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Cloning and Structural Analyses of Partial Nuclear rDNA from *Bupleurum euphorbioides* (Apiaceae)

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For the cloning of nuclear ribosomal DNA (rDNA) from *Bupleurum euphorbioides* (Apiaceae), ten clones were screened by DNA-DNA hybridization method. Among them, two clones were strongly hybridized with a heterologous probe of rice rDNA and with an autologous probe of an internally-transcribed region of *B. euphorbioides* amplified by PCR. We sequenced both ends of the two genomic clones aligned with a known sequence of rDNA. ITS2 sequences of the two clones showed 98% and 83% homology with the ITS2 sequence of *B. euphorbioides*. Our clones showed 1 bp and 3 bp nucleotide substitutions in the 25S and intergenic spacer regions, respectively, and the ITS1 and 18S regions were both missing. Restriction enzyme sites and the orientation of both clones were analyzed for physical mapping purposes. Apart from the length difference between the two clones, we found restriction site variations in the 25S and intergenic spacer regions.

Keyword: *Bupleurum euphorbioides*, PCR, rDNA, restriction mapping, intergenic spacer

Molecular variation in species level can be analyzed by restriction fragment length polymorphism (RFLP) and direct DNA sequencing. Nuclear rDNA has several advantages for this kind of molecular analysis to assessment of phylogenies. Molecular systematists have commonly used the ITS regions for phylogenetic construction of plant groups at low taxonomic levels because they show useful variations (Suh et al., 1996, 1997).

The nuclear genes that code for rRNA are reiterated thousands of times within a typical plant genome. They account for as much as 10% of total plant DNA. Nuclear rDNA is arranged in tandem repeats in one or a few chromosomal loci (Soltis et al., 1992). Each repeating unit consists of a transcribed region separated from the next unit by an intergenic spacer (IGS). A transcribed region is divided into three genes: 18S, 5.8S, and 25-28S. Both sides of the 5.8S gene are linked to internal transcribed spacers (ITS1 and ITS2).

Because rDNA is the richest and least ambiguous source of genetic variability, information on its evolution is fundamentally important to evolutionary biology (Soltis et al., 1992; Dowling et al., 1996). The nuclear rDNA pattern of restriction fragment length polymorphism is useful for the identification of geographical populations (Rogers and Bendich, 1987). Within a population of *Hordeum spontaneum* K. Koch (wild barley), a six-fold range in rDNA copy number was detected between different individuals, and among a large population of *Vicia faba* L. (broad bean), the copy number ranged from 250 to 22,000 per haploid cell (Rogers and Bendich, 1987).

Nuclear rDNA polymorphism so-called ribotype has also been found in populations of *Bupleurum falcatum* L.(Mizukami et al., 1993), although RFLP patterns in *Bupleurum* species are too complex to analyze the structure of rDNA repeating units. The nuclear rDNA structure of *Bupleurum* species was reported to consist of at least three types of repeating units (Choi et al., 1996).

The focus of our study was the cloning of the nuclear rDNA repeating units of *Bupleurum euphorbioides* L. The genomic clones were analyzed by DNA-DNA hybridization, nucleotide sequencing, and restriction mapping to confirm the structure of rDNA fragments. By comparing the nucleotide substitution ratio of the transcribed and nontranscribed regions of rDNA, we tried to estimate the divergence among its ITS, 25S and IGS regions.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

For the cloning of rDNA from Apiaceae, we extracted DNA from the leaves of *B. euphorbioides* Nakai collected on Mt. Snezhanaya in Russia (Voucher specimen: Russia, Primory, Mt. Snezhanaya,

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To extract total genomic DNA, the method of Doyle and Doyle (1987) was followed with minor modification (Choi et al., 1996). DNA was purified by ultracentrifugation in CsCl-ethidium bromide gradients after alcohol precipitation (Sambrook et al., 1989).

Cloning rDNA

In cloning rDNA from *B. euphorbioides*, total genomic DNA was digested with HindIII restriction enzyme. The DNA insert was directly ligated onto HindIII-digested pZErO™-2 cloning vector and transformed into *Escherichia coli* competent cell Top10F' by high-voltage electroporation method (Mitra et al., 1989; Sambrook et al., 1989).

Southern Blotting and DNA-DNA Hybridization

Recombinant plasmid DNA was prepared by the alkaline method and its insert size was determined by electrophoresis after restriction digestions (Sambrook et al., 1989). Approximately 1 μg of determined recombinant plasmid DNA was digested with restriction enzyme HindIII. The digested DNAs were run on 1% agarose gel, and blotted onto Hybond-H+ membrane (Amersham Co.) with a vacuum system (VacuGene XL; Pharmacia Co.), according to the manufacturer's guidelines.

DNA-DNA hybridizations of the insert with probes were performed using the random primer labelling and detection system (Amersham Co.). The membrane was hybridized with PCR products of rDNA cloned from rice (pRR217; Takaiwa et al., 1985) as heterologous probe and *B. euphorbioides* ITS regions as homologous probe (Sambrook et al., 1989).

Polymerase Chain Reactions

All PCR reactions were performed using a Progene thermal cycler (Techene Co.). The standard procedure for DNA amplification involved 30 cycles of the following steps: denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C (Eom and Lee, 1996). A final extension was carried out at 72°C for 9 min 30 s, followed by indefinite cooling to 4°C. Each reaction in a typical 20 μL volume contained 10 mM Tris-HCl (pH 8.4), 1.9 mM $MgCl_2$, 25 mM KCl, 2 μg of bovine serum albumin, 200 μM of each dNTP, 4 pmol of the appropriate primers, and variable amounts of template DNAs. Reactions were initiated after addition of *Taq* DNA polymerase at a final concentration of 0.025 unit/μL.

Universal primers for the ITS regions of plant rDNA were used: ITS1 (5'-GGAAGGGAAGTCGTAACAAGG-3') and ITS3 (5'-GCATCGATGAAGAACGCAGC-3') as forward primers, and ITS2 (5'-GCTGCGTTCTTCATCGTGC-3') and ITS4 (5'-GCTGCGTTCTTCATCGATGC-3') as reverse primers (Choi et al., 1996; Downie and Katz-Downie, 1996; Suh et al., 1996).

Sequencing Cloned DNA Fragments and Data Analysis

We used an ABI377 Automatic DNA Sequencer (Deoxyterminator FS cycle sequencing) to obtain sequences of our clones. Accession numbers of the Genenuri in BRIC (Biological Research Information Center of Korea) are KS101677, KS101678, KS101679, KS101680, KS101681, and KS1011682. Nei's one parameter model was adopted to estimate the nucleotide substitution rate (Li and Graur, 1991). For local alignment and comparison of nucleotide sequences we used the BLAST algorithm (Altschul et al., 1997) available on the World Wide Web (http://www.ncbi.nlm.nih.gov). As sample comparisons, we used known rDNA sequences of carrot (*Daucus carota* L., accession number X17534), *Sinapsis alba* L. (accession number X66325), and tomato (*Lycopersicon esculentum* Miller, accession number X13557) available in GenBank.

RESULTS

Cloning rDNA and DNA-DNA Hybridization

Ten strains of DNA were extracted to confirm whether their inserts included rDNA repeating units (Fig. 1). In restriction digestion of recombinant plasmid DNAs with HindIII, the lengths of the various insert DNA fragments were measured as 1.5 Kb, 1.9 Kb, 6.7 Kb, and 6.9 Kb, as shown in Figure 1. As a result of DNA-DNA hybridization with probes, we selected two strains as tentative clones which contained genomic rDNA units from *B. euphorbioides* (Apiaceae) (Fig. 2). These clones contained 6.7 kb and 6.9 kb rDNA fragments and showed strong bands of hybridization with PCR products of the ITS regions of *B. euphorbioides* (Fig. 2) as homologous probe. The 6.7 Kb and 6.9 Kb insertions were named pZCK7 and pZCK12, respectively.

DNA-DNA hybridization was carried out using rice rDNA (pRR 217; Takaiwa et al., 1985) as heterolo-

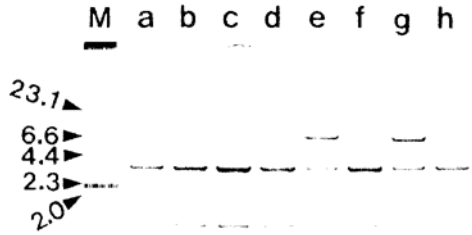


Figure 1. Electrophoresis of recombinant plasmid DNAs after restriction digestions with HindIII. pZErO^{TM}-2 vector and recombinant plasmids were prepared by mini-preparation method of plasmid DNA, digested with restriction enzyme completely and electrophoresed to estimate its insert size (Sambrook et al., 1989). Lane M, size marker(Kb); lane a, pZErO^{TM}-2 vector as a control; lanes b-k, digested DNAs of recombinant plasmids.

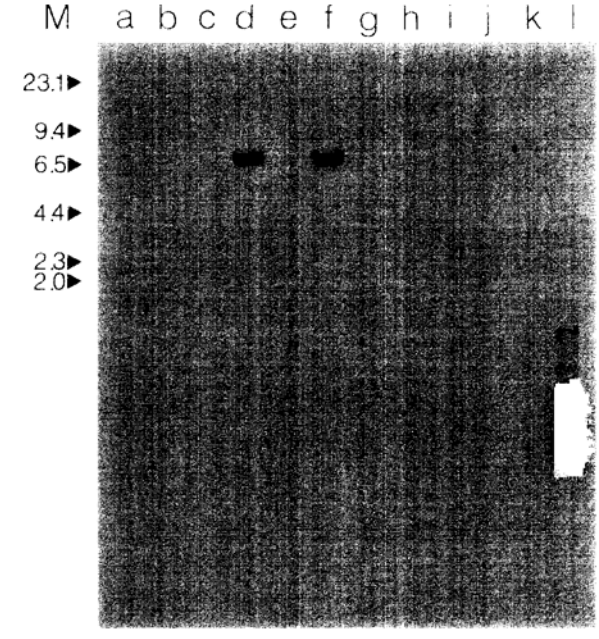


Figure 2. Southern hybridization of the recombinant plasmid DNA with the PCR product of ITS regions of *B. euphorbioides* as homologous probe (Sambrook et al., 1989; Choi et al., 1996). Only two clones (b and f) strongly hybridized with the probe. Lane M, size marker (Kb); Lane a, pZErO^{TM}-2 as a negative control; lanes b-k; lane l as a positive control, PCR products of ITS region from *B. euphorbioides* genomic DNA.

gous probe. DNA extracted from the two clones showed strong bands after hybridization with BamHI-digested DNA from pRR217. Our two clones hybridized well with the 17S region of rice rDNA (Fig. 3A), the ITS and 5.8S (Fig. 3B), and the 25S region (Fig. 3C) eluted from rice rDNA (Takaiwa et al., 1985).

DNA Sequences of Clones and Analysis of Data

To confirm that our clones contained an rDNA repeating unit from *B. euphorbioides*, we sequenced the inserted DNA fragments at both ends (Fig. 4). The nucleotide sequences of the inserted fragments from the 5' end were readable up to 705 bp and 715 bp (Fig. 4, B and C). The 3' end sequences of the inserted DNA were scored up to 554 bp and 555 bp (Fig. 4C).

After alignment and comparison of the clone sequences with known rDNA sequence data from GenBank, we found that the 5' end of these clones contained only the ITS2 and 25S regions of the rDNA repeating unit (Choi et al., 1996; Downie and Katz-Downie, 1996; Downie et al., 1998) (Fig. 4, A and B). The 3' nucleotide sequences at the end of the clones corresponded to the IGS region in rDNA (Fig. 4C).

The nucleotide lengths of the ITS2 regions of the two clones were 231 bp and 221 bp, respectively (Fig. 4A). The nucleotide sequences of the ITS2 region from the pZCK7 and pZCK12 clones were shown to have 98% and 83% homology with the known ITS2 sequences of *B. euphorbioides* (Choi et al., 1996), respectively. The ITS2 region of pZCK7 differed by 1 bp from the reported ITS2 sequence of *B. euphorbioides*. The ITS2 sequence of pZCK12 was 10 bp shorter than that of pZCK7 (Fig. 4A) with two gaps of 4 bp and 7 bp in length. G + C content of ITS2 in known sequences of *B. euphorbioides* rDNA was slightly higher than those of clones pZCK7 and pZCK12 (Table 1). The ratio of nucleotide substitutions differed from each other by one transition and three transversions. According to the two-parameter model (Li and Graur, 1991), we calculated the ratio of nucleotide substitution in ITS2 per site as $K=1.3 \times 10^{-2}$ (Table 1). The nucleotide sequences of the pZCK7 and pZCK12 25S coding regions differed by 1 bp (Fig. 4B). The 3'-end of our clone nucleotide sequences was aligned with known sequences of rDNA to identify the starting point of ITS region in an entire repeating rDNA unit (I don't understand this clause). This region belonged to the IGS region of rDNA (Fig. 4C). In this IGS region from clones pZCK7 and pZCK12, the difference of nucleotide sequence was 3bp (Table 1). The ratio of nucleotide substitution between the two clones was $K=5.43 \times 10^{-3}$ and 1 bp of indel was found in this IGS region of *B.*

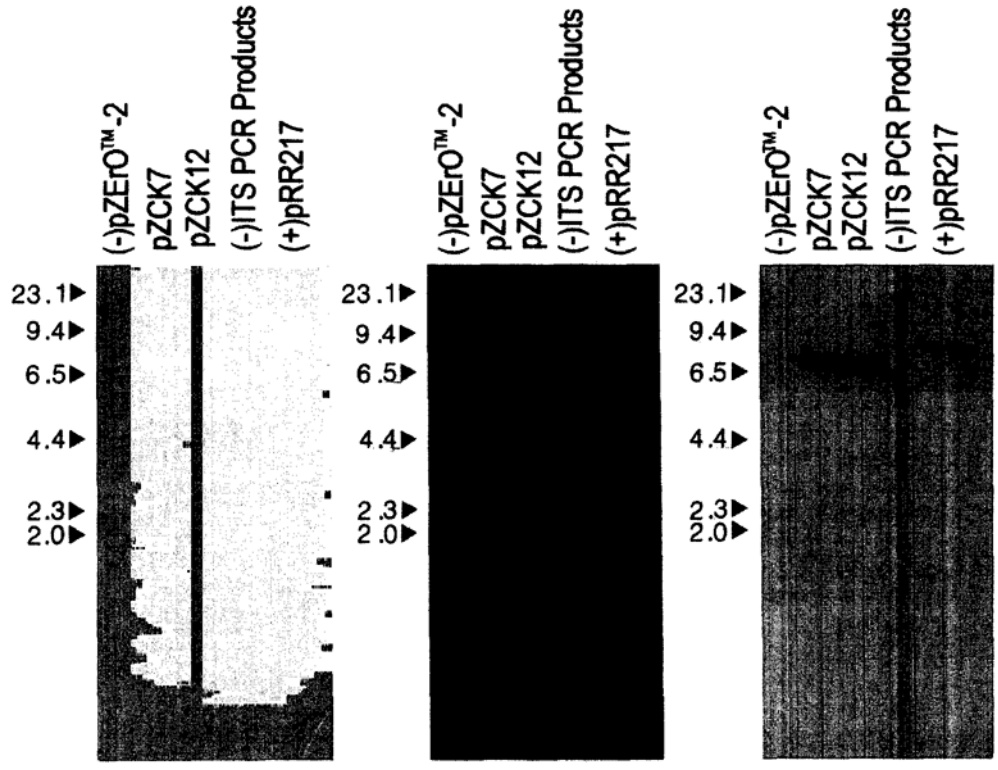


Figure 3. Southern hybridization of rDNA clones (pZCK7 and pZCK12) with the 17S (A), 5.8S (B), and 25S (C) regions of rice rDNA (pRR217; Takaiwa et al., 1985) as heterologous probes. Three probes showed considerable signals with pZCK7 and pZCK12.

euphobioides rDNA (Fig. 4C).

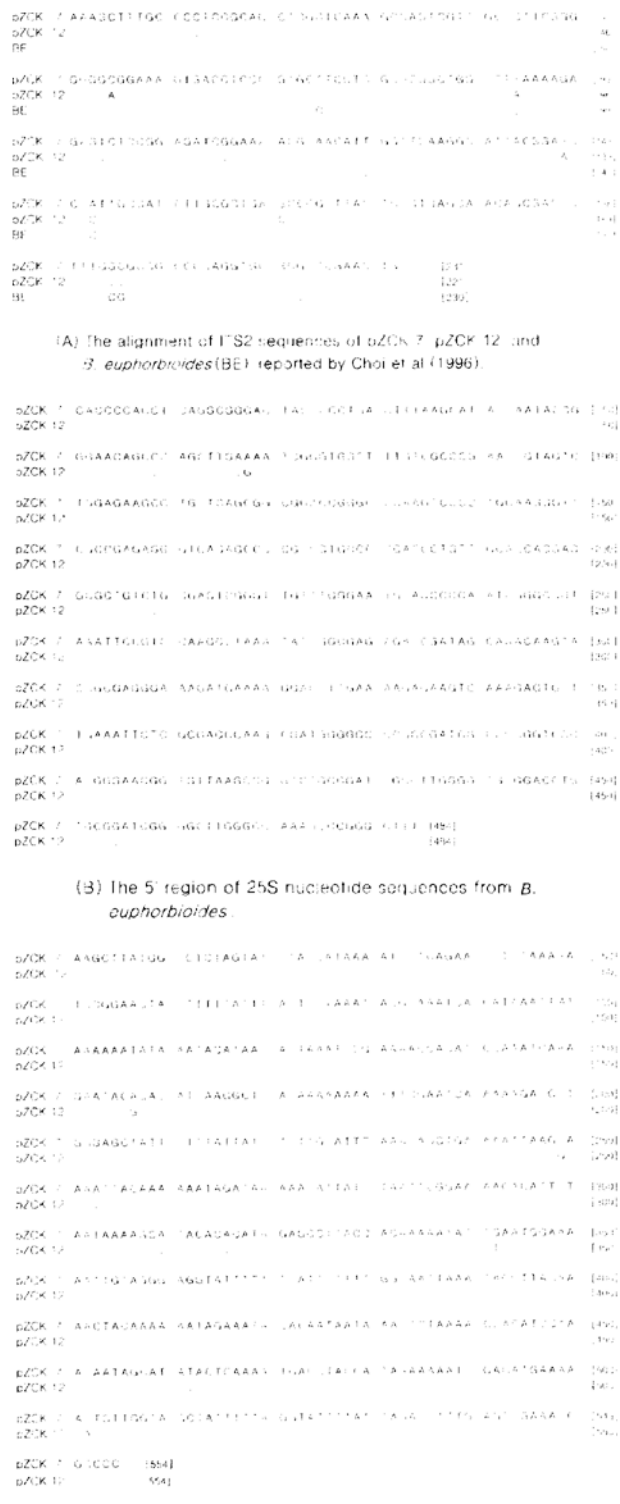
Physical Mapping of Two Clones

The pZCK7 and pZKC12 clones were digested with several restriction enzymes to map their restriction sites and lengths. There were two restriction sites and one length variation between the two clones (Fig. 5). pZCK7 was digested with six restriction enzymes (BamHI, SacI, SphI, EcoRI, ApaI, and PstI) (Fig. 5A). pZCK12 clone was digestible with one additional restriction enzyme, BstEII (Fig. 5B). BamHI and SacI cut both cloned rDNA fragments twice. Restriction sites of BamHI, SacI, SphI, EcoRI, ApaI and SacI were estimated to cut the same region of the repeating unit of rDNA from *B. euphorbioides*. We found a length variation of about 200 base pairs in the IGS region of pZCK12 (Fig. 5B).

Table 1. Analyses of nucleotide sequences of pZCK7 and pZCK12 cloned from genomic DNA of *B. euphorbioides* after HindIII digestion.

rDNA	Strains	Nucleotides (231bp)*	G+C (%)	Nucleotide substitution**	K***
ITS2	pZCK7	221	58.9	3 ($o^S/3^V$)	1.3×10^{-2}
	pZCK12	484	58.4		
25S	pZCK7	484	59.9	1 ($1^S/0^V$)	2.1×10^{-3}
	pZCK12	484	60.1		
IGS	pZCK7	554	22.9	3 ($1^S/2^V$)	5.4×10^{-3}
	pZCK12	555	23.0		

*rDNA region estimated by comparing the nucleotide sequences with rDNA sequence of GenBank as explained in the text.
**S, transition; V, transversion.
***K, nucleotide substitution ratio by Kimura's two-parameter model (Li and Graur, 1991).



(A) The alignment of ITS2 sequences of pZCK 7, pZCK 12, and *B. euphorbioides* (BE) reported by Choi et al (1996).

(B) The 5' region of 25S nucleotide sequences from *B. euphorbioides*.

(C) The aligned nucleotide sequences of IGS regions of rDNA repeating unit in *B. euphorbioides*

Figure 4. Comparison of sequences of ITS2, 25S 5' and IGS region sequences between pZCK7, pZCK12 and reported *B. euphorbioides* (Choi et al., 1996).

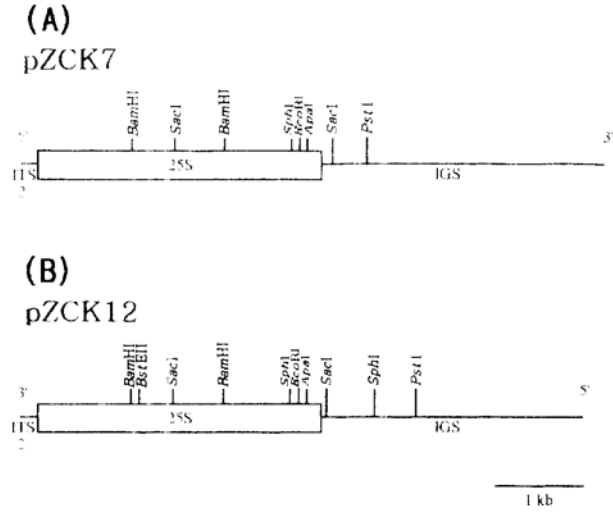


Figure 5. Physical map of pZCK7 (A) and pZCK12 (B) rDNA clones from *B. euphorbioides*. The two clones were digested with restriction enzymes including HindIII, EcoRI, BamHI, PstI, SacI, BstEII, SphI, and ApaI. Two restriction site variations and length variations are detectable in the pZCK12 clone compared to the pZCK7 clone.

DISCUSSION

rDNA repeating units are composed of transcribed and nontranscribed sequences. The transcribed region has 18S, ITS1, 5.8S, ITS2, 25S, and IGS regions. The IGS region is functionally divided into two spacers, *i.e.* nontranscribed spacer (NTS) and external transcribed spacer (ETS). The nucleotide conservativities in terms of the nucleotide substitution ratio (Li and Graur, 1991) among rDNA repeating units of *B. euphorbioides* were ordered as 25S > 5'-IGS region > ITS2. Baldwin and Markos (1998) concluded that the ETS region (3'-IGS sequences) yielded a consensus tree almost identical to the ITS tree in *Calycadenia* (Compositae). Because the ETS and ITS regions have the same control mechanism of transcription, resolution of the ETS sequences for phylogenetic analysis may not be higher than the ITS regions (Baldwin and Markos, 1998). Our data suggest that phylogenetic resolution of the 5'-region in IGS can be more powerful for the analysis of infrageneric and intergeneric comparisons than that of the ITS2 region (Table 1).

Nucleotide variations in the ITS2 sequence of pZCK7 compared to pZCK12 scored 12 gaps and 5 nucleotide substitutions. Although both clones were screened from HindIII-digested genomic DNA of *B. euphorbioides*, ITS2 sequences of our clones matched

less than 50 base pairs of the GenBank ITS2 sequences except for *Bupleurum* species (Accession numbers AJ131344, U88141, and U88139 in GenBank). The 25S gene of our clones from *B. euphorbioides* has more than 90% homology with most sequences registered in GenBank (data not shown). But there is a gap of about 93 base pairs compared to the 5' region of 25S rDNA in *D. carota*. The IGS region of our clones (Fig. 5) does not show any significant homology with the same rDNA region in carrot, *S. alba*, or tomato. Homology of the IGS region sequence in *B. euphobioides* with other organisms is less than 50 base pairs (<10%) based on BLAST searches (Altschul et al., 1997), as shown in Figure 4C. The reason for such low level homology among the IGS regions in rDNA repeating units remains unknown.

We hybridized our clones with three fragments of rice rDNA (=pRR 217; Takaiwa et al., 1985) as probes in which each fragment contained one of three coding genes, respectively. Although our results of DNA-DNA hybridization are very positive (Fig. 3), the 18S and ITS1 regions are missing from our clones (Fig. 5). Recently, we cloned a 3 Kb genomic DNA fragment from *B. euphorbioides* which showed strong hybridization signals with the ITS1 region of pRR217. We expect that one rDNA repeating unit in *B. euphorbioides* is roughly 10Kb in length. After sequencing the 18S and ITS1 regions we will present the full-length structure of rDNA in *B. euphorbioides*.

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