecular variance (AMOVA) indicated that about 74.8% of the variations resulted from genotypic variations within region and the remaining 25.2% were due to variances among regions. Base transition of ITS-rDNA sequence revealed low intraspecific variation in rDNA of *H. avenae*. The alignment of 16 sequences in *H. avenae* covered altogether 1045 nucleotide positions with 4 polymorphic bases. ISSR dataset grouped all populations into two clusters according to their geographical origin. These results suggested two different pathotypes or biological entities existing in the *H. avenae* species. Hybridization, multiple introductions, passive dispersal by anthropogenic activities and some natural means would probably be responsible for the genetic variation of *H. avenae*.

Key words: *Heterodera avenae*, polymorphic bands, genetic diversity, nucleotide positions, anthropogenic activities

INTRODUCTION

The cereal cyst nematode, *Heterodera avenae* Wollenweber is distributed worldwide in most wheat growing areas, including Asia, Europe, America and Pacific Northwest states of the USA (Rivoal and Cook, 1993; Nicol *et al*., 2003). *Heterodera avenae* parasitized over 30 plant species and caused considerable yield losses of wheat (Chen *et al*., 1992; Rivoal and Cook, 1993; Smiley *et al*., 1994). The nematode was first detected in Hubei province, China, in 1989 and now it is widespread in Anhui, Beijing, Hebei, Henan, Jiangshu, Shandong, Shanxi, Sanxi, Qinghai, Ningxia and Inner Mongolia provinces (Chen *et al*., 1992; Zheng *et al*., 2000; Ou *et al*., 2008). Pathotypes of the nematode detected in China are different from those reported in Europe and Australia (Abdollahi, 2008; Peng and Cook, 1996; Zheng *et al*., 1997). The wide distribution and different

pathotypes of *H. avenae* has required a series of management methods, such as rotational control, biological control and resistant cultivars (Zheng *et al*., 1997). Furthermore, it is difficult for most farmers to apply chemical pesticides because of the cost and environmental concern in China. In the search for ecological and economically suitable management strategies, the identification of genetic relationships at the species level and intra and interspecific variability is an essential step (Kaplan *et al*., 1999; Castagnone-Sereno, 2002).

Molecular information, mainly based on specific parts of the genome, was shown to provide significant insights into the degree of diversity existing among individuals and populations of plant-parasitic nematodes (Patra *et al*., 2011; Hlaoua *et al*., 2008; Powers *et al*., 1997; Peng *et al*., 2003; Madani *et al*., 2004). Several molecular techniques had been applied to study genetic diversity of cyst nematode populations (Zhang *et al*., 1998; Rivoal *et al*., 2003; Swofford, 2002; Madani *et al*., 2007). The Inter-simple Sequence Repeats (ISSR) PCR technique has been shown to have a wide application for the analysis of genetic variation at subspecies level, particularly in investigations of population structure and differentiation (Zietkiewicz *et al*., 1994). The ISSR technique was applied to study genetic diversity of populations of plant-parasitic nematodes of *Bursaphelenchus xylophilus* (Metge and Burgermeister, 2006), *Nacobbus aberrans* (Lax *et al*., 2007), *Ditylenchus destructor* (Huang *et al*., 2010) and to analyze the population structure of entomopathogenic nematodes and crop resistance of nematodes (Zietkiewicz *et al*., 1994; Berner and Schnetter, 2002; Metge and Burgermeister, 2006; Dayteg *et al*., 2008).

The Internal Transcribed Spacer region (ITS) of ribosomal DNA (rDNA), a well-conserved region controlled by concerted evolution (Baldwin and Mundo-Ocamp, 1991; Sridhar *et al*., 2010), has been demonstrated to be highly informative as a taxonomic marker at species level within nematodes (Powers *et al*., 1997; Subbotin *et al*., 2001). The phylogenetic relationships of cyst-forming nematodes were analyzed by ITS rDNA gene sequences (Subbotin *et al*., 2001; Peng *et al*., 2003; Maafi *et al*., 2003). The heterogeneity of the ITS-rDNA was revealed by RFLP analysis in many species of cyst forming nematodes (Szalanski *et al*., 1997; Zheng *et al*., 2000; Peng *et al*., 2003; Madani *et al*., 2004; Ou *et al*., 2008). It was revealed that intraspecific polymorphism of *H. avenae* populations in different countries can be distinguished by rDNA-RFLP studies of rDNA-ITS regions (Subbotin *et al*., 1999). Peng and Cook (1996) concluded that pathotypes of cereal cyst nematode in China may differ from those of other countries and regions but they did not allocate numbers for pathotypes using the scheme of, Andersen and Andersen (1982) because some host reactions still need to be validated. Zheng *et al*. (1997) showed the Taigu and Guzhen populations were distinctive new pathotypes. Several management methods may have specific genotype-level interactions with nematodes from different origins (Blok, 2005; Castagnone-Sereno, 2002). Therefore, evaluation of polymorphism among the populations of *H. avenae* by combining ISSR with ITS-rDNA gene analysis is needed to generate effective control strategies.

In the present study, the ISSR technique and ITS-rDNA gene sequences were used to analyze the genetic variation of *H. avenae* populations in China at two different levels. Based on molecular phylogenetic relationships, the genetic structure of this nematode was analyzed at regional level.

MATERIALS AND METHODS

Nematode populations: The sample set used in the present study included *H. avenae* populations from 16 different locations of 4 provinces throughout wheat-producing regions in China (Table 1,

Table 1: Location and genetic diversity of 16 populations of *H. avenae* and one population of *H. filipjevi*

Species	Region	Locality	Code	Latitude (N)	Longitude (E)	Altitude (m)	PPB of population (%)	Average PPB of region (%)	Average heterozygosity (H)	Shannon information index (I)
Heterodera avenae	Anhui	Bangbu	AHBB	N33° 18.473'	E117° 20.152'	17	78.60	45.2	0.168	0.250
		Chuzhou	AHCZ	N32° 40.923'	E117° 17.579'	40	61.90			
		Huaibei	AHHB	N33° 49.501'	E116° 52.334'	27	57.10			
		Huainan	AHHN	N32° 41.679'	E117° 06.864'	19	73.80			
		Suzhou	AHSZ	N33° 45.816'	E116° 57.751'	30	66.67			
	Hebei	Baoding	HBBD	N38° 53.362'	E115° 32.271'	10	66.67	33.3	0.140	0.203
		Shijiazhuang	HBSJ	N38° 04.695'	E114° 33.958'	39	69.10			
		Xingtai	HBXT	N37° 00.069'	E114° 30.377'	71	69.10			
	Henan	Anyang	HNAY	N36° 10.560'	E114° 21.775'	76	76.20	40.5	0.154	0.227
		Puyang	HNPY	N36° 02.454'	E115° 17.904'	51	71.40			
		Xuchang	HNXC	N33° 58.279'	E113° 48.357'	60	73.80			
	Shandong	Dongying	SDDY	N37° 01.615'	E118° 25.946'	19	762.00	54.8	0.231	0.333
		Laiwu	SDLW	N36° 14.637'	E117° 44.973'	194	81.00			
		Linyi	SDLY	N34° 56.683'	E118° 36.508'	73	59.50			
		Shouguang	SDSG	N36° 57.196'	E118° 33.986'	10	78.60			
		Zibo	SDZB	N36° 43.945'	E118° 00.546'	52	78.60			
Heterodera filipjevi	Henan	Luohe	HNLH	N33° 47.415'	E113° 55.261'	53	69.10	-	-	-

Altitude, latitude and longitude data were recorded by a GPS locator, PPB: Percentage of polymorphic bands

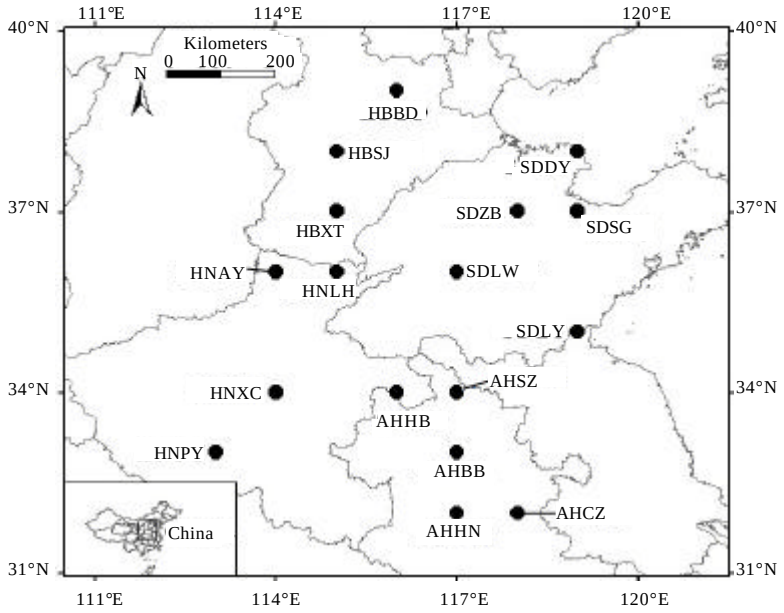

Fig. 1: Geographic location of the 16 *H. avenae* populations and one *H. filipjevi* from 4 provinces in China

Fig. 1). One population of *H. filipjevi* from Henan province was also included as an outgroup. Ten soil cores (10 cm diameter and 25 cm deep) were collected from each location using a cylindrical sampling tube and mixed uniformly. Cysts were isolated from soil by the

routine sieving-decanting method and identified by examining the vulval cone as described by Yan and Smiley (2010). Five cysts were selected randomly from each population and put under 4°C for six weeks then under 15°C for five weeks for the hatching of second-stage juveniles (J2s).

DNA isolation: For each population, genomic DNA was isolated from 50 J2s of the mixed cysts. DNAs of J2s from the same population were used to test the repeatability of the amplified products obtained. Each specimen was crushed with a glass pestle inside the tube after it was immersed in liquid nitrogen for 30 sec. Then 32 μL Worm Lysis Buffer (500 mM KCl, 10 mM Tris-HCl, 15 mM $MgCl_2$, 1.0 mM DTT, 4.5% Tween20) and 8 μL Proteinase K (20 mg mL^{-1}) were added to each tube. The tubes were incubated at 65°C for 2 h, followed by 10 min at 94°C in a Master cycler 5332 PCR thermal sequencer (Eppendorf Inc). DNA extracted from each population that produced reproducible bands selected for the next analysis and they were kept at -20°C until use.

ISSR-PCR amplification: Several DNA and primer concentrations used in the reaction mix, number of cycles, annealing temperature and evaluation of multiple 2 μL aliquots from DNA extraction were tested to optimize PCR reaction conditions and guarantee the repeatability of the amplified products obtained. A total number of 10 ISSR primers were tested (Zietkiewicz *et al*., 1994; Tikunov *et al*., 2003) those that produced clear, reproducible and polymorphic bands were selected for the analysis. Each ISSR reaction was carried out as the system of Lax *et al*. (2007) with the programme as follows. An initial denaturation at 94°C for 5 min, followed by 38 cycles of 30 sec at 94°C, 45 sec at 52°C, 1 min at 72°C and final extension of 10 min at 72°C. The PCR products were separated electrophoretically on 2% agrose gels in 0.5×TAE buffer. Gels were stained with ethidium bromide and photographed with Gel Doc XR image analysis system (Bio-Rad).

ISSR data analysis: Amplified bands were scored as 1/0 (presence/absence) of homologous bands for all samples. Only bands that were reproducible were included in the study. Similarity matrices were analyzed using the unweighted pair group arithmetic average (UPGMA) clustering method with the NTSYS program, version 2.1 and dendrograms were created (Rohlf, 2000). The robustness of the dendrogram was tested by bootstrapping with 1000 permutations. The resulting presence/absence data matrix of regional group of *H. avenae* was analyzed using POPGENE version 1.32 (Yeh *et al*., 1999) to estimate the level of genetic diversity by the Percentage of Polymorphic Bands (PPB), average Heterozygosity (H) and Shannon information index (I).

ITS-rDNA-PCR and sequencing: The protocol for ITS-rDNA-PCR was essentially performed as described by Madani *et al*. (2007) with some minor modifications. The PCR reaction consisted of 2 μL of DNA, 2.5 μL 10×reaction buffer (500 mM KCl, 100 mM tris-Cl, pH 9.0; 25 mM $MgCl_2$), 2 μL of each dNTP (2.5 mM each), 2 μL of each primer (10 μM) and 0.5 μL of Taq DNA Polymerase (5 U μL^{-1}) and distilled water to a total volume of 25 μL. PCR reactions were programmed for an initial denaturation at 94°C for 5 min, followed by 34 cycles of 30 sec at 94°C, 45 sec at 55°C, 1 min at 72°C and final extension of 10 min at 72°C.

Gel purified ITS-rDNA-PCR products were ligated into a pMD-18T vector and transferred to *Escherichia coli* DH5a according to the manufacturer's instructions (Invitrogen, America). Both DNA-strands were sequenced from all samples by an ABI 377 sequencer in Beijing Genomic Institute.

Phylogenetic data analysis: A total 16 newly obtained ITS-rDNA sequences of *H. avenae* and one sequence of *H. filipjevi* were used for the final analysis. Four ITS Sequences (AY148360, AY148362, AY148367, EF153843) of *H. avenae* from other countries were downloaded from GenBank for the phylogenetic analysis. Sequences were aligned with Clustal (W) and Minimum-Evolution (ME) analyses were performed with Mega4 software by Tamura *et al.* (2007). The robustness of the dendrogram was tested by bootstrapping with 1000 permutations.

RESULTS

The cuticle of mature cysts was dark brown to black for *H. avenae* in color and golden to light brown for *H. filipjevi*. A distinct underbridge with bifurcated arms was present in vulval cones of the cysts of *H. filipjevi* whereas no underbridge was found in *H. avenae*.

ISSR analysis: Sequences of the three anchored primers selected to amplify ISSR in all populations were shown in Table 2. High polymorphism and genetic variability were observed using selected primers (Table 1, Fig. 2). A total number of 42 unambiguous fragments were scored, among which 33 were polymorphic (78.6% polymorphism). For the three selected primers (Table 2), the number of scored bands were 15, 14, 13 and the polymorphism was 80.0, 78.6, 76.9%, respectively. No population-specific band of *H. avenae* was detected. Similarity matrix generated by Jacard's coefficient showed that the average genetic similarity of *H. avenae* was 0.69 and the genetic similarity of *H. filipjevi* was 0.65. The highest genetic similarity coefficient was observed between HNPY and HNXC with the value of 0.93. The lowest genetic similarity coefficient was observed between HNAY and HBXT with the value of 0.48.

Within the four studied geographical regions, the PPB of *H. avenae* was varied from 33.3 and 54.8% in Hebei and Shandong province, respectively (Table 1). Average heterozygosity (H) value and I index showed similar trends, which ranged from 0.14 to 0.23 and 0.21 to 0.33 in Hebei and Shandong province, respectively. At the species level of *H. avenae*, the average PPB was 78.6%, heterozygosity (H) was 0.30 and Shannon information index (I) was 0.44. At the region level of *H. avenae*, the hierarchical AMOVA revealed small genetic divergence among regions from different

Table 2: Sequence of ISSR primers used for the analysis of *H. avenae* populations and polymorphism of primers

Primer	Sequence(5'→3')	Number of loci scored	Polymorphism (%)
ISSR2	$(GACA)_4$	15	80.0
ISSR10	$(GA)_8TT$	14	78.6
ISSR16	$(AC)_8C$	13	76.9

Table 3: Hierarchical analysis of molecular variance (AMOVA) of the 16 *H. avenae* populations

Source of variation	df	Sums of squares	Variance component	Total variance (%)	p-value
Among regions	3	36.81	1.7116	25.2	0.001
Within regions	13	66.13	5.0872	74.8	0.001

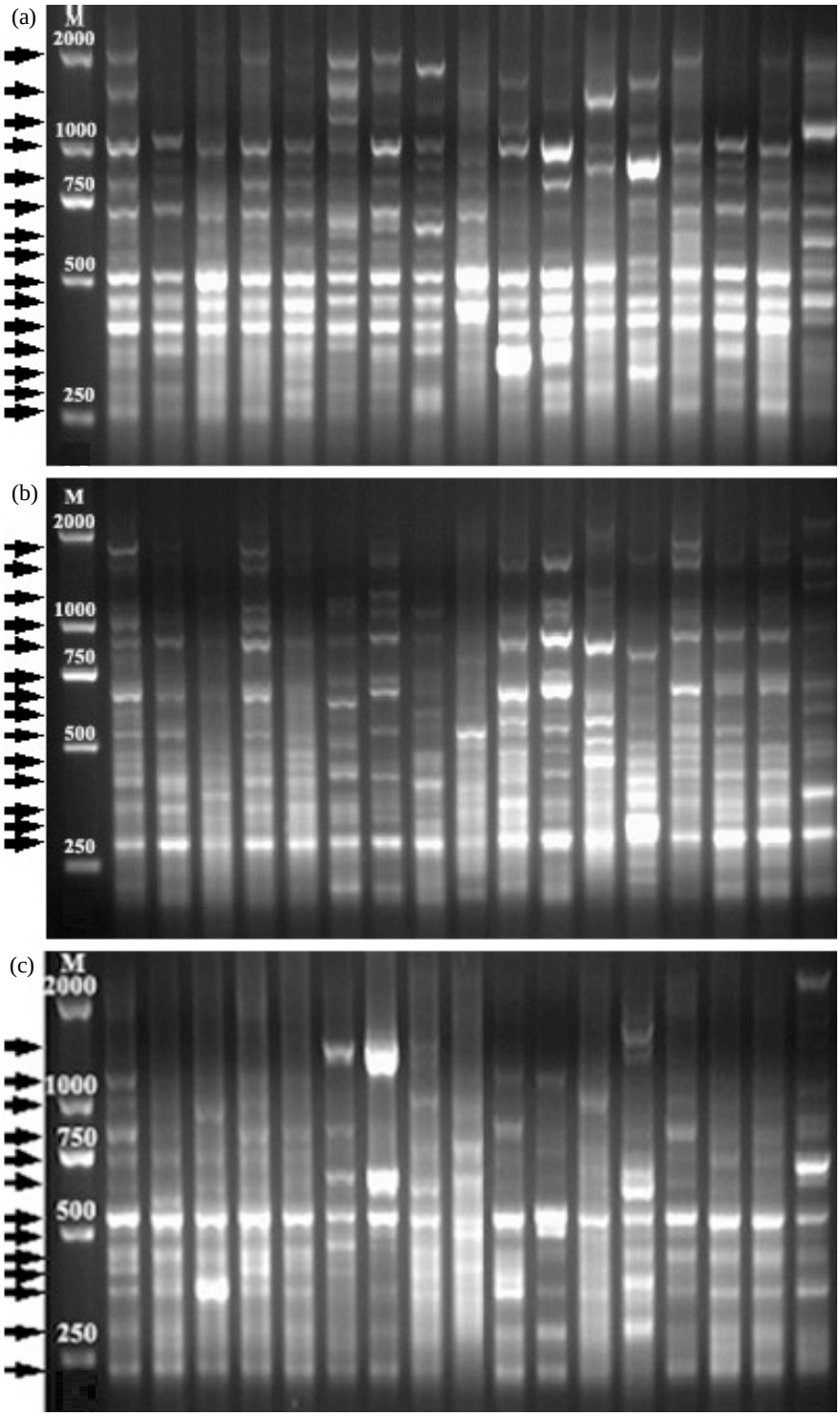

Fig. 2(a-c): ISSR bands of 16 different populations of *H. avenae* and one population of *H. filipjevi* to indicate the selected bands (arrows) for the polymorphism analysis, (a) Primer ISSR2, (b) Primer ISSR10 and (c) Primer ISSR16

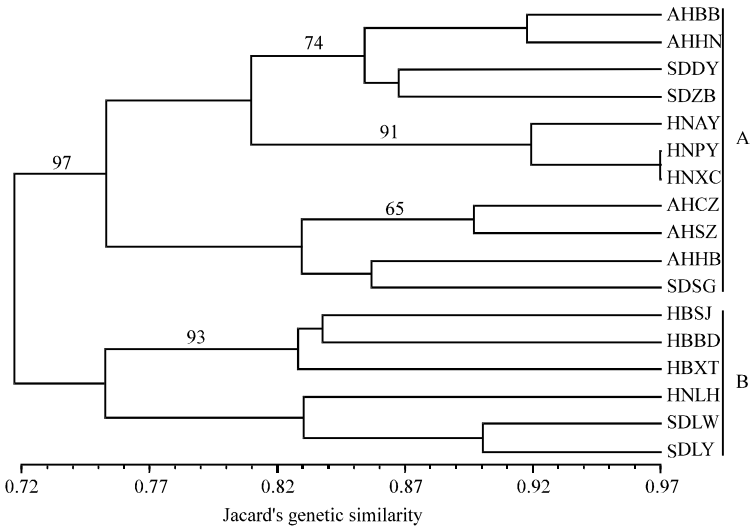

Fig. 3: Phylogenetic relationships among *H. avenae* populations based on their Jacard's genetic similarity values using ISSR markers. UPGMA clustering was constructed using the NTSYS program, version 2.1. Confidence levels over 60% from bootstrap analysis (1000 permutations) are indicated at the nodes. A: Clade A, B: Clade B, Grouped on the basis of Jacard's diversity level of 0.75

locations. The results also showed that the majority of genetic variation (74.8%) occurred within regions, whereas small genetic variation (25.2%) was due to regional differences (Table 3). Values for two hierarchical levels were significantly different.

Sixteen populations of *H. avenae* were grouped into two main clades at the Jacard's diversity level of 0.75 (Fig. 3). Clade A comprises all the populations of *H. avenae* in Anhui and Henan province and three populations in Shandong province. The SDLW, SDLY population and all populations of Hebei province of *H. avenae* were grouped in clade B. Most *H. avenae* populations were grouped in the same clade which was corresponded to their geographic distributions. However, five populations in Shandong province were clearly separated to two different clades.

ITS analysis: The ITS sequences of *H. avenae* and *H. filipjevi* reported here have been deposited in GenBank: HM370406-HM370411, HM370414-HM370420, HM370426-HM370429. The ITS-rDNA sequence length of *H. avenae* was 1045 bp and the ITS-rDNA sequence length of *H. filipjevi* was 1054 bp. Except for ten extra bases existing in *H. filipjevi*, thirty parsimony-informative sites occurred between sequences of *H. avenae* and *H. filipjevi*. The alignment of 16 sequences in *H. avenae* covered 1045 nucleotide positions with 4 polymorphic sites.

With the outgroup of *H. filipjevi*, seventeen equally most parsimonious trees was generated by ME analysis of ITS-rDNA gene alignment. Sixteen *H. avenae* populations were grouped into two main clades, which were distinctively divergent from the *H. filipjevi* population (Fig. 4). The clade I contained all the populations of China and the clade II contained populations from India, Gemany, Morocco and USA. Within clades I, close relationship was

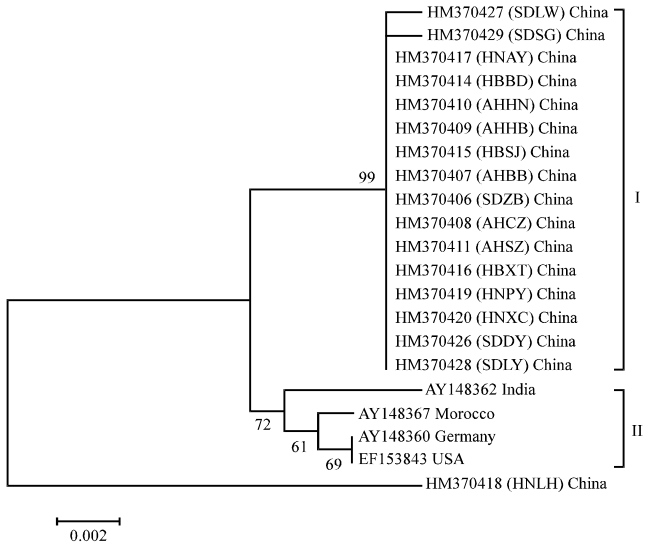

Fig. 4: Phylogenetic relationships with the ITS-rDNA sequences of *H. avenae*. Posterior probabilities greater than 60% are given for appropriate clades. I: Clade I containing all the population of China, II: Clade II containing population of India, Morroco, Germany and USA

observed according to their geographical origin in most subclades. However, SDLW and SDSG were somewhat divergent from other *H. avenae* populations.

DISCUSSION

ISSR results: To our knowledge, this is the first study for the genetic structure of *H. avenae* populations revealed by ISSR markers at a macro geographical level. It was proved that ISSR markers were efficient for detecting genetic variation among the different geographic populations of *H. avenae* in China. At the species level, genetic diversity within population of *H. avenae* was higher than those of *H. glycines*, *H. schachtii* and *Bursaphelenchus xylophilus*, regardless of molecular markers (Zhang *et al.*, 1998; Plantard and Porte, 2004; Madani *et al.*, 2007; Vieira *et al.*, 2007). Genetic similarity index is reliable for the evaluation of genetic variation level among different populations. In the present study, the genetic similarity index ranged from 0.41 to 0.86, which indicated *H. avenae* had high genetic diversity. One possible source of the high genetic polymorphisms found in *H. avenae* populations might be related to the reproduction mode. Because of their small body size, active dispersal is probably limited to a few centimeters or decimeters. Individual male could mate with different females and occasionally several males would attempt to mate with the same female at the same time (Anderson and Darling, 1964). The multiple matings would favor offspring with higher genetic diversity of *H. avenae*. Based on the investigation of Zheng *et al.* (1997) and Peng *et al.* (2003) on the race of *H. avenae*, we speculate there are at least two different pathotypes within *H. avenae* in China. The outcrossing of different pathotypes at the same field will favor the increase of the genetic variation among populations. In addition, the genetic variation may largely result from multiple introductions of *H. avenae* from different geographical origins (Da Conceicao *et al.*, 2003; Plantard *et al.*, 2008). In addition, the nematodes can move only short distances in the soil and have no natural means of long-range movement, gene

flow among populations could be generated by passive transport of nematodes across fields by human activities (e.g. transport of soil by farm machinery, combine harvester and cultivation) or by water (flood, irrigation or drainage) and wind (Meagher, 1977; Plantard and Porte, 2004). These dispersal mechanisms would be favored for the maintenance of highly effective population sizes which contribute to the increase of the genetic diversity of natural populations within this species (Lax *et al*., 2007).

ITS results: Although, ITS repeat units are generally considered to be rapidly homogenized by concerted evolution, the occurrence of intra-specific and intra-individual, ITS polymorphism were reported for *Meloidogyne, Globodera* and *Heterodera* spp. (Szalanski *et al*., 1997; Subbotin *et al*., 2000). Our sequence analysis of ITS-rDNA clone variants revealed low intraspecific variation in rDNA of *H. avenae*. Base transition and transvrersion of rDNA may result from different individuals of the same population or from different operons of the same nematode. A more possible explanation for the observed ITS variation might be related to the presence of several major rDNA gene loci in the genome of *H. avenae* (Madani *et al*., 2007). Since it was demonstrated that intrachromosomal homogenization was faster than interchromosomal recombination (Parkin and Butlin, 2004), the processes of concerted evolution might not have enough time to homogenize rDNA sequences across different loci. In addition, the outcrossing nature of *H. avenae* would also lead to hybridizations between nematodes and subsequently resulted in homogenization of the variants by concerted evolution.

Comparison between ISSR and ITS data: From the results of this study, it was clear that the ISSR molecular marker, which covers multiple loci across the genome, can provide a higher resolution of clustering between the analyzed populations of *H. avenae* at large scale. Based on the ISSR and ITS data we conclude that *H. avenae* showed some degree of differentiation at large geographical scale. High level of variability and gene flow within and between populations of different regions in China was also observed. ISSR and ITS-rDNA dataset grouped most populations into two clusters separately. These results would be confirmed by two different pathotypes or biological entities existing within the species. Given the clear genetic differentiations between these two groups considered, it would be interesting to conduct intensively investigation to make a genetic comparison between these two biological entities.

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