USE OF RAPD FOR DETECTING AND IDENTIFYING *PORPHYRA* (BANGIALES, RHODOPHYTA)*

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Abstract Randomly amplified polymorphic DNA (RAPD) technique was applied to assess the genetic variations and phylogenetic relationships in 4 species of *Porphyra*. The samples were collected from the coast of Canada, Vietnam, Zhoushan, Fujian and Qingdao in China. Amplifications with 20 primers were carried out under predetermined optimal reaction conditions (samples were first heated at 94℃ for 5 min. and followed by 45 cycles of 1 min at 94℃, 1 min at 36℃, and 2 min at 72℃, then held at 72℃ for 10 min). The amplified products were scored as present (1) or absent (0) for each DNA sample and an index of genetic similarity (F) was calculated by using Nei & Li's matching coefficient method (1979). The value of (1 - F) was used to quantify the genetic distances between species and construct a phylogenetic tree. The relationship indicated by the UPGMA and NJ cluster analysis on the values of the genetic distance is in good overall agreement with classical taxonomy. The obvious differences between natural and cultivated population of *P. haitanensi* suggest that variation or hybridization with other species occurred during the culture.

Key words: *Porphyra*, RAPD, identification, phylogenetic relationship

INTRODUCTION

Many molecular biology based DNA techniques provide powerful means for studying of genetic variation and phylogenetic relationships. A molecular technique based on the polymerase chain reaction (PCR) is an effective tool for rapid identification of genetic markers known as Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990; Welsh & McClelland, 1990). Such markers, derived from priming sites randomly distributed throughout the genome, are inherited in a Mendelian pattern. These polymorphisms allow the analysis of complex genomes without prior knowledge of the DNA sequence (Hadrys et al., 1992). Potential uses of RAPD in phylogenetic studies and population genetics have been widely documented in a large variety of organisms(Dawson et al., 1995; Ho et al., 1995; Huff et al., 1993; Liu et al. 1993; Patwary et al. 1993, 1994; Van Oppen et al., 1995, 1996). Due to the cryptic diversities between *Porphyra* species and strains,

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work must be done to gain further understanding of the genetic background and the phylogenesis of the species. The objectives of our study are to identify the different species of *Porphyra*, detect the variations, and reveal the phylogenetic relationships among, and genetic background of, the species and strains.

MATERIALS AND METHODS

1. Tissues purification and culture

Four species of *Porphyra* were collected: *P. linearis* from Canada coast natural habitats, *P. vietnamensis* from Vietnam natural habitats, and *P. yezoensis* from Qingdao natural habitats. The conchocelis were isolated and preserved in the our lab. A strain *P. haitanensis*-1 was collected from the cultivated population in Zhoushan, Zhejiang Prov., and another strain *P. haitanensis*-2 was collected from the natural habitat population in Fujian Prov. Subculture of conchocelis was conducted for two weeks after isolation of the specimens from other alga.

2. Genomic DNA isolation

DNA extraction was carried out after the biomass reached a certain volume. Plastic pestles were used to grind 100 mg biomass in a microcentrifuge tube containing 100 mmol/L EDTA, 10 mmol/L Tris (pH 7.5), 1% SDS, and 50 μg/ml proteinase K. Samples were incubated at 55℃ for 2 h and extracted with phenol, phenol/chloroform (1:1), then chloroform/isoamyl alcohol (24:1). The resulting aqueous fraction was mixed with two volumes of ethanol and 1/10 volume 3 mol/L sodium acetate. Following centrifugation at 12000 g for 10 min, the DNA pellet was vacuum dried and dissolved in TE buffer.

3. PCR amplification and electrophoresis

PCR reaction mixtures (25 μl final volume) contained approximately 25 ng genomic DNA, dATP, dCTP, dGTP, dTTP each at 100 μmol/L final concentration, 1 × standard Taq polymerase buffer, 0.2 μmol/L primer, 2 mmol/L $MgCl_2$, and 1 unit of Taq polymerase (S_{ABC}). The 20 random sequence 10-mer primers used in this study were products of Operon Company. Forty five cycles were run as follows: 1 min at 94℃, 1 min at 36℃ and 2 min at 72℃. After the final cycle, samples were incubated for a further 10 min at 72℃, then held at 4℃ prior to analysis. Fragments generated by amplification were separated according to size on 1.5% agarose gels run in 1 × TBE (89 mmol/L Tris-HCl, pH 8.3, 89 mmol/L boric acid, 5 mmol/L EDTA), stained with ethidium bromide and observed under ultraviolet light.

4. Data analysis

Different fragments produced with each primer were numbered sequentially, and the presence or absence of a fragments in each sample was recorded in a binary matrix as 1 if present, or 0 if absent. The matrix was analysed using the polymorphism parsimony

method in the phylogenetic inference package PHYLIP (V3.5 phylogenetic inference software). The data were used to calculate Nei's similarity index: $S_{AB} = 2N_{AB}/(N_A + N_B)$, where N_{AB} is the number of shared fragments, N_A the number of fragments from species A, and N_B the number of fragments from species B (Nei & Li, 1979). The similarity index matrices were then converted into distances (d) using the formula $d = 1 - S$ (Swofford & Olsen, 1990). The distance matrices were subjected to cluster analysis using the program NEIGHBOR in PHYLIP with the UPGMA (unweighted pair group method with arithmetic mean) algorithm and *NJ* (neighbor joining) method. Phenograms were plotted with the program DRAWGRAM in PHYLIP.

RESULTS

1. Amplification of DNA from porphyra

The amplifications were carried out twice independently using the same PCR machine (PE9600), yielding reproducible results. Amplifications with 20 primers under predetermined optimal reaction conditions yielded 164 reproducible amplified fragments ranging from 230 to 2800 bp. Polymorphic fragments (141) comprised 86% of the number of amplified fragments. The primers yielded different species-specific patterns of length and intensity of the amplification fragments (Fig. 1 shows RAPDs obtained with primers OPL-02, OPL-03, OPL-11 and OP-12).

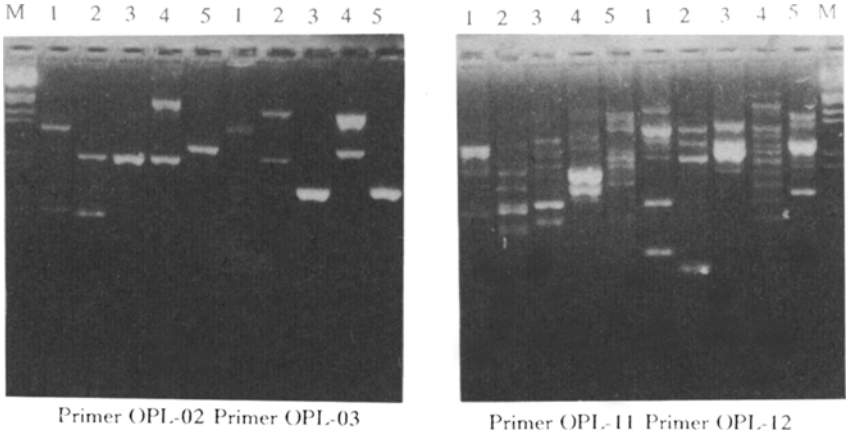

Fig. 1 Electrophoresis patterns of RAPD from four species of *Porphyra* after random amplification with primers OPL-02, 03, 11 and 12

1: *P. haitanensis*-1; 2: *P. vietnamensis*; 3: *P. haitanensis*-2; 4: *P. yezoensis*; 5: *P. linearis*. M: DNA Ecor I/Hind

2. Analysis of RAPD data

The fragments in each sample were recorded in a binary matrix as 1 (present) or 0 (absent), and the data on band sharing were collected and analysed. The generated similarity index matrix and distance matrix are shown in Table 1, in which values of S, the

similarity index (also called the proportion of shared fragments) between the species, are shown to the right of the diagonal, and the values of d, the genetic distance, are shown to the left of the diagonal. Using the methods of UPGMA and NJ in the program NEIGHBOR in PHYLIP, the best trees for these data were generated and are shown in Fig. 2 and Fig. 3. They are in good agreement with each other.

Table 1 Matrix showing values of S (similarity index) to the right of the diagonal, and the values of d (the genetic distance) to the left of the diagonal

	P. haitanensis - 1	*P. vietnamensis*	*P. haitanensis* - 2	*P. yezoensis*	*P. linearis*
P. haitanensis - 1	—	0.32373	0.25000	0.05128	0.09523
P. vietnamensis	0.68627	—	0.25532	0.21739	0.20408
P. haitanensis - 2	0.85000	0.74468	—	0.28571	0.26315
P. yezoensis	0.94872	0.78261	0.71429	—	0.32432
P. linearis	0.90476	0.79592	0.73684	0.67568	

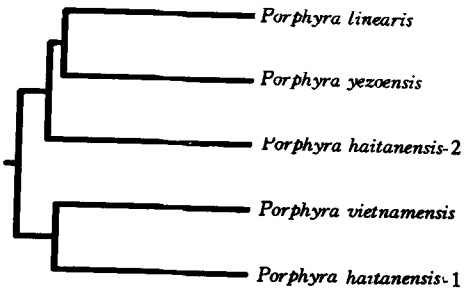

Fig. 2 Phylogenetic tree based on the genetic distance matrices with the method of UPGMA

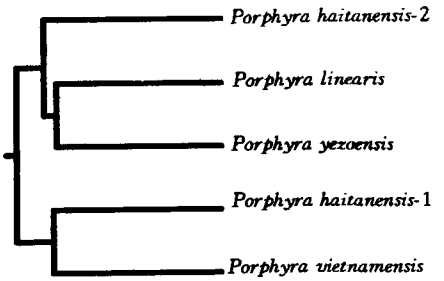

Fig. 3 Phylogenetic tree based on the genetic distance matrices with the method of NJ

DISCUSSION

Despite the continuous accumulation of morphological and habit data, systematic and phylogenetic relationships among many *Porphyra* are not yet completely understood. Advances in biochemical and molecular methods greatly help us to observe the variability of the genes and gene products among species, strains, and populations.

New methods (such as RAPD, RFLP, AFLP, and DNA fragment sequencing) can reveal the variations much more extensively. The analysis of nucleotide sequence variability has been revolutionized by the development of the polymerase chain reaction (PCR) technique (Saiki et al., 1988). Random amplified polymorphic DNA (RAPD) (Welsh and McCelland, 1990; Williams et al., 1990) is a PCR based method used for identification purpose, and this technique is rapid and useful for detecting a large number of poly-

morphisms. RAPD assay is essentially a novel PCR technique using a single oligonucleotide primer (of arbitrary nucleotide sequence and length) to initiate DNA strand synthesis. This primer will anneal to a number of complementary binding sites scattered throughout the target genome. The ensuing thermal cycling generates several amplification products in a single reaction. Advantages of the technique are that no prior knowledge of the molecular biology of the investigated organisms is required, cost-effectiveness, and theoretically larger number of DNA polymorphisms that can be detected compared with traditional methods.

We applied RAPD analysis to study the systematic relationships among four species of *Porphyra*, collected from different regions of the world: cold water species *P. yezoensis*, from Qingdao, and *P. linearis*, from Canada; and warm water species *P. haitanensis* and *P. vietnamensis*. The dendrogram constructed from the RAPD analysis shows clearly the clustering of *P. haitanensis* and *P. vietnamensis* and of *P. yezoensis* and *P. linearis*, and that species living in the same environmental conditions share a higher similarity. The relationships revealed by RAPD markers are also in good agreement with classical taxonomy, indicating RAPD markers can feasibly be used to detect and identify the strains of *Porphyra*.

Even though *P. haitanensis* – 1 and *P. haitanensis* – 2 belong to the same species (the former came from the cultivated population in Zhoushan, China, the latter from the natural population in Fujian) the similarly index between them is low (0.25000), even lower than the similarly index between *P. haitanensis* – 1and *P. vietnamensis* (0.32373). In the dendrogram, *P. haitanensis* – 1 clustered with *P. vietnamensis* first, then with *P. haitanensis* – 2. As *P. haitanensis* – 1 is the cultivated strain, and the environment and other factors in Zhoushan are different from those in Fujian, we considered that genetic mutation, differentiation and hybridization with other species must have occurred during the cultivation.

The results suggested that RAPDs can clearly show the differences between species and strains in *Porphyra*, and can provide an efficient and sensitive method to detect the variability, and determine the status of *Porphyra* in the phylogenetic tree and skirt the controversial systematics. As variations of *Porphyra* occur frequently with changes of environment and other factors, strains verification is still important for determing if the still traditional classification of *Porphyra* is correct for some species, and so must be studied further in detail. The results of our study suggest that RAPD approaches will be as useful in providing markers for *Porphyra* genetics as they have been for other species.

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