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Morphometric and Genetic Variability Among *Tylenchulus semipenetrans* Populations from Citrus Growing Area in Korea

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***Tylenchulus semipenetrans*, citrus nematode is an important phytopathogenic nematode and responsible for serious damage on citrus. However, little information is available about genetic variability of *T. semipenetrans* among different populations with variation of conventional diagnostic characteristics. In this study, we compared the morphometric and genetic characteristics among different populations. The mature female of *T. semipenetrans* collected in this study had thicker cuticle than those in the previous studies. In comparative sequence analysis of *T. semipenetrans* populations obtained from Jeju in Korea, we observed genetic variations within clones generated from single individuals. To determine whether variability among copies of nuclear ribosomal DNA sequences exists in the genome of *T. semipenetrans*, PCR-RFLP technique from individuals of Korean isolates with *Mse*I and *Msp*I restriction enzymes was used to prove experimentally that all populations have intraspecific variations. Restriction enzyme digestion created several fragments on 3.0% agarose gel corresponding to several haplotypes in all populations, though some populations displayed fragment deletion. The total length of fragments was larger than before digestion, indicating sequence heterogeneity within the genome of *T. semipenetrans*.**

Keywords : heterogeneity, ITS region, morphometrics, rDNA, *Tylenchulus semipenetrans*

The genus *Tylenchulus* Cobb, 1913, belongs to subfamily Tylenchulinae Skarbilovich 1947, of which four species have been described until now. One of *Tylenchulus* species, the citrus nematode, *Tylenchulus semipenetrans* Cobb, 1914, is an economically important pest occurring citrus-growing areas throughout the world, causing yield loss of 10% to 30% depending on the level of damage thresholds (Verdejo-Lucas and Mckenry, 2004). It was first reported on citrus root in California in 1912 and reproduced by parthenogenesis (Cobb, 1914). In USA, according to field survey, more than 50% of citrus orchards were infested by *T. semipenetrans* (Cohn and Duncan, 1990). Therefore, various management strategies to cope with this nematode pest have been progressed including non-infected nursery stock, resistant rootstocks, nematicides and also quarantine measures (Kaplan, 1981; McClure and Schmitt, 1996; Verdejo-Lucas and Mckenry, 2004).

Recently, a rapid movement of agricultural products with an extensive international trading has led to an increased attention to nematodes as having regulatory significance. One of them is the pine wilt nematode, *Bursaphelenchus xylophilus* that has shown a wide dispersal with the movement of timbers contaminated with the nematode. With the increase of global dispersal of pests, new diagnosis methods are required for a rapid and reliable species and/or biotype identification to restrict introduction of the pests (Baines et al., 1974). Ecological and control studies of phytopathogenic nematode species have often required a concurrent analysis of a wide range of taxonomic characteristics. However, the conventional analysis of taxonomic criteria including quantitative and qualitative characters has been laborious, and need highly trained taxonomists with intensive reliance on gross morphological and morphometric characterization. Recently, novel molecular diagnostic techniques provide clues to solve taxonomic problems associated with conventional species identification (Al-Banna et al., 2004; Szalanski et al., 1997). Ribosomal DNA analyses are commonly accepted as alternative methods including polymorphism of restriction enzyme recognition sites (PCR-RFLP), length variation and comparative sequence analysis of rDNA array such as ITS1 and ITS2 (Cherry et al., 1997; Power et al., 1997; Thiery and Mugniery, 1996).

However, barrier in using this genetic region as a taxonomic marker may arise from existence of heterogeneity within the genome of a nematode (Bae et al., 2008; Faten et al., 2002; Zheng et al., 2000). This is probably because of the presence of unique reproduction ability, different location of rDNA within chromosome, or heterozygote. It is important to know genetic variations among and/or within populations to develop resistant varieties, and also

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obtain information on the extant of gene flow. Unfortunately, a wide study about genetic structure of *T. semipenetrans* has not been conducted. The objective of this study was to compare morphometric characteristics with those in the previous reports and study genetic polymorphism based on PCR-RFLP and sequences of ITS region and D1-D3 expansion segments of 28S gene.

Materials and Methods

***Tylenchulus semipenetrans* populations.** Six populations of *T. semipenetrans* were collected from citrus fields in different localities; three populations from the southeast area, one from the west, one from the north and one from the south area in Jeju (Table 1). Nematode samples were collected from citrus fields in 2008.

Measurements of female morphometrics. Individual nematodes were obtained from citrus roots using a dissecting microscope, and the nematodes extracted were fixed and dehydrated in hot (80°C) TAF fixative: (7 ml formalin (40% formaldehyde), 2 ml triethanolamine and 91 ml distilled water) dehydrated by Seinhorst's rapid glycerin method (Hooper, 1986). Identification of specimens followed the *Tylenchulus* species identification key proposed by Inserra et al. (1988). Measurements and drawings were made with a light microscope attached with a drawing tube.

DNA Extraction. Mature females of *T. semipenetrans* were selected for extraction of total DNA. Three individuals from each population were hand-picked and transferred individually into a 0.5-ml- microcentrifuge tube with 0.5-µl RNA- free water. DNA was extracted with RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich Co., St. Louis, MO, USA). Other females collected from the same population were placed in 0.5 ml of 1 M NaCl in a microcentrifuge tube and stored at –80°C for future study.

Sequencing of the ITS region and D1-D3 expansion segments of 28S gene: The sequence variability of nuclear ribosomal DNA (rDNA) internal transcribed spacer region (ITS) and D1-D3 expansion segments was analyzed by polymerase chain reaction (PCR). The whole ITS region including the 3' portion of the 18S gene, the entire ITS1 region, the 5.8S gene, the ITS2 and 5' portion of the 28S gene was amplified using primers Hoc-1f (5'-AACCTGCTGCTGGATCATTA-3') and LSUD-03r (5'-TATGCTTAAGTTCAGCGGGT-3'). The primer Hoc-1f was designed by comparative sequence alignment of *Scutellonema bradys* (Steiner and LeHaw, 1933) Andrassy, 1958 sequence found in the GenBank (AY 271722) and LSUD-03r primer of *Globodera tabacum* (Lownsbery and Lownsbery, 1954) Behrens, 1975 sequence from GenBank (DQ 097515). The primers used to amplify the D1-D3 expansion segments of 28S gene were LSUD-1f (5'-ACCCGCTGAACTTAAGCATAT-3') which was designed using comparative sequence alignment with *Globodera tabacum* (Lownsbery and Lownsbery, 1954) Behrens, 1975 sequence from GenBank (DQ 097515) and LSUD-2r PCR primer (5'-TTTCGCCCCTATACCCAAGTC-3') with *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 sequence from GenBank (AY592993). Amplification was carried out in a thermal cycler with the following protocol: after initial denaturation at 95°C for 3 min, there was 35 cycles of 95°C for 45 s, 57°C for 1 min and 30 s, 72°C for 2 min, and final extension step at 72°C for 10 min. Each reaction included a negative control without a DNA template. After amplification, 10 µl of each reaction product was loaded onto 1.5% agarose gel for electrophoresis at 120V for 50 min and photographed under UV light. PCR products were resolved on a 1% agarose gel. This amplified fragment was purified using the Quantum Prep PCR Kleen Spin Columns (BIO-RAD, USA) and cloned into pGEM-T Easy vector (Promega). XL1-Blue competent cells (Stratagene) were transformed with the ligated DNA, and the resulting plasmid DNA was isolated using Wizard plus SV minipreps DNA Purification System (Promega). Positive clones were obtained through blue/white color selection, and further identified by *Eco*RI restriction digestion at 37°C for 1 h. The Marcrogen DNA sequencing and synthesis facility (Seoul, Korea) sequenced PCR product of the ITS and D1-D3 expansion segments using ABI 3730xl DNA sequencer (PE

Table 1. Populations of *Tylenchulus semipenetrans* used in this study

Sample code	Host	Location	ITS Accession Number	28S Accession Number
JSE12	Citrus	Samdal-ri Seongsan-eup Seogwipo-si, Jeju	FJ969705	FJ969712
JN31	Citrus	Bukchon-ri Jocheon-eup Jeju-si, Jeju	FJ969707	FJ969713
JSE34	Citrus	Samdal-ri Seongsan-eup Seogwipo-si, Jeju	FJ969708	FJ969714
JSE44	Citrus	Pyoseon-ri Pyoseon-myeon Seogwipo-si, Jeju	FJ969709	FJ969715
JW77	Citrus	Jeoji-ri Hangyeong-myeon Jeju-si, Jeju	FJ969706	FJ969711
JS87	Citrus	Sanghyo-dong Seogwipo-si, Jeju	FJ969704	FJ969710

Applied Biosystems, Foster city, CA, USA). DNA sequences were submitted to GenBank as accession numbers FJ969704 to FJ969715.

Restriction enzyme digestion: PCR-RFLP for ITS region was performed with restriction enzymes to verify micro-heterogeneity in the ITS region. Ten μl of PCR product was digested overnight in a total volume of 20 μl using each of the following restriction enzymes according to manufacturer's recommendations (Bioneer): *Eco*RI, *Hae*II, *Mse*I, *Msp*I and *Rsa*I. Restriction fragments were separated in 3% TBE agarose gel (Sigma) along with a 100-bp DNA ladder (Invitogen) to measure fragment size and stained with ethidium bromide. Gels were photographed under UV light.

Results

Morphometric comparison of mature females and males. The general shape of the mature female was a more or less elongate anteriorly but swollen posteriorly (Fig. 1). Mature females of *T. semipenetrans* occurring in Korea were compared with those in Florida (Table 2). The PVSC (postvulval section cavity of body) of Korea was shorter than that of Florida (1.31-5.24 μm vs. 1.8-7.1 μm) and the PVSW (postvulval section width of body) was similar (10.5-14.4 μm vs. 9.1-13.2 μm) each other. The cuticle thickness of *T. semipenetrans* of this study was larger than that of Florida (3.9-9.2 μm vs. 2.9-5.6 μm) and also than that previously reported (2.5-5.5 μm) by Choi (2001). When mature males were compared with the previous report (Choi, 2001), body length was relatively longer (343.97-973.5 μm vs. 330-410 μm), but stylet length was shorter (3.93-7.86 μm vs. 10-12 μm) (Table 3).

Genetic variation. PCR amplification of the ITS region using primers Hoc-1f and Hoc-2r yielded a single PCR fragment of approximately 830 bp in size for all populations. The DNA sequencing of the ITS1, 5.8S, ITS2, with partial 18S and 28S genes displayed no length variations among the different populations examined. Consequently, the determination of the ITS1, 5.8S, and ITS2 regions was conducted by sequence similarity search using BLAST and multiple sequence alignments using known species; the length of the ITS1 was from 424 to 426 bp with 49.7-51.1% GC contents; the length of the 5.8S was 155 bp with 52.2-52.9% GC contents; the length of the ITS2 was from 182 to 183 bp with 53.6-54.45% GC contents. The DNA sequenc-

Table 2. Measurements of *T. semipenetrans* mature females

Character[a]	Present population (n=15)			Florida[b] (1988) (n=25)	
	Range	Mean±SD	CV[c] (%)	Range	Mean
BWV (μm)	13.1-32.75	23.89±4.94	1.38		
PVSC (μm)	1.31-5.24	3.58±1.35	2.52	1.8-7.1	4.3
PVSL (μm)	23.58-44.54	30.92±6.18	1.33	26.5-52.0	40.0
PVSW (μm)	10.48-14.41	12.84±1.23	0.64	9.1-13.2	10.9
VEPD (μm)	11.79-27.51	17.59±4.56	1.85		
CT (μm)	3.93-9.17	6.29±1.50	1.59	2.9-5.65	3.7

[a] BWV; body width at vulva, PVSC; postvulval section cavity of body, PVSL; postvulval section length of body, PVSW; postvulval section width of body, VEPD; vulva excretory pore distance, CT; cuticle thickness
[b] Refer to Inserra et al., 1988b
[c] Coefficient of variation

Table 3. Measurements of *T. semipenetrans* males

Character[a]	Present population (n=13)			Choi (2001)
	Range	Mean±SD	CV (%)	
L (μm)	343.97-973.5	554.60±211.09	38.06	330-410
stylet (μm)	3.93-7.86	6.43±1.24	19.23	10-12
a	29.17-104.04	60.20±26.62	44.21	29-39
b	3.24-9.17	5.70±2.07	36.32	3.28-4.23
c	9.05-30.96	18.13±6.88	37.96	7.89-10.1
Spic. (μm)	9.83-19.00	13.10±2.88	21.96	14-18
Gub. (μm)	2.36-8.38	5.22±2.02	38.77	3-4

[a] L; Body length, a; body length/maximum body width, b; body length/oesophageal length, c; body length/tail length, Spic; Spicule length, Gub; Gubernaculum.

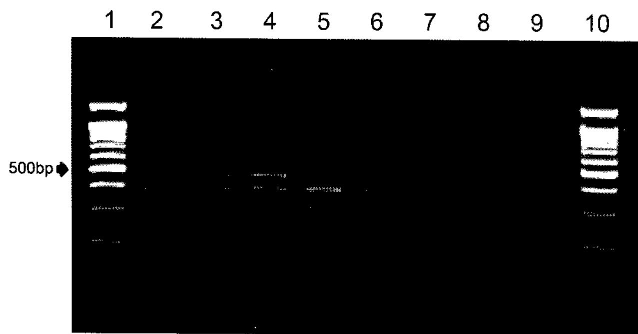

Fig. 1. Restriction fragments variability among populations of *Tylenchulus semipenetrans* (*Mse*I) lane 1 and lane 10, 100 bp ladder, JSE12; lane 2, and lane 3, JN31; lane 4, JSE34; lane 5, JSE44; lane 6, JW77; lane 7, and lane 8, JS87; lane 9.

ing of the D1-D3 expansion segments of 28S gene displayed that it has 690 bp with 55.1% GC contents; the length of D1 is 150 bp with 56% GC contents; the length of D2 is 373 bp with 54% GC contents; and the length of D3 is 167 bp with 58% GC contents. No genetic variation was observed among the six populations for the D expansion segment sequences. However, according to DNA sequence electropherograms, several distinct double peaks in both directions were observed in the D expansion segments of 28S and also after cloning of the ITS product, heterogeneity with multiple bases at some positions within individuals of *T. semipenetrans* was detected. Sequence variations were observed in the ITS1, 5.8S and ITS2 regions within clones obtained from all populations.

To verify heterogeneity existing in the sequences of *T. semipenetrans*, several restriction enzymes were selected. For some of restriction enzymes, the *Eco*RI, *Hae*II, and *Rsa*I digestion profiles were identical one another among all populations and showed no extra digestion fragments (data not shown). Two restriction enzymes, *Mse*I and *Msp*I, however, revealed intraspecific variations existing in the

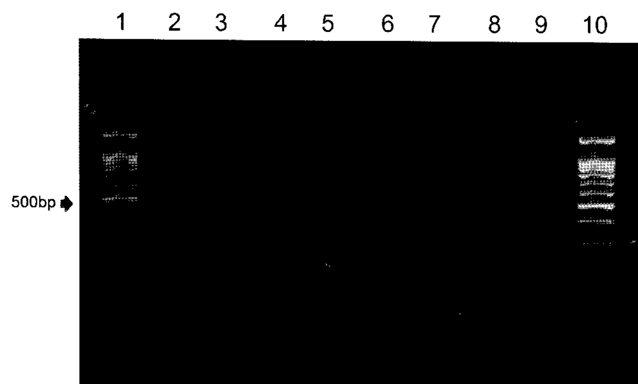

Fig. 2. Restriction fragments variability among populations of *T. semipenetrans* (*Msp*I) lane 1 and lane 10, 100 bp ladder, JSE12; lane 2, and lane 3, JN31; lane 4, JSE34; lane 5, JSE44; lane 6, JW77; lane 7, and lane 8, JS87; lane 9.

genome of *T. semipenetrans*. With enzyme *Mse*I digestion, it yielded four fragments that were shown on two different profiles: the first consisting of 440 bp and 380 bp; the second consisting of 380 bp, 300 bp, and 140 bp (Fig. 1). With enzyme *Msp*I, we also found that *T. semipenetrans* had two profiles corresponding to two haplotypes coexisting in the genome; the first consisting of 215 bp, 209 bp, 152 bp, 134 bp, 67 bp and 43 bp; the second consisting of 392 bp, 209 bp, 152 bp, and 67 bp (Fig. 2). The total lengths of restriction fragments digested with these enzymes were larger than those of undigested PCR products, indicating the existence of heterogeneity. We also observed that the *Mse*I digestion of some Jeju populations displayed 440 bp fragment deletion.

Discussion

The cuticle outgrowths around the excretory pore are unique characteristics of the genus *Tylenchulus* (Inserra et al., 1988a; Van den Berg and Spaull, 1982). A large extent of morphological variability among populations of a species was reported from economically important nematodes (Han et al., 2006; Handoo et al., 2005; Inserra et al., 1996). It is important to know variability of morphometric characters within a taxon to delimit species boundary (Duncan et al., 1998). In the previous studies (Inserra et al., 1996; Duncan et al., 1998), the important criteria of nematode taxonomy, such as body length and width, were influenced by environmental conditions such as, food availability and age of females in the population, or unknown environmental conditions. In our study, some specimens showed that the mean value of cuticle thickness exceed that of previous reports. Therefore, we need to examine more measurements and conduct additional experiments to verify potential environment effects on the morphometric variation.

Regulatory concerns grow higher every year with the increased import of agricultural products. To develop a reliable and rapid nematode diagnostic method is an important step to detect or prohibit introduction of pests from infested areas. DNA sequence variation in the ITS region of the ribosomal DNA is useful to identify nematode taxa. Heterogeneity, however, has been detected from many nematode species through comparative sequence analysis and PCR-RFLP (Bae et al., 2008; Han et al., 2006; Szalanski et al., 1997; Zheng et al., 2000). In this study, ITS-RFLP with *Mse*I, and *Msp*I presented multiple bands exceeding the length of the unrestricted PCR products suggesting the coexistence of two ITS haplotypes in the same genome. These results may be related with reproduction ability of *T. semipenetrans*. According to Hugal et al. (1999), obligatory mitotic (apomictic) parthenogenetic species, *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* showed a high level

of intraspecific genetic variation due to reticulate evolution. A previous study by Faten et al. (2002) also revealed two haplotypes in the genome of *Myzus persicae* and suggested two hypotheses, heterozyotic and gene duplication in another region of the genome. To verify some characters of nematode evolution, we need to obtain and compare multiple sequences from amphimictic and parthneognetic species. In case of some species, the heterogeneity of the ITS region or D expansion segments of 28S gene didn't exist in all populations of the same species. The emergence of heterogeneity within limited geographical populations of same species may suggest existence of new local geographic subtype (Cherry et al., 1997; Szalanski et al., 1997; Zheng et al., 2000). We cannot conclude that the deletion of restriction digestion fragment came from emergence of new regional subtype or intra-individual ITS polymorphism within an individual (Block et al., 1998; Subbotin et al., 2000).

In conclusion, the sequence results obtained from this research presented the first full-size sequence data of the ITS region and partial sequences of D expansion segments of *T. semipenetrans* found in Korea. A number of examinations of many populations collected from distantly located origins should be conducted to calculate exact genetic variation existing within the species and also develop molecular diagnostic techniques.

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