**Repetitive DNA sequence families in *Hemitaxonus*
minomensis and *H. athyrii*
(Hymenoptera; Tenthredinidae)***

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ABSTRACT

Families of the repetitive DNA sequences from *Hemitaxonus minomensis* and *H. athyrii* were characterized. pHMS family and pHME family in *H. minomensis* consist of tandemly arranged arrays whose basic repeat units are 260 bp and 330 bp, respectively. pHAE family in *H. athyrii* consists of a tandemly arranged array whose basic repeat unit is 330 bp. pHMS family and pHME family occupy approximately 4.8% and 0.07% of the genome of *H. minomensis*, respectively. By contrast, in *H. athyrii*, pHAE family comprise 0.04% of the genome. Nucleotide sequence comparison of these three repetitive families showed very little homology. Southern blot hybridization using six species of *Hemitaxonus* showed that these repetitive families are species specific.

1. INTRODUCTION

Tandemly arranged repetitive DNA sequences constitute a substantial portion of the eukaryotic genome. These sequences, sometimes referred to as satellite DNA, have been found in varieties of plant and animal species in association with constitutive heterochromatin (Brutlag, 1980; Singer, 1982). Remarkable variation is observed between highly repetitive DNA sequences from different species, indicating a fast rate of evolution and dynamic component of the eukaryotic genome. Sequence comparisons between different repetitive DNA sequences from the same species indicate that sequence divergence have occurred through a random mutation or some unknown amplification mechanism (Brutlag, 1980; Singer, 1982). Much of the evolutionary history of eukaryotic genomes reflects

* The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under accession numbers of pHMS2, pHME7 and pHAE2 are D38084, D38085 and D38086, respectively.

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the turnover of repetitive sequences, and speciation may be a consequence of such turnover (Rose and Doolittle, 1983).

In the previous study, we reported a family of tandemly repetitive sequences from a sawfly, *Hemitaxonus japonicus* (Sonoda et al., 1992). The repetitive sequences consist of the exact multiples of a basic repeat unit, having a length of 270 bp with AT rich sequences. Only a sibling species, *H. sasayamensis*, has the homologous repetitive sequence.

Twelve species of *Hemitaxonus* exist in the Old World and they can be split into four species groups which respectively possess some unique features in morphological, ecological and cytological aspects (Naito, 1971). Each species group possesses sibling species, and therefore *Hemitaxonus* is an important material for insect genetic study. However, little work has been done on its genome structure and function at the molecular level. With the exception of Sonoda et al. (1992), there is no reports on any repetitive sequences from the genome of *Hemitaxonus*.

In this study, we report the cloning and characterization of the families of three repetitive DNA sequences from *H. minomensis* and *H. athyrii*.

2. MATERIALS AND METHODS

Insect materials

Repetitive DNA sequences analyzed in this study were cloned from *H. minomensis* and *H. athyrii*. In addition to the two species, *H. japonicus*, *H. sasayamensis*, *H. struthiopteridis* and *H. melanogyne* were used for Southern blot hybridization analysis. The taxonomical relationship deduced by Naito (1971) was shown in Table 1.

Table 1. List of *Hemitaxonus* species used in this study

Species group	Species
paucipunctatus	<i>Hemitaxonus melanogyne</i>
struthiopteridis	<i>H. japonicus</i>
	<i>H. sasayamensis</i> □ a
	<i>H. struthiopteridis</i>
minomensis	<i>H. minomensis</i>
	<i>H. athyrii</i>

a. Sibling species.

Genomic DNA isolation and cloning of the repetitive DNA sequences from H. minomensis and H. athyrii

High molecular weight DNA was prepared from the final instar larvae of these sawflies, according to Sambrook et al. (1989).

After digestion with restriction endonucleases, *EcoRI* or *SacI* for *H.*

minomensis and *EcoRI* for *H. athyrii*, 10 μ g of the digests were fractionated on a preparative 1.0% agarose gel. Following ethidium bromide staining, visible bands corresponding to fragments of about 260 and 330 bp in *H. minomensis* and 330 bp in *H. athyrii* were cut out and the DNA were eluted using Prep-A-Gene Kit (Bio Rad) according to the manufacturer's recommendation. The resulting DNA was ligated into the *EcoRI* or *SacI* site of the plasmid vector pBluescript SK- (Stratagene), using *Escherichia coli* strain XL1-blue as a host. Recombinant clones were selected on ampicillin (50 μ g/ml) plates containing X-gal and IPTG.

Plasmid DNA was extracted by SDS-alkaline lysis method (Sambrook et al., 1989). The inserts of the three recombinant plasmids, pHMS2, pHME7, and pHAE2, were used as probes in the subsequent experiments (see RESULTS AND DISCUSSION).

Southern and slot blot hybridizations

All restriction enzymes were used according to the manufacturer's recommendations. Completely digested DNA were fractionated on 1.0% agarose gels in TBE buffer (0.09 M tris base, 0.09 M boric acid, 0.002 M EDTA, pH 8.0). After ethidium bromide staining, DNA fragments were transferred onto Hybond-N+ filters (Amersham), followed by Southern blot hybridization (Southern, 1975). Filters were prehybridized at 42°C for 2 h or longer in 6 $\times$ SSC (0.9 M NaCl, 0.09 M sodium citrate), 50% formamide, 0.5% SDS, 5 $\times$ Denhardt's solution (0.1% BSA fraction V, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400), and 0.1 mg/ml of sonicated herring DNA. Hybridization was carried out by the addition of ³²P-labeled probes and incubated for 12 h. DNA was labeled using Random Primed Labeling Kit (Boehringer). After hybridization, the membrane was washed twice with 2 $\times$ SSC, 0.1% SDS at room temperature followed by washing with 1 $\times$ SSC, 0.1% SDS at 60°C. After drying, the membrane was exposed to X-ray film (KODAK O-MAT) for autoradiography.

Slot blots were obtained by spotting variable amounts of denatured genomic DNA onto Hybond-N+ filters; denatured pHMS2, pHME7, and pHAE2 were used as positive controls. Prehybridization and hybridization conditions and washes were those used for genomic Southern blot hybridization and the specific radioactivity was determined using FUJIX BAS 2000 System.

DNA sequencing

The nucleotide sequences were determined by chain termination method (Sanger et al., 1977) using Sequenase Version 2.0 (United States Biochemical). Sequence data were analyzed using GENETYX (Software Development).

3. RESULTS AND DISCUSSION

Molecular structure of repetitive sequences

When genomic DNA from *H. minomensis* was digested with *Sac*I, fractionated on 1.0% agarose gel and stained with ethidium bromide, prominent bands of 260 bp and the multimers were observed. *Eco*RI digests of *H. minomensis* and *H. athyrii* also showed bands of 330 bp. The three bands were cut out and their DNA cloned in the plasmid vector pBluescript SK⁻. From each experiment we selected clones for subsequent analysis: pHMS2 and pHMS3 contained 260 bp *Sac*I fragments from *H. minomensis*, pHME7 contained a 330 bp *Eco*RI fragment from *H. minomensis*, pHAE2, pHAE3 and pHAE7 contained 330 bp *Eco*RI fragments from *H. athyrii*.

To determine whether the cloned repetitive DNA sequences were dispersed or tandemly repetitive sequences, genomic DNA of *H. minomensis* and *H. athyrii* was examined by Southern blot hybridization (Fig. 1). pHMS2 insert hybridized to multimers of a basic 260 bp unit that were generated by digestion with *Sac*I, *Dra*I, and *Alu*I, indicating that the cloned DNA belonged to a tandemly repetitive DNA sequences. The strongest intensity of the hybridization bands was at dimer (520 bp) position and the band intensity gradually declined with an increase of the length. Signals on the *Sau*3AI pattern did not correspond to the basic 260 bp unit because, as shown below by sequencing, most units have more than one *Sau*3AI site. Hybridization patterns of the *Eco*RI and *Ssp*I digests revealed that a fraction of the homologous DNA remained undigested, since DNA bands corresponding to high molecular weight multimers which showed nearly the same mobility as undigested DNA were observed (Fig. 1). This signifies the existence of certain subfamilies that have escaped from homogenization which occur through mechanisms such as gene conversion and unequal crossing-over.

Hybridization pattern with ³²P-labeled pHME7 insert is shown in Fig. 2. *Eco*RI internally cleaved all the repeat units, resulting in a monomeric unit of approximately 330 bp and the respective dimer and trimer. The band intensity gradually declined with an increase of the length. This hybridization pattern is indicative of a tandemly arranged repetitive DNA family. *Sac*I digests revealed the hybridization pattern showing that the majority of homologous DNA remained undigested. Signals on the *Ssp*I pattern did not correspond to the basic 330 bp unit because, as shown below by sequencing, most units have more than one *Ssp*I site. *Dra*I, *Sau*3AI, and *Alu*I digests displayed deviations of the regular ladder pattern. These hybridization patterns appear to represent the repeat units within the array that contained some RFLP (restriction fragment length polymorphism).

pHAE2 insert hybridized to multiple genomic *Eco*RI and *Sau*3AI fragments from *H. athyrii* DNA that were multimers of a basic 330 bp unit, indicating that the cloned DNA also belonged to a tandemly repetitive DNA sequences (Fig. 3).

Fig. 1. Southern blot analysis of *H. minomensis* genomic DNA digests with different restriction enzymes using the insert of pHMS2 as a probe. Genomic DNA extracted from *H. minomensis* were digested with restriction enzymes (lane 1, *Eco*RI; 2, *Ssp*I; 3, *Sac*I; 4, *Dra*I; 5, *Sau*3AI; 6, *Alu*I), size-fractionated on 1.0% agarose gel, blotted on Nylon membrane and hybridized with the probe. The numbers at the far right indicate the size of DNA fragments in bp.

Fig. 2. Southern blot analysis of *H. minomensis* genomic DNA digests with different restriction enzymes using the insert of pHME7 as a probe. Genomic DNA extracted from *H. minomensis* were digested with restriction enzymes (lane 1, *Eco*RI; 2, *Ssp*I; 3, *Sac*I; 4, *Dra*I; 5, *Sau*3AI; 6, *Alu*I), size-fractionated on 1.0% agarose gel, blotted on Nylon membrane and hybridized with the probe. The numbers at the far left indicate the size of DNA fragments in bp.

Fig. 3. Southern blot analysis of *H. athyrii* genomic DNA digests with different restriction enzymes using the insert of pHAE2 as a probe. Genomic DNA extracted from *H. athyrii* were digested with restriction enzymes (lane 1, *Eco*RI; 2, *Ssp*I; 3, *Sac*I; 4, *Dra*I; 5, *Sau*3AI; 6, *Alu*I), size-fractionated on 1.0% agarose gel, blotted on Nylon membrane and hybridized with the probe. The numbers at the far left indicate the size of DNA fragments in bp.

The strongest intensity of the hybridization band was at monomer (330 bp) position and the band intensity gradually declined with the increase of the length. In *Eco*RI digests, in addition to the main ladder of fragments based upon the 330 bp monomer, a minor ladder with a different size was observed. Furthermore, *Eco*RI, although digesting largely within the repetitive family to bring about monomer and low-size multimers, shows a subset of the repetitive family that remains undigested as relic DNA. *Ssp*I, *Sac*I, *Dra*I, and *Alu*I digests also revealed the hybridization patterns that a fraction of the homologous DNA remained undigested. These also signify the existence of certain subfamilies that have escaped from homogenization as are the cases in *H. minomensis*.

Slot blot analysis allowed us to estimate the amounts of pHMS and pHME families within the *H. minomensis* genome and that of pHAE family within the *H. athyrii* genome. Fifty ng of *H. minomensis* genomic DNA gave about the same signal as 30 ng of entire pHMS2 plasmid as a control. Since pHMS2 insert is 8% of the entire plasmid, we concluded that pHMS family represented about 4.8% of the genome of *H. minomensis*. Using the same procedure and calculations, it was estimated that pHME family comprises about 0.07% of the genome of *H. minomensis* and pHAE family comprises about 0.04% of the genome of *H. athyrii*.

Sequence analysis of the clones

pHMS family, represented by the clones pHMS2 and pHMS3, was composed of a repetitive DNA sequences with a length of 255 bp and 254 bp, respectively (Fig. 4A). These repetitive sequences showed a homology of 93% with average AT content of 69%. Sequence comparison of the two repetitive sequences implicated that variations might have been occurred due to point mutations and deletions. The repetitive sequence in pHMS2 could be further divided into two smaller sub-domains between nucleotides 1 and 128 and 129 and 255 with 78% homology (Fig. 5A). Repeat units of the clones, pHMS2 and pHMS3, showed much higher homology (93%) than between two adjacent internal repeats (78% in pHMS2, 76% in pHMS3). We consider, with respect to the evolution of pHMS family, that the repeat unit might probably have been arisen by duplication and the divergence of an ancestor unit with approximately 130 bp. An inverted subrepeat in pHMS family is also shown in Fig. 4A.

The pHME (Fig. 4B) and pHAE (Fig. 4C) families, represented by the clones pHME7 and pHAE2, pHAE3 and pHAE7, respectively, are also rich in AT sequences. pHME7 insert is 334 bp long that contains 68% AT. The repetitive sequences in pHAE2, pHAE3, and pHAE7 are 329 bp fragments with AT rich (65%) sequences. pHME7 insert contains imperfect direct repeats as shown in Fig. 4B. pHAE family represented by three cloned inserts showed average homology of 97%. The 329 bp insert of pHAE2 contains a large internal subrepeat of 98 bp between nucleotides 17 and 114 and 197 and 294 with 76% homology (Fig. 5B). No inverted repeats were observed in both families.

A

pHMS2 CATTTTGGCG GAAATCACC TATTTTGAC CGAATTTTGG ACAATTTTTT TTTTGCCTA 60
 pHMS3 *****T *****A *****A* -----***** 59

 pHMS2 AAAACGACCA TATCTTGGCT TCTATCAATC GTACGTTATT TTTATCCATA TTTTCGGTTT 120
 pHMS3 ***** *****A ***** ***** **C***** ***** 119

 pHMS2 TGTGAGATCA TTTTGACATA GAATCACCCA TTTTGTACTG AATTTTGTAC ATTTTGTATT 180
 pHMS3 ***** ***** ***** ***** **CAAA*T T***** 179

 pHMS2 TTTTCTAAAA ATGATCATAT CTTGCTCCT GTAGATTCTGA CGTCATTTC AACTATATTT 240
 pHMS3 ***C***G** *C*CT*** ***** ***** *****T ***** 239

 pHMS2 TCGGTACTTT GAGCT
 pHMS3 ***** *****

B

AATTCTGTAT TTTCTCAGAA TAGACTCTGA AAAATGGAAT CGCAATACCG CTATTTTATG 60
 CAAATCAATC TTGTGGATTC GAGGACGTTA GTAAAATAAT GTGAGAGCAA TAAAAAAAAG 120
 AAATCAATGA TTTCATACAT TTTCAATTTA TGCAATTTTT TTTTTCACGA AAATATTCCG 180
 AGGAATGGGT TTGTGACGAC ACTTTCTCAT GAAGAATGAT TCCAGTAATT CGAAAATATG 240
 ATCAAAATCA CCGTACGACC AAAAGAAGCT GAGTATTCGA TGATTTCATG TCTTTTCGTA 300
 TTTACGCAA AATATTTCAA AAAATGGGTC GCTG

C

pHAE2 AATTCTATCG CCTATAGGTG TTTCTTATGC AGAATTATCT CAGGAATGCA ACAAACCGAG 60
 pHAE3 ***** ***** ***** *****C ***** G*****
 pHAE7 ***** ***** ***** *****C *****

 pHAE2 TCTCATTGTT GTGCGATACT CAGGGAATAA GATATCGACA ATTTTCAGGA TTCTCGATT 120
 pHAE3 ***** ***** **C***** ***** *****
 pHAE7 ***** ***** ***** ***** *****

 pHAE2 TCGCAAAAT CATAAGGGT TAGCCTTACT TTTTTCGA CGAAAAAA AGTTGACTGG 180
 pHAE3 ***** ***** ***** C***** ***G***** **T***
 pHAE7 ***** ***** ***** ***** ***** **T***

 pHAE2 AATTTATTTT TTTCCGGGTG TTTCTTGTAG AGAATAATCT CACGAATCCA AAAACGCG 240
 pHAE3 ***** ***** ***** **C*G**** *****
 pHAE7 ***** ***** ***** ***G**** ***G*G** *****

 pHAE2 TCTCGTTGTT GTGCGATAAT TAGGAGAAAA GATATTCAAG ATTCAGTAA TTTTATTATT 300
 pHAE3 ****A***** ***** ***** ***** **T****C* *****
 pHAE7 ****A***** ***** ***** ***** *****C* *****

 pHAE2 TTTCTAAAA TCGGAGAAAT GTAGGTCAG
 pHAE3 ***** *****
 pHAE7 ***** *****

Fig. 4. Nucleotide sequences of the inserts of pHMS2 and pHMS3 (A), pHME7 (B), and pHAE2, pHAE3 and pHAE7 (C). Direct and inverted repeats are shown by solid and broken arrows, respectively. Identical nucleotides are represented by asterisks. A dash indicates a deletion.

A

pHMS2 H=78.1%

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1 CATTTCGCGG GAAAATCACC TATTTTGAC CGAATTTTGG ACAATTTTTT TTTTGCCTA
129 *****AT A*G***** C***** T*****T* *-***** *****TT**

61 AAAACGACCA TATCTTGGCT TCTATCAATC GTACGTTATT TTTATCCATA TTTTCGGTTT
188 ****T**T** *****C*** C**G*AG**T CG***C*** **C*A*T*** *****AC

121 TGTGAGAT
248 *T****C*
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B

pHAE2 H=75.5%

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17 GGTGTTTCTT ATGCAGAATT ATCTCAGGAA TGCAACAAAC CGAGTCTCAT TGTGTGCGCA
197 ***** G*AG*****A *****C*** *C***** GC*****G* *****

78 TACTCAGGGA ATAAGATATC GACAATTTTC AGGATTCT
257 **A*T***AG *A*****T C*AG***CA* T*A***T*
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Fig. 5. Sequence comparisons between two internal subrepeats from pHMS2 (A) and pHAE2 (B) inserts. The percentage homology (H) is given for each alignment.

The nucleotide sequences of the three families analyzed above did not show any significant homology among them, indicating that they represented different repetitive DNA families. Including a family of tandemly repetitive sequences from *H. japonicus* (Sonoda et al., 1992), no nucleotide sequences with significant homology were identified by a EMBL Data Library search. Despite the difference in base constitution, the size of the repeat units is strongly conserved between different repetitive families from different sawfly species. The conservation of the size of the repeat unit among sawfly species implicates the hypothesis that these repetitive sequences may have some common property. The size of the repeat units from the alphoid satellite and from several other plant satellites is conserved in between 150 and 180 bp (Brutlag, 1980; Schmidt and Heslop-Harrison, 1993). In these cases, there exists a striking similarity between the size of the repeat unit and the size of the DNA in a chromatin nucleosome containing tandemly repetitive sequences, suggesting their involvement in determining chromatin structure (Grellet et al., 1986; Martinez-Zapater et al., 1986; Schmidt and Heslop-Harrison, 1993).

Evolution and distribution in the genus

To determine whether sequences homologous to pHMS and pHME families from *H. minomensis* and pHAE family from *H. athyrii* were also present in other related species, the genomic DNA from *H. minomensis*, *H. athyrii*, *H. japonicus*, *H. sasayamensis*, *H. struthiopteridis*, *H. melanogyne* were digested with *Sac*I (for pHMS family) or *Eco*RI (for pHME and pHAE families), size-fractionated on 1.0% agarose gel and hybridized with ³²P-labeled probes of the three representative repetitive sequences. The results showed a lack of sequences homologous to

Fig. 6. Presence of the repetitive DNA sequences in the genus *Hemitaxonus* genomic DNA. The genomic DNA from *H. minomensis*, *H. athyrrii*, *H. japonicus*, *H. sasayamensis*, *H. struthiopteridis* and *H. melanogyne* were used for Southern blot analysis. (A) *Sac*I digests probed with pHMS2 insert. (B) *Eco*RI digests probed with pHME7 insert. (C) *Eco*RI digests probed with pHAE2 insert. The numbers at the far left indicate the size of DNA fragments in bp.

the three families in any of the related species (Fig. 6). It is considered that tandemly repetitive DNA sequences occur through mechanisms such as rolling circle replication or unequal crossing over, and diverged rapidly by accumulation of mutations or through process such as conversions and crossing over (Singer, 1982; Halldén et al., 1987). The function of repetitive DNA sequences is highly speculative. The association of tandemly repetitive sequences with centromeric and telomeric heterochromatin has led to a suggestion that they might play a role in chromosomal pairing (Flavell, 1982; Rose and Doolittle, 1983). On the other hand, Orgel and Crick (1980) suggested that repetitive sequences are parasitic element and have no meaningful function.

Analyses of *H. minomensis* and *H. athyrrii* in this study and *H. japonicus* (Sonoda et al., 1992) revealed the presence of families of tandemly repetitive sequences whose repeat units are similar in size but consist of different nucleotide sequences. This fact suggests that other *Hemitaxonus* species also have such repetitive sequences. Further comparative molecular studies on such repetitive sequences might provide insight into the evolution of genomes and species in *Hemitaxonus*.

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