Genes & Genomics (2010) 32: 129-135
DOI 10.1007/s13258-009-0804-0

RESEARCH ARTICLE

Independent chromosomal localization of two different size 5S rDNA of *Allium victorialis* var. *platyphyllum* by sequential fluorescence *in situ* hybridization in accordance with sequence polymorphism

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Received: 31 August 2009 / Accepted: 19 January 2010 / Published online: 30 April 2010

Abstract
5S rRNA gene repeat units in a species are usually organized as either one relatively close size with numbers of intraspecific variations in NTS region or two different sizes with completely different sequence in NTS. *Allium victorialis* var. *platyphyllum* revealed two different size products of approximately 0.39 kb and 0.51 kb with highly conserved coding region of 120 bp. However, an extra sequences of approximately 120 bp between at 324 and 443 bp in long NTS region revealed, besides the remaining sequences of two NTS regions of short and long size were highly conserved giving the identity of 94.9%. To identify whether two different size 5S rDNA are occupied by a mixed state as random repeat or an independent group by each size in a particular locus, two rounds of FISH was sequentially performed using two probes of independent different size 5S rDNA and additional probe of only extra sequences of 120 bp in long NTS. Due to the highly conserved coding regions of both 5S rDNA, two different size 5S rDNA were detected in 3 loci in short arm of chromosome 6, however, extra sequences of long NTS was shown only in one locus within detected 5S rDNA from all examined chromosomes and interphase cells. This independent localization of two different size 5S rDNA suggests that 5S rDNA may be organized as a tandem repeat with random positions in a molecular level, but of cytogenetic view in chromosomes and interphase cells, they are organized as an independent group in a significant loci consisting of own size by the patterns of nucleotide variations.

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Keywords 5S rRNA gene; *Allium victorialis* var. *platyphyllum*; Sequential fluorescence *in situ* hybridization; Informative factor; Independent localization

Introduction

5S rRNA gene is organized with hundreds or thousands of tandem repeat units and located at one or more loci in one or more chromosomes independently of 45S rRNA gene (Seo et al., 2007a). It contains nontranscribed spacer (NTS) region as ITS of 45S rRNA gene that is also found with a variety of variations in nucleotide sequence and lengths of the complete region (Goldsborough et al., 1981; Samson and Wegnez, 1984; Lee et al., 1999; Baum and Bailey, 2001). While the 120 bp of coding sequence of 5S rDNA is highly conserved in its length and sequences to be a component of large subunit of ribosome, NTS retain high diversity in variable length and sequence. The NTS regions within plant species varies from 100 to 900 bp, giving rise to different classes of repeat units in inter- and intra-specific sequence (Appels et al., 1980; Rogers and Bendich, 1987; Specht et al., 1990; Maughan et al., 2006). Based on the sequence polymorphism of 5S rDNA within species, the phylogenetic relationship and the localization of 5S rRNA gene within *Allium* (Do and Seo, 2000) species have been studied using several points of molecular markers such as sequences from the nuclear ribosomal ITS region (Mes et al., 1999), and also on behalf of RAPDs, RFLPs, and AFLPs (Klass, 1998; Fritsch and Friesen, 2002). Cronn et al. (1996) reported that most of the nucleotide positions in the spacer region are expected to be free to vary, because the variants are selectively neutral and consequently they can become fixed which causes the accumulation of fixed in-

traspecific differences. On the other hands, most mutations in the 5S rRNA coding sequence are expected to be selectively neutral only when they occur in a subcritical position repeats in the array. Consequently, fixed difference between species fails to accumulate in 5S rRNA coding sequence despite their possibility of undergoing mutation equal to those variants of the NTS sequences.

Due to the highly conserved sequences in 5S rRNA coding region, it is known that 5S itself can be possible to study and classify in the phylogenetic relationships among diverse species, but incapable of studying within species in the same genus (Hori and Osawa, 1986). The discrimination of closely related species or individuals of the same species shows some ground of variation in DNA sequence of NTS in which neutral selection can be occurred in spite of homogenizing force to reduce the heterogeneity (Maughan et al., 2006). On the basis of these described characteristics of 5S rRNA gene unit, neutral DNA such as NTS has been used as an important factor in phylogenetic and molecular studies among closely related species (Appels et al., 1989; Yang et al., 1998; Baum et al., 2004). But on the other hands, due to the sequence heterogeneity within 5S rRNA repeat units among the genomes of species, homogenizing forces have not been strong enough to overcome processes of generating the sequence variations (Kellogg and Appels, 1995; Cronn et al., 1996; Campbell et al., 1997). By comparing and putting all account together with the reasonable data of 5S rRNA gene within numbers of genera, 5S rRNA coding sequence itself as well as NTS region revealed some polymorphic sequence variations which can be preferred as informative factor in genera of higher plants (Do and Seo, 2000; Park et al., 2000; Kim et al., 2006; Seo et al., 2007b).

In a molecular and phylogenetic study, polymorphic sites of sequence variations are mainly considered as an important factor for the analysis of the molecular relationship among the species and genus. The sequence variations such as informative factor or nucleotide transitions are very useful not also in intra- but also in inter-specific variations. Two typical types of polymorphic sites can be described. One is autapomorphic site that only a single nucleotide is unique within other sequences aligned. The other is synapomorphic site that usually preferred as an informative factor in phylogenetic study which is mainly considered as the main molecular factor to classify the relationships between species, which gives the variation of sequence in nucleotide at least 2 or more of the accessions from the aligned sequences (Maughan et al., 2006).

Many authors have reported the physical mapping of multigene families by using species-specific repetitive, ribosomal genes or a unique sequence such as 5S rDNA, 18S-5.8S-26S rDNA and highly repetitive DNA sequences to identify more significant molecular factors corresponding to the chromosomal relationships between species using fluorescence *in situ* hybridization (Lapitan et al., 1989; Mukai et al., 1991; Leitch and Heslop-Harrison, 1993; Do et al., 1999). Chromosomal localization of multigene families in some *Allium* species has been reported, however, no significant information of *Allium victorialis* has been revealed either in sequence variations or in chromosomal localization due to two different size 5S rDNA (Ricroch et al., 1992; Lee and Seo, 1997; Lee et al., 1998; Do et al., 1999; Seo et al., 2007a). The information of physical localization with the corresponding repetitive sequences can be very useful to identify the corresponding chromosomes of other *Allium* species which may lead to classify more significant phylogenetic relationships.

In this study, we investigated the sequence analysis of two different size 5S rRNA genes of *A. victorialis* and verify the independent localization of 5S rDNA corresponding to sequence polymorphism to identify whether they are localized in same locus as a mixed state or as an independent localization in a significant group by each size. First, the sequence polymorphisms and significant informative factors for two different size 5S rDNA in *A. victorialis* were examined to identify a significant informative factor between different sizes. And second, FISH was sequentially performed with specific identified probe of informative factor from NTS region with two different size 5S rDNA independently based on sequence analysis.

Materials and Methods

Plant materials, chromosome preparation and DNA extraction

Forty wild species of *Allium victorialis* var. *platyphyllum* grown in Ulengdo, Korea were collected and grown in greenhouse for plant materials. For accumulation of metaphase chromosomes, the fresh root tips of *A. victorialis* were excised and then transferred to ice water for 28 hrs followed by fixation in ethanol-acetic acid of 3 : 1. Chromosome preparation was obtained followed by the procedure of Mukai et al. (1991), and genomic DNA of *A. victorialis* was extracted from fresh young leaves according to Rogers and Bendich (1987).

Amplification and cloning of 5S rRNA genes

To amplify the 5S rRNA genes including the coding and the non-transcribed spacer (NTS) regions, polymerase chain reaction (PCR) was performed. According to the previous report (Do and Seo, 2000), primer sequences of the forward and the reserve were designed to amplify the complete sequences for

5S rRNA gene. Total genomic DNA was used as template DNA for the amplification of the 5S rRNA genes. The reaction mixture (25 ㎕) contained 10 ng of template DNA, 5 pM each of primers, 200 μM each dATP, dGTP, dCTP, and dTTP, and 2.5 U Ex Taq polymerase in 1× Ex Taq buffer with 2 mM MgCl, and performed the amplification in a PCR thermal cycler (Takara, Japan). Amplification involved a preliminary 2 minute denaturation at 94℃, 30 cycles for 30 seconds at 94℃ (denaturation), 30 seconds for 55℃ (annealing) and 1 minute for 72℃ (extension), and a final extension at 72℃ for 7 minutes followed by gradual cooling to 4℃. PCR products were visualized by electrophoresis in 1% agarose gels followed by staining with ethidium bromide. Among fragments of amplified DNA in a set of discrete sizes with integral multiples of the smallest size fragment, prominent bands corresponding to the full length of single unit of each of the 5S rRNA genes were isolated using a Gel extraction kit (Takara, Japan), ligated into pMD 18-T vector (Takara, Japan), introduced into DH5α *E. coli* competent cells by transformation, and obtained recombinant plasmids with Miniprep kit (Qiagen, USA).

Sequencing Analysis and Alignments

DNA sequences of the inserts originated from randomly selected colonies were determined by the chain termination method through commercial sequencing service to identify nucleotide sequence of both of coding and NTS regions of the 5S rRNA gene unit (Genotech, Korea).

For the sequence analysis, sequences of the coding and NTS regions of 5S rRNA gene were aligned by multiple alignment programs Clustal X and W, and used Bio-edit program to aid dissecting more significant homologies and specific informative factors (Thomson et al., 1994; Reddy et al., 2003).

Sequential Fluorescence *in Situ* Hybridization

Fluorescence *in situ* hybridization (FISH) was sequentially performed to identify the specific physical location of two different size 5S rRNA gene units of *A. victorialis*, respectively with a specific probe of an extra sequence region of long NTS. For probe labeling of 5S rDNA, each extracted size 5S rRNA gene was amplified with Biotin-16-dUTP for short size 5S rDNA and Digoxigenin-11-dUTP for long size (Roche Diagnostics, Germany) by PCR. The reaction mixture and the condition of the PCR amplification was same as the PCR performed for 5S rRNA gene amplification except the following substitutions as 140 μM of dTTP and 60 μM of digoxigenin-11-dUTP or Biotin-16-dUTP instead of 200 μM dTTP. To amplify the extra sequences of long NTS, two specific primer sequences of the forward and reserve (Fig. 1) from the consensus sequence of long size 5S rRNA gene were designed and amplified with Biotin-16-dUTP with the same contents and conditions as other probes except the PCR condition of annealing at 40℃. Before the FISH began, chromosomal DNA fixed on slides were denatured in 70% formamide- 20× SSC (1× SSC: 0.15 M NaCl with 0.015 M sodium citrate, pH 7.0) for ≥ 3 minutes at 68℃ before an immediate dehydration in an ethanol series. The slides were incubated at 37℃ followed by applying the 1st probe mixture of extra sequences of long NTS onto each slide (probe mixture with digoxigenin-11-dUTP / biotin-16-dUTP, formamide, 50% dextran sulfate, 20× SSC and 500 μg/ml salmon sperm DNA). The hybridized slides were washed in SSC series before incubation at 37℃ for an hour followed by applying with avidin-FITC and anti-digoxigenin-rhodamine (Roche diagnostics, Germany). They were washed in SSC series with 0.1% Tween 20 and overlaid a cover slip followed by counter staining by 4, 6-diamino-2-phenylindole (DAPI) with mounting medium, Vectashield (Vector laboratory, USA). Signal detection of each slide was examined and recorded by an epifluorescence microscope (Zeiss Axiophot, Germany). After the 1st round, the slides were washed twice in 2× SSC + 0.05% Tween 20 for 15 min to remove the cover slip, immersion oil and antifade solution. The slides were briefly rinsed in PBS and distilled water before denaturation in 70% formamide for ≤ 2 min and dehydration in an ethanol series to remove the previous hybridized probes and denaturation. In the 2nd round, probes of short and long size 5S rDNA were deposited on the slides and followed hybridization, washing and staining procedures performed in the first round. The signals were observed and recorded at the same positions where the slides were taken in the first round.

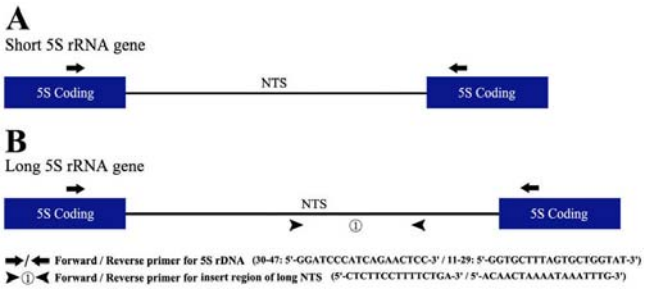

Figure 1. Positions and sequences of primers of two different size 5S rDNA of *A. victorialis* amplified for PCR and sequential FISH. (A) Short 5S rDNA (B) Long 5S rDNA.

Results

A. victorialis produced two different size products of 5S rRNA genes of approximately 0.39 kb and 0.51 kb with a multiple.

Figure 2. Consensus sequence comparison of two different size 5S rRNA genes. The first 120 bp sequence presents the coding region of 5S rDNA. Polymorphic sites are indicated by gray box and deletion of 120 bp by black background.

Two reasonable consensus sequences of each size 5S rRNA gene were constructed by reading the major group of polymorphic sites. A significant informative factor revealed as an extra sequences of 120 bp at between 324 and 443 bp of long NTS, whereas the remaining sequences of both 5S rDNA were highly conserved (Fig. 2). To indicate a significant physical localization of two different size 5S rDNA of *A. victorialis*, FISH was sequentially performed by 2 rounds as 1st round with the probe of extra sequences of 120 bp in long NTS and 2nd with independently amplified probes of two different size 5S rDNA (Fig. 1). From three detected loci of both size 5S rDNA in short arm of chromosome 6, only one locus for extra sequences of 120 bp of long NTS revealed in both of chromosomes and interphase cells (Fig. 3).

Nucleotide polymorphism of autapomorphie and synapomorphie in sequence analysis

Each group of sequences from two different size 5S rRNA gene; short size (AvicA) and long size (AvicB) was aligned independently to analyze which repeat units are more stable ribosomal. From both of the different size 5S rRNA gene, numbers of polymorphic sites revealed in both of coding and NTS regions. Table 1 shows the size, nucleotide polymorphisms and numbers of clones examined in this study. Aligned 120 bp of coding sequences of two different size 5S rRNA gene were highly conserved, even a few polymorphic sites were found. All the polymorphisms found in both coding sequences were autapomorphic sites which is unique to a single nucleotide. AvicA revealed only 4 sites, whereas 10 autapomorphic sites were found in AvicB. Besides highly conserved sequences of coding regions revealed for both 5S rRNA genes, a very large number of differences in polymorphic sites were found in a comparison of both NTS regions. 15 polymorphic sites were found in AvicA. All 15 polymorphic sites were autapomorphie which especially showed unstable including deletion between 295 and 305 bp. On the other hands, AvicB revealed 64 polymorphic sites in NTS region even the numbers of sequences aligned were less than AvicA. A total of 64 polymorphic sites, 61 autapomorphic sites revealed with 1 synapomorphie and 2 deletions which showed very unstable sequences between 252 and 262 bp.

To indicate a significant sequence homology of two different size 5S rRNA genes of *A. victorialis*, two reasonable consensus sequences of each size 5S rRNA genes were constructed by reading the major group of the polymorphic sites. While some large numbers of polymorphic sites were found by independently aligning each size of 5S rDNA, 5S rRNA coding sequence from the consensus comparison revealed a complete homologue, however, 10 polymorphic sites which were all single nucleotide substation and an informative factor were found to distinguish a significant sequence analysis in NTS regions. Besides the polymorphic sites of nucleotides substitution revealed in NTS region, approximately 120 bp between at 324 bp and 443 bp of long NTS region was deleted in short NTS region (Fig. 2). Putting all a count of coding and NTS sequences of 5S rRNA gene of *A. victorialis* from two consensus sequence comparison, the identity of homology

Table 1. Summary of clone isolated numbers, the range of sizes of repeat unit, size of consensus sequence, and sequence analysis of nucleotide polymorphism of autapomorphic, synapomorphic and deletion occurrence in coding and NTS region with numbers of unique clones observed.

Taxon		No. of clones examined	Size of repeat (bp)	Size of consensus sequence	Unique clone	Nucleotide polymorphism (coding region / NTS)		
						Autapomorphic	Synapomorphic	Deletion
Allium victorialis var. *platyphyllum*	AvicA	7	392–395	395	7	4 / 15	0 / 3	0 / 0
	AvicB	3	503–511	510	3	10 / 61	0 / 1	0 / 2

revealed 94.9% excluding the extra sequences of 120 bp of long NTS region.

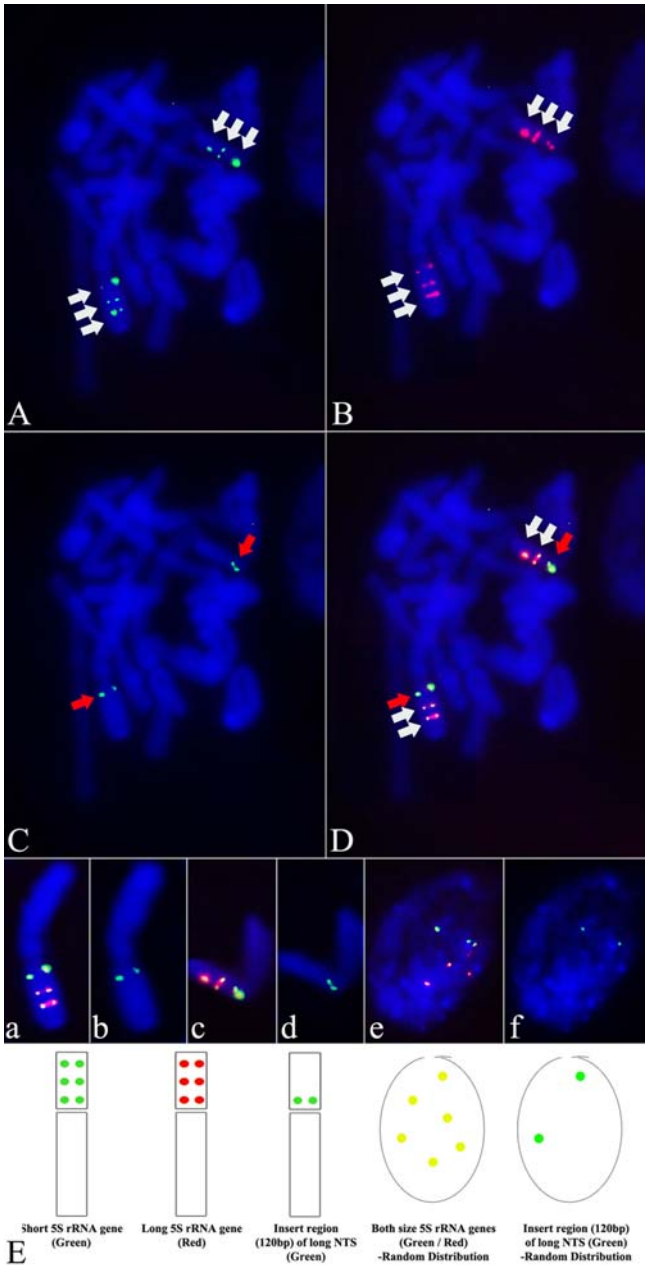

Figure 3. Localization of two different size 5S rRNA genes and an extra sequences of long NTS in chromosomes and interphase cells of *A. victorialis*. (A) Biotin-16-dUTP labeled on short 5S rDNA (Green). (B) Digoxigenin-11-dUTP labeled on long 5S rDNA (Red). (C) Biotin-16-dUTP labeled on extra sequences (Green). (D) Merged image of A-C. (E) Signal detected chromosome and interphase cell isolated with the idiogram on the bottom. (a and c) Merged signals of both 5S rDNA. (b and d) Extra sequences of long NTS. (e) Merged signals of both 5S rDNA in interphase cell. (f) Extra sequences in interphase cell. White arrows indicate the detected signals of 5S rDNA and red for extra sequences of long NTS.

Localization of two different size 5S rDNA by FISH

Sequential FISH was carried out to identify a significant localization of two different size 5S rRNA gene locus in *A. victorialis* by independently applying probe of extra sequences of 120 bp of long NTS. Even with the independent probes of two different size 5S rDNA of *A. victorialis*, the signals by FISH were expected to be identical at the same positions due to the highly conserved coding region of 5S rDNA. Thus, to identify a significant localization of each 5S rRNA gene unit, two unique primers were designed from consensus sequence, only containing the extra sequences of approximately 120 bp in long NTS, and amplified for an additional independent probe to investigate whether the long 5S rRNA genes repeat as an independent group on each size or tandem with short 5S rRNA gene as a mixed state. Figure 3 shows the signal patterns by FISH on the metaphase chromosomes and interphase cells of 5S rRNA genes. From all detected chromosomes, two different size 5S rRNA genes revealed in 3 loci at the same positions on the short arm of chromosome 6 as expected due to the highly conserved coding regions. However, the extra sequences of long NTS revealed only in one locus at the location close to centromeric region within 3 loci of 5S rRNA gene units (Fig. 3A-D). Interphase cells were also found with two signals of extra sequences, whereas a total of 6 signals of two different size 5S rRNA genes revealed (Fig. 3E, e and Fig. 3f).

Discussion

The studies for highly conserved consensus sequences on genus *Allium* has been reported by many researchers. Although 5S rDNA possesses the same functional role as ribosomal large subunit, considerable heterogeneity in sequence among different genus was report in *Hordeum* (Baum and Johnson, 1999), *Capsicum* (Park et al., 2000) and *Caenopodium* (Maughan et al., 2006). Hori and Osawa (1986) proposed that 5S rRNA coding sequence is informative in inferring phylogenetic relationships among diverse species but too highly conserved to differentiate between species in the same genus, respectively with the molecular level. But even between a very closely related species in the same genus, non-transcribed spacer (NTS) of 5S rDNA is another informative to identify the relationship phylogenetically because of the rapid evolving of nucleotides by neutral selection (Yang et al., 1998; Baum et al., 2004).

By amplifying the highly conserved sequence of 5S rRNA coding region, two different size 5S rRNA genes revealed with a pattern of integral multiples of approximately 0.39 and 0.51 kb in *Allium victorialis*. In sequence analysis from examined

clones, the coding region of 120 bp beginning with GGGT GCGATC to ending TTGCACTCCT (Do and Seo, 2000) was identified from all examined clones that showed two different size 5S rRNA genes are tandem repeating in *A. victorialis*. Due to the organization of two different size 5S rRNA genes in *A. victorialis* not likely other *Alliums*, the sequence analysis was first purposed to identify whether which size 5S rDNA is more stable in genomic DNA considering the polymorphic sites or any relative relationship of two different size as an hybrid from two different ancestries of other *Allium* species. By analyzing each size 5S rRNA genes as an independent group, numbers of sequence variations revealed in some positions as autapomorphic or synapomorphic phenomenon but with some different numbers between coding and NTS region of 5S rDNA. From 120 bp of coding region of both size 5S rDNA, only 4 polymorphic sites revealed from short size 5S rDNA (AvicA), whereas 10 sites were found in long size 5S rDNA (AvicB) which were all autaopmorphic providing that the coding regions were highly conserved. However, a large number of differences of sequence polymorphism were identified between two different NTS regions. Fifteen autapomorphic sites revealed in NTS region of AvicA. However, 61 polymorphic sites including 1 synapomorphic were identified in AvicB. Due to the differences of examined numbers of clones, we cannot assure which size 5S rRNA genes are more stable. But by reading the number of polymorphic sites and knowing that AvicB showed more sequence variations even with the shorter numbers of examined clones, we could assume that short size 5S rRNA gene (AvicA) may tandem repeats as a more stable group. In a comparison of consensus sequences (Fig. 2), the coding sequence of two different size 5S rDNA showed a complete homology, but a significant informative factor revealed from the sequence of NTS regions. We identified that approximately 120 bp at between 324 and 443 bp was deleted in AvicA, whereas the remaining sequences of both NTS regions were highly conserved revealing only 14 polymorphic sites. From the analysis of two different size 5S rDNA, we could assume that the informative factor, extra sequences of 120 bp in long NTS region may has been changed either from long or short through the evolutionary period in accordance with many other environmental factors.

Genes encoding 5S rDNA which is a major component of large subunit of ribosome are located independently from other rDNA clusters and organized into tandem repeat units with altenative arrays of coding and non-transcribed spacer in one or more loci in genome (Lee and Seo, 1997; Do et al., 1998; Lee et al., 1999; Baum and Bailey, 2001; Seo et al., 2007a, b). Because of the 5S rDNA family's characteristic of tandem repeats with many unit copies, probe preparation for detection by FISH has been very useful to localize and map the gene loci, and identify the phylogenetic relationship between species in the metaphase chromosomes. However, due to the highly conserved coding region of 5S rDNA, it was capable to map the complete 5S rDNA tandem repeats, but incapable to isolate the individual sequence variation site in NTS regions by FISH.

In this study, the results were obtained by using two different size 5S rDNA independently with the 120 bp of extra sequences in long NTS by performing sequential FISH. The signal distributions of two different size 5S rRNA gene loci were completely homologous in the short arm of chromosome 6 due to the highly conserved coding region of 5S rDNA as 3 detected loci (Fig 3A and Fig. 3B), respectively with 6 signals in interphase cells. By sequentially applying FISH with extra sequences of 120 bp in long NTS, more significant molecular cytogenetic detection of 5S rDNA was analyzed to identify whether two different size 5S rDNA repeats as a mixed state or independently groups with each length in chromosomes and interphase cells. From the 3 loci of both 5S rDNA detected in the short arm of chromosome 6, only one locus of extra sequences of long NTS revealed (Fig. 3C). All examined chromosomes showed the same pattern of the signal distribution of 5S rRNA genes and extra sequences. In a comparison between metaphase chromosomes and interphase cells, the distribution numbers of 5S rDNA and extra sequences in interphase cells were identified as 6 and 2 which were same as the numbers revealed in chromosomes. Both 5S rRNA genes were indicated in a significant localization of 3 loci in the short arm of chromosome 6, and only locus of extra sequences close to centromere was detected within 3 loci from all the examined chromosomes. However, the signal pattern of interphase cells were found as a random distribution (Fig. 3Ee and Fig. 3F). Thus, two different size 5S rRNA genes of *A. victorialis* may be described as tandem repeat units in one large group by sequence analysis. But analysis of the detected loci by sequential FISH with the extra sequences of long NTS and two different size 5S rDNA with the highly conserved 5S rRNA coding region showed that two different size 5S rDNA are localizing in tandem repeat units as a significant independent group in chromosomes and interphase cells by an extra sequences of 120 bp.

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