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Random Amplified Polymorphic DNA (RAPD) Variation in *Porphyra yezoensis* and *P. tenera*

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The random amplified polymorphic DNA (RAPD) technique was used to analyze six isolates of two species of *Porphyra*, *P. yezoensis* and *P. tenera*. Four 21-mer primers were combined randomly into six groups of double primers and screened for DNA amplification using nuclear and chloroplast template DNA. The RAPD patterns resulting from RnRc and CnCc primers provided evidence for both genetically homo- and heterogeneous populations of *P. yezoensis* and *P. tenera*. Similarity values obtained by RnRc primer analysis of nuclear DNA varied from 0.364 to 0.714 and those of chloroplast DNA were high, ranging from 0.727 to 1.000, except for *P. yezoensis* (Enoura).

Key words : AP-PCR, *Porphyra* isolates, Random amplified polymorphic DNA (RAPD)

Introduction

Species of *Porphyra* have been classified by means of the morphological features of the blade, such as shape, size, color, thickness and division pattern of the reproductive cells. Distinguishing *P. tenera* from *P. yezoensis* is important to research on the management and breeding of *Porphyra* species. Based only on the morphological features, it is however, very difficult to unmistakably identify and distinguish *Porphyra* cultivars, because of the different existing growth conditions (light, temperature, salinity etc.). Therefore, there is current interest in investigating new approaches, such as genomic DNA analysis, to identify *Porphyra* cultivars.

In recent years, the chloroplast genome map and genomic DNA analysis through restriction fragment length polymorphism and random amplified polymorphic DNA of *Porphyra* spp. have been reported (Shivji, 1991 ; Shivji et al., 1992 ; Araki et al., 1992 ; Reith and Munholland, 1993 ; Dutcher and Kapraun, 1994). Arbitrarily primed polymerase chain reaction(AP-PCR) and DNA fingerprinting has also been used to distinguish cultivars and somatic hybrids (Welsh and McClelland, 1990 ; Mizukami et al., 1995).

It was reported the results of RAPD analysis generated by AP-PCR that discriminate both between and within species of *P. yezoensis* and *P. tenera* using chloroplast and nuclear DNA.

Materials and Methods

Plant materials

The following samples were used : *P. yezoensis* cultivars Noma-1, Enoura and NBD (isolated in Ariake farm, Japan), and *P. tenera* cultivars Ariake-1, Minomi, and *P. tenera* wild type (from Kumamoto region, Japan). For indoor culture, conchospores were adjoined on synthetic fibers (cremona monofilament) and cultured. Unialgal cultures were maintained at 100 μmole $m^{-2}s^{-1}$, using daylight fluorescent lamps with a 12 : 12 LD photoperiod,

and at 18℃. Thalli grown to about 10 cm in length were used for this study.

DNA isolation

Chloroplast and nuclear DNA were extracted from protoplasts of indoor cultured thalli. Protoplasts were prepared according to the method of Fujita and Saito (1990). For the choloroplast DNA isolation(Uchimiya et al., 1984), protoplasts suspended in TE buffer A (50 mM Tris-HCl, 20 mM EDTA, pH 8.0, 5 mM 2-mercaptoethanol) were poured onto the top of a 20, 45 and 60% sucrose gradient solution and centrifuged at 3000×g for 30 min. The chloroplasts were isolated from the 45% gradient fraction. The chloroplasts were resuspended in lysis buffer (TE buffer A containing 3% L-lauroylsarcosin and proteinase K at 50 $\mu g \cdot mL^{-1}$) and incubated at 37℃ for 3 h. This mixture was extracted with phenol/chloroform and then chloroform/isoamylalcohol. The DNA was precipitated with 100% cold ethanol, rinsed with 70% ethanol and dried. For the nuclear DNA isolation, protoplasts were lysed in lysis buffer and incubated at 37℃ for 3 h as described above. After phenol/chloroform purification, total DNA was precipitated with 100% cold ethanol. The organellar DNA was purified by the equilibrium density gradient centrifugation (170000×g, 20℃, 24 h with a Hitachi Ti 70 angle rotor) in a cesium chloride solution containing Hoechst 33258 (Sambrook et al., 1989). The nuclear DNA was isolated from the second band (Goff and Coleman, 1988; Roell and Morse, 1991). The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20℃ until used.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in 50 μℓ volumes containing reaction buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 40 pmole of each primer, 2 units of Taq DNA polymerase (Promega) and 20 ng template DNA. The reaction mixture was overlaid with a drop of mineral oil. The four primers, designed from the chloroplast conserved sequences of RuBisCo and phycoerythrin (Valentin and Zetsche, 1989 ; Douglas et al., 1990 ; Reith and Douglas, 1990 ; Hasebe et al., 1992), were synthesized with an Applied Biosystem 394 DNA synthesizer. Amplification was performed in a Program Temp. Control System Model PC-700 (Astec Co., Japan) programmed for 35 cycles of 1 min at 94℃, 1 min at 40℃, and 1 min at 72℃. Amplification products were treated with equal volumes of chloroform and precipitated with 2.5 volumes of 100% cold ethanol. The products were dissolved in 10 μℓ sterile distilled water and stored at -20℃ until used. The amplified DNA was electrophoresed on a 0.8% agarose gel.

Band-sharing analysis of RAPD banding pattern was carried out using pairwise comparison according to the formula of Nei and Li (1979) for similarity value calculation.

Results and Discussion

Theoretically PCR is so sensitive that a single DNA molecule may be amplified, and single copy genes are routinely extracted out of complex mixtures of genomic sequences and visualized as distinct bands on agarose gels. Selection of primers is the most important in PCR conditions. The approach to the selection of efficient and specific primers remains somewhat empirical, there are no discrete rules that will guarantee the choice of an effective primer pair.

As shown in Fig. 1, PCR-primers were combined arbitrarily in different combinations into six groups. The primer sets were screened for genomic DNA amplification using nuclear DNA from *P. tenera* wild type (Fig. 2). Of the six double primers tested, CnCc, RnRc and RnCc primers were able to generate reproducible amplification products. Subsequently the primers CnCc and RnRc were tested for six isolates of two *Porphyra* species.

A : 5′ATGCTTGA(C/T)GC(A/T)TTTTCTAGA3′
B : 5′ATCAAAGTAGCTTGCCTCACG3′
C : 5′GA(C/T)(C/T)TATTTGAAGAAGGATCG3′
D : 5′TAAAGTACC(A/T)CC(A/T)CCAAATTG3′

CnCc : AB primer	RnRc : CD primer
CnRn : AC primer	RnCc : BC primer
CnRc : AD primer	RcCc : BD primer

Fig. 1. Sequences and combination of primers.

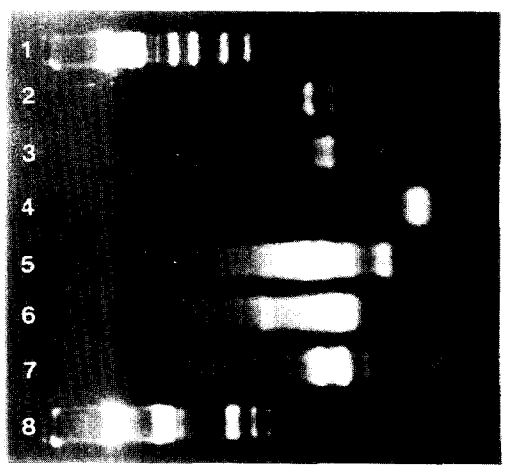

Fig. 2. Random amplified polymorphic DNA patterns of nuclear DNA from *Porphyra tenera* (wild type from Kumamoto region, Japan) with various primers. Lane 1, λ DNA(Sty I)+pUC19(Hae III) ; Lane 2, CnCc primer ; Lane 3, CnRn primer ; Lane 4, CnRc primer ; Lane 5, RnRc primer ; Lane 6, RnCc primer ; Lane 7, RcCc primer ; Lane 8, λDNA(Hind III/Eco RI).

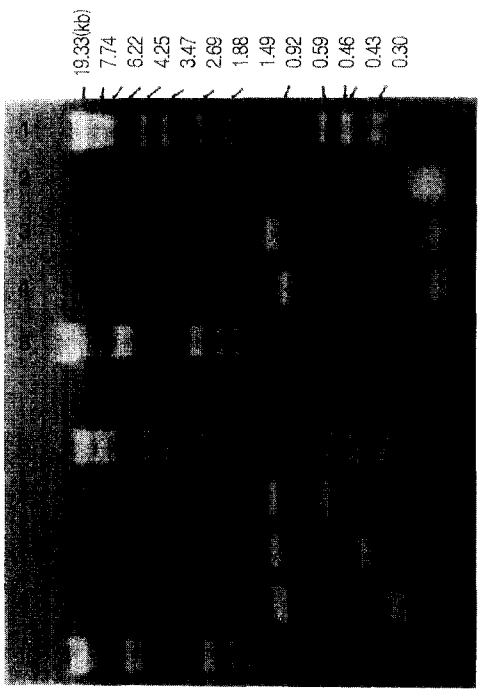

Fig. 3. Random amplified polymorphic DNA patterns(CnCc primer) of chloroplast DNA from *Porphyra.* Lanes 1,6, λDNA(Sty I)+pUC19 (Hae III) ; Lane 2, *P. yezoensis* (NBD) ; Lane 3, *P. yezoensis* (Noma-1) ; Lane 4, *P. yezoensis* (Enoura) ; Lane 7, *P. tenera* (wild type) ; Lane 8, *P. tenera* (Minomi) ; Lane 9, *P. tenera* (Ariake-1) ; Lanes 5,10, λDNA(Hind III/Eco RI).

The RAPD patterns of *P. yezoensis* (Enoura) chloroplast DNA distinguished a unique band at approximately 0.9kb generated by the CnCc primer (Fig. 3, lane 4). RAPD patterns of *P. tenera* chloroplast DNA also revealed three distinct genotypes, 0.75kb, 0.6kb and 0.55kb (Fig. 3, lanes 7 to 9). *Porphyra yezoensis* (Enoura) chloroplast DNA amplified with the RnRc primers generated one characteristic band (Fig. 4, lane 4). The nuclear DNA amplified with RnRc primer revealed two distinct genotypes, 0.7kb and 0.3kb (Fig. 5, lanes 2 to 3). Similarities among various RAPD patte-

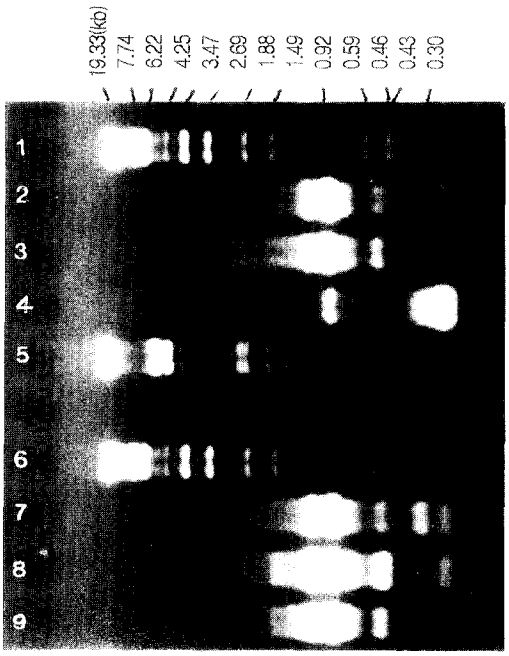

Fig. 4. Random amplified polymorphic DNA patterns (RnRc primer) of chloroplast DNA from *Porphyra*. Lane 1,6, λDNA(Sty I)+pUC19(Hae III) ; Lane 2, *P. yezoensis* (NBD) ; Lane 3, *P. yezoensis* (Noma-1) ; Lane 4, *P. yezoensis* (Enoura) ; Lane 5, λDNA(Hind III/Eco RI) ; Lane 6, *P. tenera* (wild type) ; Lane 8, *P. tenera* (Minomi) ; Lane 9, *P. tenera* (Ariake-1).

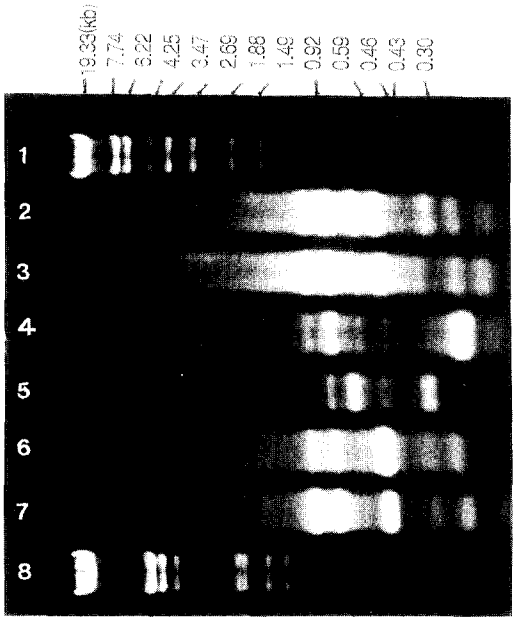

Fig. 5. Random amplified polymorphic DNA patterns (RnRc primer) of nuclear DNA from *Porphyra*. Lane 1, λDNA(Sty I)+pUC19(Hae III) ; Lane 2, *P. yezoensis* (NBD) ; Lane 3, *P. yezoensis* (Noma-1) ; Lane 4, *P. yezoensis* (Enoura) ; Lane 5, *P. tenera* (wild type) ; Lane 6, *P. tenera* (Minomi) ; Lane 7, *P. tenera* (Ariake-1) ; Lane 8, λDNA(Hind III/Eco RI).

rns from chloroplast and nuclear DNA of the six *Porphyra* isolates are shown in Table 1. Interestingly, similarity values of *P. tenera* (Ariake-1) nuclear DNA were closer to those of *P. yezoensis* and *P. tenera* (Minomi) than of *P. tenera* (wild). Miura et al. (1992) were also of the view that *P. tenera* (Ariake-1) is not a strain of *P. tenera*, but could

Table 1. Relation both between and within *Porphyra* species based on band sharing

	PY(NBD)	PY(,oma-1)	PY(Enoura)	PT(Wild)	PT(Minomi)	PT(Ariake-1)
PY(NBD)	–	0.714	0.714	0.364	0.462	0.714
PY(Noma-1)	0.889	–	0.714	0.364	0.769	0.714
PY(Enoura)	0.333	0.286	–	0.364	0.615	0.714
PT(Wild)	0.800	0.727	0.500	–	0.600	0.364
PT(Minomi)	0.889	0.800	0.571	0.727	–	0.615
PT(Ariake-1)	0.889	0.800	0.571	0.727	1.000	–

The value of 1.000 indicates that the two samples were identical. The upper matrix is RAPD analysis of nuclear DNA and the lower matrix is RAPD analysis of chloroplast DNA from *Porphyra*. PY, *P. yezoensis* ; PT, *P. tenera* ; PT(Wild), *P. tenera* wild type from Kumamoto region in Japan.

be a strain of *P. yezoensis*. Similarity values of *Porphyra* chloroplast DNA, except for *P. yezoensis* (Enoura), were high and ranged from 0.727 to 1.000.

To date, analysis of isozymes (Fujio et al., 1988) and restriction fragment length polymorphism have been used to distinguish *Porphyra* species (Araki et al., 19 92 ; Shivji et al., 1992 ; Mizukami et al., 1995). Dutcher and Kapraun (1994) reported genetic variation in three species of *Porphyra* using RAPD results from the total DNA with a single 20-mer M13 bacteriophage primer. We analyzed both chloroplast and nuclear DNA, and used 21-mer primers, especially double primers for the reproducibility of banding patterns. For RAPD analysis, primer selection is very important to differentiate individuals within a population. This study suggests that AP-PCR analysis from chloroplast and nuclear DNA can be useful in distinguishing both between and within *Porphyra* species.

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