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Genetic variation of *Hoplolaimus columbus* populations in the United States using PCR-RFLP analysis of nuclear rDNA ITS regions[1]

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Abstract: Hoplolaimus columbus is an important nematode pest which causes economic loss of crops including corn, cotton, and soybean in the Southeastern United States. DNA sequences of the ITS1-5.8S-ITS2 region of ribosomal DNA from *H. columbus* were aligned and analyzed to characterize intraspecific genetic variation between eleven populations collected from Georgia, Louisiana, North Carolina, and South Carolina. In comparative sequence analysis with clones from either one or two individuals obtained from the eleven populations, we found variability existed among clones from an individual and that clonal diversity observed from within individuals was verified by PCR-RFLP. PCR-RFLP analysis with *Rsa* I and *Msp* I restriction enzymes yielded several fragments on 3.0% agarose gel that corresponded to different haplotypes in all populations and the sum of digested products exceeded the length of undigested PCR products, which revealed that ITS heterogeneity existed in a genome of *H. columbus*. This indicates that heterogeneity may play a role in the evolution of this parthenogenetic species.

Key words: ITS region, PCR-RFLP, haplotypes, *Hoplolaimus columbus*, lance, nematode.

The Columbia lance nematode, *Hoplolaimus columbus*, (Sher, 1963) has an endoparasitic or semi-endoparasitic relationship with cotton and soybean. The nematode causes severe damage to the cortical parenchyma and to the endodermal-vascular region of the roots of both plants, resulting in the hosts being stunted and chlorotic (Fassuliotis et al., 1968; Lewis et al., 1976). *Hoplolaimus columbus* was first collected in Richland County, South Carolina. *Hoplolaimus* presently has 29 species according to Sher (1963) and Handoo and Golden (1992), or 32 species in three subgenera, *Basirolaimus* (10), *Hoplolaimus* (18), and *Ethiolaimus* (4) according to Siddiqi (2000). In addition to cotton and soybeans, *H. columbus* is an important yield-limiting pest of corn, (*Zea mays* L.), and has a wide host range including weeds, vegetables, and other crops (Fassuliotis., 1974; Nyczepir and Lewis, 1979; Noe, 1993; Koenning et al., 2004).

The mode of reproduction of *H. columbus* is parthenogenesis. Parthenogenetic lineages of animals are generally recognized to be genetically identical and have short-term evolutionary life spans and a distribution pattern known as "geographic parthenogenesis" when compared to sexual lineages (Kearney, 2003). Parthenogenetic species lack the ability to reproduce new genotypes for adaptation to new environmental conditions but have the advantages of rapid colonization and long term preservation of their genotype (Little and Hebert, 1997; Maraun et al., 2003; Castagnone-Sereno, 2006). Other species, however, have shown an "ancient asexual status" that has survived for a long time with proof from fossils (Poinar and Ricci, 1992).

In recent years, nuclear ribosomal DNA (nrDNA) has been widely used for phylogenetic analysis, population genetics, and taxonomic studies in nematology based on sequence variation (Gasser and Hoste, 1995; Al-Banna et al., 1997; Power et al, 1997; Szalanski et al., 1997; Blok et al, 1998; Subbotin et al., 2000; De Luca et al., 2004). Ribosomal DNA is a multiple gene family that contains coding regions for the 18S, 5.8S, and 28S ribosomal subunits, as well as two internal transcribed spacer (ITS) regions, ITS1 and ITS2. Different rates of evolution exist, with coding regions being more conserved than spacer regions (Hillis and Dixon., 1991). Eukaryotic rDNA is arranged in hundreds of tandem repeating gene copies in one or several chromosomal loci with each copy being identical or very similar due to the fact that rDNA does not evolve independently as a result of concerted evolution. This means that a mutation occurring within repeating units will be homogenized through all units (Dover and Coen 1981; Hillis and Dixon., 1991). Concerted evolution results not only in complete homogeneity in different copies of the repeat within a species, but also divergence between species (Dover., 1982; Arnheim., 1983). However, heterogeneity of rDNA within individuals was detected from multiple copies and each mutation was confirmed experimentally using restriction enzyme digestion (Cherry et al., 1997; Hijri et al., 1999; Zheng et al., 2000). According to a previous study by Hugall et al. (1999), it was revealed that the ITS region possessed several genetically distinct ITS groups by using ITS sequence analysis of rDNA in the investigated obligatory mitotic (apomictic) parthenogenetic species and the presence of divergent sequences may be due to recent hybrid origins from genetically distinct species. Voger and Desalle (1994) found sequence divergence in the ITS1 of the Tiger beetle, *Cicindela dorsalis*, and suggested that the presence of two different genotypes in one genome was probably because each region was located on a different chromosome. Ibrahim et al. (1994) obtained two PCR amplification products from within an individual *Aphelenchoides arachidis* and ITS heterogeneity arose from the existence of different size ITS1 repeat regions in the same genome.

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Comparative sequence analysis of the ITS region and D expansion region of the 28S gene within and between populations can provide information on the extent of gene flow which can then be used for the development of resistant varieties, as well as provide information on host-race formation and speciation. So far, no genetic studies evaluating intraspecific variation of ITS region of rDNA in *H. columbus* have been conducted. The objectives of this research were to characterize nuclear rDNA ITS genetic variation within and between populations of *H. columbus* to improve the understanding of the genetic structure of this parthenogenetic species.

Materials and Methods

Hoplolaimus columbus populations: Eleven populations of *H. columbus* were collected from different geographical origins and host plants; six populations from SC, three populations from LA, one population from NC and one population from GA (Table 1). Nematode samples were collected from field soil samples or living specimens in water between 2002 and 2006. Individual adult females were selected for extraction of total DNA.

DNA Extraction: One or two individuals from each population were hand-picked and transferred into an individual 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).

Amplification, cloning and sequencing of the ITS region: The sequence variability of the nuclear ribosomal DNA ITS region was analyzed by polymerase chain reaction (PCR). The amplified region included the 3' portion of the 18S gene, the entire ITS1 region, the 5.8S gene, the ITS2 region, and the 5' portion of 28S gene. The primers for PCR were Hoc-1f (5'-AACCTGCTGCTGGATCATTA-3') and LSUD-03r (5'-TATGCTTAAGTTCAGCGGGT-3'). The primer Hoc-1f was designed by comparative sequence alignment of *Scutellonema bradys* sequence found in the GenBank (AY271722), and the primer LSUD-03r was designed from a *Globodera tabacum* sequence from GenBank (DQ097515). Amplification was carried out in a thermal cycler with the following protocol: after initial denaturation at 95°C for 3 min, there were 35 cycles of 95°C for 45 sec, 57°C for 1 min 30 sec, 72°C for 2 min, and the 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 was loaded onto 1.5% agarose gel (120 V, 50 min) and photographed under UV light.

The PCR product was resolved on a 1% agarose gel. This amplified fragment was purified using the Quantum Prep PCR Kleen Spin Columns (BIO-RAD) and cloned into pDrive cloning vectors (Qiagen, Valencia, CA). The plasmids were transformed into *Escherichia coli* Qiagen EZ Competent cells according to the manufacturer's protocols. Positive clones (White) were obtained through blue/white color selection, and further identified by *Eco R*I restriction digestion at 37°C for 1 hr. The plasmid preparation was extracted and purified using the QIAquick Spin Miniprep Kit (Qiagen, Valencia, CA) and were sent to the University of Arkansas DNA sequencing and Synthesis Facility (Little Rock, AR) for direct sequencing in both directions.

Restriction enzyme digestion: The ITS PCR product was digested with restriction enzymes to verify microheterogeneity in the ITS1 region. Restriction enzymes were from several commercially available varieties chosen from the NEB cutter Software (http://www.neb.com). Eight µl of PCR product was digested overnight in a total volume of 20 µl using each of following restriction enzymes according to manufacturer's recommendations (Promega, and New England BioLabs): *Dde* I, *Eco R*I, *Hae* III, *Hae* II, *Hha* I, *Hinf* I, *Mbo* I, *Msp* I, and *Rsa* I. Restriction fragments were separated using a 3% TBE agarose gel (Sigma) or 6% polyacryamid gel (Invitrogen) along with a 100 bp DNA ladder (Invitogen), and stained with ethidium bromide. Gels were photographed under UV light.

Results

For the ITS region amplified from all eleven populations, each population yielded one fragment of

Table 1. Populations of the Columbia lance nematode, *H. columbus* used in this study.

Sample code	Collection year	Host	Location	GeneBank Accession numbers
LA 67	2003	Corn	Pointe Coupee County, LA	FJ485610, FJ485611, FJ485612, FJ485613
LA92	2003	Cotton	Franklin County, LA	FJ485614, FJ485615, FJ485616
LA94	2003	Cotton	Pointe Coupee County, LA	FJ485617, FJ485618,
SC103	2003	Cotton	Lee County, SC	FJ485619, FJ485620,
GA105	2003	Cotton	UGA research station Midville, GA	FJ485621, FJ485622, FJ485623, FJ485624, FJ485625, FJ485626
SC144	2004	Corn	Dorchester County , SC	FJ485627, FJ485628
SC147	2004	Soybean	Dorchester County , SC	FJ485629, FJ485630, FJ485631
SC195	2005	Cotton	Blackville, SC	FJ485608, FJ485609
SC196	2005	Cotton	Florence, SC	FJ485632, FJ485633, FJ485634
SC198	2005	Soybean	Blackville, SC	FJ485635, FJ485636
NC247	2006	Cotton	Johnston , NC	FJ485637, FJ485638, FJ485639

approximately 1.1 kb in size, suggesting a lack of ITS size polymorphism within and between populations. In general, average nucleotide composition of the ITS region among clones was 24.1% (A), 23.2% (C), 27.2% (G), 25.5% (T), 50.4% (G+C), and 49.6% (A+T). Consequently, the determination of the ITS1, 5.8S, and ITS2 regions were conducted by sequence similarity search using BLAST and multiple sequence alignments using known species. The length of the ITS1 is from 567 to 569 bp, the length of the 5.8S is 158 bp, and the length of the ITS2 is from 233 to 235 bp. We found little or no size variation of rDNA within *H. columbus*, but sequence variation was observed in the ITS1 and ITS2 sequence within clones obtained from all populations. The nucleotide sequences of the Hoc-1f/LSUD-3r PCR product are presented in Fig. 1.

Based on nucleotide sequences variation within and between populations, PCR-RFLP analysis using eleven restriction enzymes that have four base recognition sites (*Hae* III, *Rsa* I, *Mbo* I, *Msp* I, and *Hha* I), five base

```
                                                                               60
                                                HinfI   HinfI                                  HhaI
H.columbus195-2-1           AACCTGCTGC TGGATCATTA CCCACGTGAT TCTGATTCAC CGACTACCTG GTGGGTTTGC
H.columbus195-2-2           .......... .......... .......... .......... .......... ..........
H.columbus105-3-3           .......... .......... .......... .......... .......... ...T......
H.columbus105-2-5           .......... .......... .......... .......... .......... .......A..

                                                                              120
                                             RsaI
H.columbus195-2-1           GCTTTACCAG TATTGTAGGG CGTACCCACC TGCCCTGAGG GTCCGTTTTT GTTGGGGTTG
H.columbus195-2-2           .......T.. .......... .......... .......... .......... ..........
H.columbus105-3-3           .......T.. .....G.AA. ....A..... .......G.. .......... ..........
H.columbus105-2-5           ...C...T.. .......... .......... .......G.. .......... ..........

                                                                              180
                                     HhaI                RsaI
H.columbus195-2-1           TTTTGGCATA GTGAGCGCTC ACTTTCCTCT ACGTACAAAC ACATACGTGT GCCACGAGGG
H.columbus195-2-2           .......... .......... ......A... .......... .......... ..........
H.columbus105-3-3           .......... A......... .......... .......... .......... .....A....
H.columbus105-2-5           .......... .......... .......... ....TT.... .......... ..........

                                                                              240
                          HaeIII
H.columbus195-2-1           TGGCCGGAGG CGAAGAGACC AAGCAAGCTG ATGACTGGCA TTGGCGTCAG GAAGCTGTCC
H.columbus195-2-2           .......... .......... .......... .......... .......... .G........
H.columbus105-3-3           .......... .......... ......T... .......... .......T.. .G........
H.columbus105-2-5           .......... .A...G.... .......... .......... .......... AG........

                                                                              300
                                                           HhaI
H.columbus195-2-1           TATGCACTGA ACAACAGCCC TCGGGGCACA AAACGGCTGC GCTGGTGTCT GTGCGTTGTT
H.columbus195-2-2           .......... .......... .......... .......... .........C ..........
H.columbus105-3-3           .......... .......... .......... .......... .......... ..........
H.columbus105-2-5           .......... .......... .......... .......... .......... ..........

                                                                              360
                                     RsaI
H.columbus195-2-1           GAGCAGTTGT TGTGTACTTT TCCGCGGAGT ATGTGGGTTG AGCATGCTGT CATAGACCTA
H.columbus195-2-2           .......... ....C..... .........A ........GA .......... .........G
H.columbus105-3-3           .......... ....C..... .........A ........GA .......... .........G
H.columbus105-2-5           .......... ....C..... .........A ........GA .......... .........G

                                                                              420
                            HaeIII                                           HhaI
H.columbus195-2-1           AAAGTGGCCC GCTGGGTCGT CTATGTCTTA CAAACCGTAA CAAGGGTGTG CTTAAACCGC
H.columbus195-2-2           .......... .T.....T.. .......... .......... .T........ ...C......
H.columbus105-3-3           .......... .T.....T.. .........G .......... .T........ ...C......
H.columbus105-2-5           .......... .T.....T.. .......... .......... .T........ ...C......

                                                                            480

                          MspI                                            HaeIII
H.columbus195-2-1           GCTCCGGCAC GAATTGTGCG CTACGTCCGT GGCTGCGATG AGATGACGCG GTAGGGCCCG
H.columbus195-2-2           ....T...G. .......C.. .......... .......... .......... ..........
H.columbus105-3-3           ....T...G. .......C.. .......... .......... .......... ..........
H.columbus105-2-5           ....T...G. .......... .......... .......... .......... ..........
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FIG. 1. Restriction enzyme recognition sites and sequence alignments of four different ITS types of *H. columbus*.

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                                                                                  540
                             HhaI          HinfI
H.columbus195-2-1      TGCTGGATTG GCGCGTGGCT TAAGACTCGA TGAGCTCGTG TAGAGAGCCG CCAGCAAACT
H.columbus195-2-2      .......... .......... .......... .......... .......... ..........
H.columbus105-3-3      ....A..... .......... .......... .......... .......... ..........
H.columbus105-2-5      .......... .......... .......... .......... .......... ..........

                                                                       600
                                          RsaI                      EcoRI
H.columbus195-2-1      TTTTTTCACT AACATTTTTT AAAGGTACCA TTGTGTGCTG AATATGATGA ATTCTAGTCT
H.columbus195-2-2      .......... .......... .......... .......... .......... ..........
H.columbus105-3-3      .......... .......... .......... .......... .......... ..........
H.columbus105-2-5      .......... .......... .......... .......... .......... ..........

                                                                       660
                          MboI             MboI
H.columbus195-2-1      TATCGGTGGA TCACTCGGCT CGTAGATCGA TGAAGAACGC AGCCAACTGC GATAAGTAGT
H.columbus195-2-2      .......... .......... .......... .......... .......... ..........
H.columbus105-3-3      .......... .......... .......... .......... .......... ..........
H.columbus105-2-5      .......... .......... .......... .......... .......... ..........

                                                                      720
                                                        HhaI
H.columbus195-2-1      GTGAACTGCA GAAACCTTGA ACACAGAACA TTCGAATGCA CATTGCGCCA TTGGAGTGAC
H.columbus195-2-2      .......... .......... .......... .......... .......... ..........
H.columbus105-3-3      .......... .......... .......... .......... .......... ..........
H.columbus105-2-5      .......... .......... .......... .......... .......... ..........

                                                                      780

H.columbus195-2-1      ATCCTTTGGC ACGCCTGGTT CAGGGTCGTA ATACAAAAAC GCACAGCTAA TGCGTGTATA
H.columbus195-2-2      .......... .......... .......... .......... .......... ..........
H.columbus105-3-3      .......... .......... .......... .......... .......... ..........
H.columbus105-2-5      .......... .......... .......... .......... .......... ..........

                                                                       840
                          MboI                                       MspI
H.columbus195-2-1      TGTTGCGAGA TCAGTGTGCG GATC-AGCTC TGTTCTCGCA TATGTGAAGC GGCTCCGGAG
H.columbus195-2-2      .......... .......... ..A.G...C. .......... .......... ..........
H.columbus105-3-3      .......... .......... ..A.G...C. .......... .......... ..........
H.columbus105-2-5      .......... .......... ..A.G...C. .......... .......... ..........

                                                                       900
H.columbus195-2-1      CAGTGGTTGG AGTGCTGGCA TGGACATCCG TATGCTTTCG TCCATGTCTT ACAAACCGTA
H.columbus195-2-2      .......... .......... .......... .......... .......... ..........
H.columbus105-3-3      .......... .......... .......... .......... .......... ..........
H.columbus105-2-5      .......... .......... .......... .......... .......... ..........

                                                                       960
                         RsaI
H.columbus195-2-1     ACTAGGGTAC GACAATCACT CTCT-AAGGC AGCCAAATGT CTCGTCAGCC AACATGTGTC
H.columbus195-2-2     .......... .......... ....T..... .......... .......... ..........
H.columbus105-3-3     .......... .......... ....T..... .......... .......... ..........
H.columbus105-2-5     .......... .......... ....T..... .......... .......... .........G

                                                                    1020
                        HaeIII                                 DdeI      RsaI
H.columbus195-2-1     GCGTTGGCCG AGCATTTATG CATACTGTTG ATTTTTCGAC CTGAACTCAG ACGTGAGTAC
H.columbus195-2-2     .......... .......... .......... .......... .......... ..........
H.columbus105-3-3     .......... .......... .......... .......... .......... ..........
H.columbus105-2-5     .......... .......... ..C....... .......... .......... ..........

                       1026
H.columbus195-2-1     CCGCTG
H.columbus195-2-2     ......
H.columbus105-3-3     ......
H.columbus105-2-5     ......
```

FIG. 1. Continued.

recognition sites (*Dde* I, and *Hinf* I) and six base recognition sites (*Eco*R I, and *Hae* II), were conducted to evaluate within-population variability in the ITS region of rDNA from the eleven populations of *H. columbus*. These restriction enzymes were selected by the Web cutter program (http://www.neb.com), showing several restriction sites for available enzymes. The results of restriction enzyme digestion including *Dde* I, *Eco R*I, *Hae* II, *Hae* III, *Hha* I, *Hin*f I, and *Mb o*I revealed that each enzyme gave identical digestion profiles for all populations. No sequence variation among populations was detected from the sites recognized by these enzymes. However, PCR-RFLP analysis with *Msp*I and *Rsa*I revealed that ITS heterogeneity existed in a population of *H. columbus* (Figs. 2 and 3). The digestion with *Rsa* I yielded eight fragments on 3.0% agarose gel corresponding to four haplotypes in all populations and the sum of digested products exceeded the length of undigested PCR products, which showed heterogeneity in the ITS region of each population and different ITS types existing in a single genome (Fig. 2). The first haplotype consisted of 412 bp, 342 bp, 110 bp, 82 bp and 72 bp. The second consisted of 412 bp, 342 bp, 154 bp, and 110 bp. The third consisted of 342 bp, 256 bp, 150 bp, 110 bp, 82 bp, and 72 bp. The fourth consisted of 484 bp, 342 bp, 110 bp, and 82 bp. With the enzyme *Msp* I, we also determined that *H. columbus* has three profiles corresponding to three haplotypes coexisting in the genome. The first consisted of 651 bp, 190 bp, and 185 bp. The second consisted of 478 bp, 190 bp, 185 bp, and 173 bp. The third consisted of 411 bp, 240 bp, and 185 bp. When resolved on 3% agarose gel, the 478 bp and 411 bp fragments were not obvious for some populations (Fig. 3). To eliminate the possibility that the differing band intensities came from partial digestion, we extended digestion periods from 1 hour to 24 hours with all repetitions, yielding identical results.

Discussion

The results obtained in this research present a genetic analysis of the rDNA ITS region of *H. columbus* populations found in the southeastern United States. In this study, considerable intraspecific variation was discovered in ITS1 sequences obtained from populations of *H. columbus*. This result was confirmed by previous reports that the spacer region has higher nucleotide substitution rate than rDNA genes (Hillis and Dixon, 1991; Ferris et al., 1995; Blok et al., 1998). In a sequence comparison between the ITS1 and ITS2 regions among populations, the ITS1 showed more nucleotide variation than the ITS2. Ferris et al. (1994) found that the maximum sequence dissimilarity between populations of *Heterodera avenae* is 5.3% in the ITS1 but 1.9% in the ITS2. We found that each individual from eleven populations possessed several unique ITS1 sequence patterns. ITS1 heterogeneity has been reported in many nematodes, insects, and fungi. (Vogler and Deselle, 1994; Cherry et al., 1997; Szalanski et al., 1997; Hijri et al., 1999; Hugall et al., 1999; Subbotin et al., 2000; Ko et al., 2002) and it has coexisted in the 18S and 5.8S genes (Carranza et al., 1996; Hosny et al., 1999). ITS1 heterogeneity has been reported either in all populations, as seen in our investigation, or in limited geographical populations within the same species (Cherry et al., 1997; Szalanski et al., 1997). The limited geographical distribution of a certain genotype may indicate the advent of a new local geographic subtype. Of the nine restriction enzymes used for intraspecific variation assays, two enzymes, *Rsa* I and *Msp* I, detected ITS heterogeneity. PCR-RFLP showed that there were differences in band intensities within RFLP profiles, suggesting ITS polymorphism within an individual (Block et al., 1998; Body et al., 2000; Subbotin et al., 2000). In phylogenetic analysis, the difference of copy numbers at single site of repetitive rDNA between populations may result in incorrect evolutionary history. Therefore, the determination of the copy number of *H. columbus* in the ITS region of rDNA is important to the estimation of nucleotide sequence variation within populations and accurate phylogenetic analysis. We also observed identical sequences in different individuals from different populations. This was probably because of the limited amount of DNA sequence data used to analyze the genetic structure of *H. columbus*.

In parthenogenetic species, genotype diversity is more significant than gene diversity because the genetic diversity is distributed among clonal lineages. According to previous studies, multiple copies existed in the rDNA and were recognized to not evolve independently (Arnheim et al., 1980; Dover and Coen, 1981). This means that by molecular turnover mechanisms,

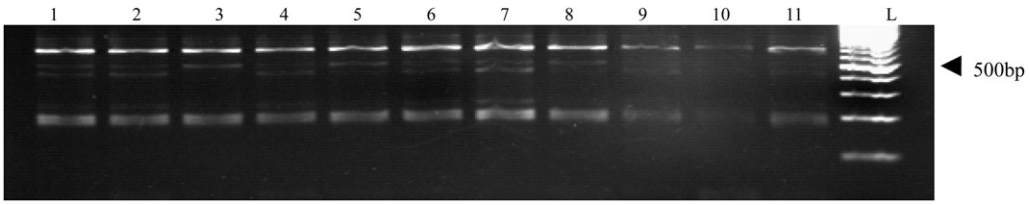

Fig. 2. Restriction fragments variability among populations of *H. columbus* (*Msp*I). lane 1; LA67; lane 2, LA92; lane 3, LA94; lane 4, SC103; lane 5, GA105; lane 6, SC144; lane 7, SC147; lane 8, SC195; lane 9, SC196; lane10, SC198; lane 11, NC; L, 100bp ladder.

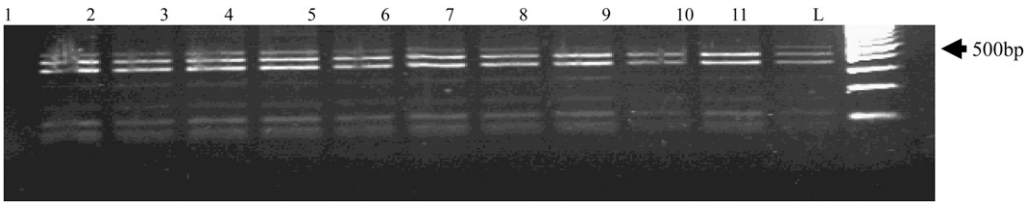

FIG. 3. Restriction fragments variability among populations of *H. columbus* (*Rsa* I). lane 1; LA67; lane 2, LA92; lane 3, LA94; lane 4, SC103; lane 5, GA105; lane 6, SC144; lane 7, SC147; lane 8, SC195; lane 9, SC196; lane10, SC198; lane 11, NC; L, 100 bp ladder.

such as gene conversion and unequal crossing over, each copy of an rRNA array was homogenized within a short period of evolutionary time. This phenomenon is called concerted evolution (Hillis and Dixon, 1991). However, Hillis et al. (1991) proposed that there is a different process for concerted evolution between parthenogenetic and sexual species as one of molecular mechanism responsible for homogenization of multiple copies is restricted in parthenogenetic species. We found that each individual in examined populations possessed several ITS sequence types and this heterogeneity is a potential obstacle for use of this region as a DNA marker. The findings of microheterogeneity from each of eleven populations in *H. columbus* suggested that there are possible explanations such as PCR error, duplication of rDNA in another genome region, or hybridization events.

The ITS sequence of ribosomal DNA has been used to infer phylogenetic relationship and as molecular marker for species discrimination. However, amplification of a paralogous gene from the ITS region can result in an inaccurate estimation of species relationship and genetic distance among species or isolates. This can also result in the discrepancy of gene phylogenies when compared with different genetic markers such as mtDNA (Szalanski et al., 1997; Ko and Jung., 2002). To explain this result, comparisons of more genetic profiles from parthenogenetic lineages and sexual species, cytological aspects, and in-situ hybridizations are required to prove the origin of different ITS variants.

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