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Genetic diversity and phylogenetic relationships in five *Porphyra* species revealed by RAPD analysis

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Summary

The genus *Porphyra* comprises red algae known as laver. They are distributed throughout Asia and used as food in Korea and other countries. Five species of *Porphyra* (*P. tenera, P. yezoensis, P. seriata, P. suborbiculata* and *P. dentata*) are regarded as ecologically and economically important in Korea. We used random amplified polymorphic DNA (RAPD) to investigate their genetic diversity and phylogenetic relationships. The analysis of ten primers revealed 97 loci, of which 70 were polymorphic (72.2%). *P. seriata* had the highest genetic diversity (0.147), and *P. dentata*, the lowest one (0.059). An indirect estimate of the number of migrants per generation ($Nm = 0.212$) indicated that gene flow was very low among the populations studied. The classification of *Porphyra* species based on the DNA markers does not fully coincide with the classification based on morphology.

Key words: *Porphyra*, random amplified polymorphic DNA, phylogenetic relationships

Introduction

Seaweeds are an important source of food in China, Korea, and Japan. According to Xia and Abbott (1987), most families in northeastern China eat a seaweed dish at least once a day and over 100 million pounds of seaweed are consumed annually all over the country. Of the 73 seaweed species listed by Xia and Abbott as food items in China, 47 are red algae, 15 are green algae, and 11 are brown algae. Of the 28 genera of red algae contributing to the Chinese diet, four genera account for half of the species: *Porphyra* (8 spp.), *Gracillaria* (6 spp.), *Gelidium* (5 spp.) and *Hypnea* (4 spp.). Although many of the same species are used in Korea, some are different. The Chinese prefer seaweeds as a hot dish, broth or soup, whereas the Koreans would rather cook them beforehand and then eat cold or dry as boiled rice wrapping or in Korean salad. Five species of *Porphyra* are abundant in Korean marine areas (Hwang and Lee, 2002). They are known as laver and eaten with boiled

rice. The Korean populations of five *Porphyra* species are typically distributed in patches. The cultivation of these species has been very popular in the south and eastern coast of Korea (Lee and Kang, 1986). Laver is known to be one of the sources of potassium and calcium in organic form as well as carotene (Pearson, 1995). Thus, these species play an important role in Asian ecology and economy.

Although molecular and biochemical approaches are now increasingly being applied to taxonomic and phylogenetic relationships within animals and plants in Korea, no population genetic studies of algal species have been conducted. Taxonomy of *Porphyra* mostly relies on morphological characteristics and allozyme analysis (Hwang et al., 1998). However, the resolving power of morphological characteristics is restricted, mainly because of the small number of characters available. Efficient methods to clarify the taxonomic status of several species are much needed.

RAPD assay has been useful in determining genetic relationships among closely related species (Demeke et al., 1992; Beebe et al., 2000). It is quick, robust and requires minimal preliminary work (Kresovich et al., 1992; Molnar et al., 2000). We expected that RAPD analyses could be used to assess the amount and structure of genetic diversity within and between natural populations and to discriminate all the tested genotypes more finely than the allozymes (Huh and Ohnishi, 2001). Hence it would be possible to characterize genetic relationships in local natural populations of *Porphyra* in Korea.

Therefore, the objectives of this study were to determine the amount of genetic diversity in the genus *Porphyra* and to describe how species-specific markers are distributed among species. Insights into the relative gene diversity within and between wild populations of *Porphyra* would be useful in plant breeding as well as for development of strategies of *ex situ* conservation of genetic resources.

Material and Methods

Plant material

Material was obtained from five species of the genus *Porphyra: P. tenera, P. yezoensis, P. seriata, P. suborbiculata*, and *P. dentata* in Korea (Table 1). The algae were identified according to Hwang and Lee (2002). Forty plants of each species were randomly gathered from each species during the period from 2004 to 2005. The population of every species was divided into two subpopulations (patches) to examine molecular variation within and between subpopulations. *Undaria pinnatifida* (Harvey) Suringar was used as the outgroup.

DNA extraction

The genomic DNA of the 240 samples including the outgroup was extracted from fresh blades using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, U.S.A.) according to the manufacturer's protocol.

RAPD analysis

Twenty arbitrarily chosen 10-mer primers, the kit D (OPD-01 to 20) of Operon Technologies (Alameda, Co.), were used. All the reactions were repeated twice and only reproducible bands were scored for analyses. To analyze the DNA of individuals, we selected 10 decamer primers that produced RAPD bands in five species in a preliminary test (Table 2).

Amplification reactions were performed in 0.6 ml tubes containing 25 μl of the reaction buffer; 10 mM Tris-HCl, pH 8.8, 50 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP, dTTP, 0.2 mM primer, 2.1 units Taq DNA polymerase, and 25 ng of genomic DNA. A 100 bp ladder DNA marker (Pharmacia) was used for the estimation of fragment size. The amplification products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light using Alpha Image TM (Alpha Innotech Co., USA).

Statistical analyses

All RAPD bands were scored by eye and only unambiguously scored bands were used in the analyses. Since RAPDs are dominant markers, it was assumed that each band corresponded to a single character with two alleles, presence (1) and absence (0) of the band, respectively.

The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh et al. (1999): the percentage of polymorphic loci (P_P); mean numbers of alleles per locus (A); effective number of alleles per locus (A_e); and gene diversity (H) (Lewontin, 1972). The degree of polymorphism was quantified using Shannon's index (H_0) of phenotypic diversity (Bowman et al., 1971).

To analyze the organization of variability within *Porphyra*, we examined the genetic variation by partitioning the total genetic diversity (H_T) into an intra-species component (H_S) and an inter-species one (G_{ST}). Furthermore, gene flow (Nm) between the pairs of populations was calculated from G_{ST} values by $Nm = 0.5(1/G_{ST}-1)$ (McDermott and McDonald, 1993).

To elucidate the extent of genetic divergence between the populations, Nei's genetic identity (GI) and genetic distance (D) were calculated for each pairwise

Table 1. Collection sites of the algae used in the study.

Species	Locality of populations
Porphyra tenera	Myeonggi-dong, Gangseo-gu, Busan Province
P. yezoensis	Heuidong-ri, Jindo-gun, Chonlanam-do Province
P. seriata	Samcheon, Sacheon-ci, Gyeongsangnam-do Province
P. suborbiculata	Gokyeumdo, Wando-gun, Chonlanam-do Province
P. dentata	Jeongdo-ri, Wando-gun, Chonlanam-do Province
Undaria pinnatifida	Jeongdo-ri, Wando-gun, Chonlanam-do Province

combination of populations (Nei, 1973). Homogeneity of variance among species was tested by Bartlett's statistics.

A phylogenetic tree was constructed by the neighbor-joining (NJ) method using the NEIGHBOR program in PHYLIP version 3.57 (Felsenstein, 1993).

Results

From the 20 decamer primers used for a preliminary RAPD analysis, ten primers yielded good amplification products both in quality and variability (Table 2). Overall, 97 fragments were generated among the *Porphyra* array tested. At the species level, 70 of the 97 loci (72.2%) showed detectable polymorphism in at least one species. Invariant fragments ranged from 1 to 6 per primer.

In a simple measure of intra-species variability by the percentage of polymorphic bands, *P. seriata* exhibited the highest variation (38.1%). *P. dentata* showed the lowest variation (15.5%) (Table 3). The mean numbers of alleles per locus (A) and effective number of alleles per locus (Ae) revealed the same trends for *P. seriata* and *P. dentata*.

The phenotypic frequency of each band was calculated and used to estimate genetic diversity (H) within species. The mean H was 0.103 across species, varying from 0.059 to 0.147. Shannon's index of phenotypic diversity (H_0) was highest in *P. seriata* (0.219), and showed significant difference (paired t test). *P. dentata* had the lowest expected diversity (0.088).

As typical populations of *Porphyra* are small, isolated, and patchily distributed, they maintain a low level of total genetic diversity ($H_T = 0.109$) (Table 4). The mean H_T was 0.109 across species, varying from 0.226 in *P. seriata* to 0.022 in *P. yezoensis*. Measures of total genetic variability (paired t test) showed significant difference.

It was shown that most of the genetic variation (64%) resided within species. Inter-species diversity accounted for 36% of the total genetic diversity (Table 4). The average number of individuals exchanged between populations per generation (Nm) was estimated to be very moderate or low (0.926).

Genetic identity (GI) based on the proportion of shared fragments was used to evaluate relatedness among five species. GI ranged from 0.490 to 0.966 (Table 5).

Clustering of species, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 1). The phylogenic tree showed three distinct groups: *P. dentata* and *P. yezoensis, P. tenera* and *P. suborbiculata*, and *P. seriata*. *P. seriata* is resolved as sister group to *P. tenera* and *P. suborbiculata.*

OPD4-01, OPD4-03 and OPD6-02 loci can be recognized as unique loci of *P. seriata.* OPE05-03, OPE05-07, and OPE08-07 loci were not found in *P. tenera, P. suborbiculata,* and *P. seriata*. Thus, these loci can be used to distinguish the group "*P. yezoensis* and *P. dentata*" from other groups. *P. yezoensis* does not have OPD10-05 locus, so it can be used to distinguish *P. dentata* from *P. yezoensis. P. tenera* does not have OPD08-04 locus that is present in *P. suborbiculata.*

Table 2. List of decamer oligonucleotide used as primers, their sequences, and associated polymorphic fragments amplified in the *Porphyra* representatives.

Primer	Sequence(5' to 3')	Number of bands	Number of polymorphic bands	Percentage of polymorphic bands
OPD01	ACCGCGAACG	7	4	57.1
OPD02	CGACCCAACC	13	12	92.3
OPD03	GTCGCCGTCA	11	8	72.7
OPD04	TCTGGTGAGG	6	4	66.7
OPD05	TGAGCGGACA	12	6	50.0
OPD06	ACCTGAACGG	10	7	70.0
OPD07	TTGGCACGGG	8	5	62.5
OPD08	GTGTGCCCA	13	10	76.9
OPD09	CTCTGGAGAC	7	5	71.4
OPD10	GGTCTACACC	10	9	90.0
Total		97	70	72.2

Table 3. Measures of genetic variation for RAPD generated among species. The number of polymorphic loci (Np), percentage of polymorphism (*P*p), mean number of alleles per locus (*A*), effective number of alleles per locus (*A*e), gene diversity (*H*), and Shannon's information index (*H*o).

Species	N_P	P_P	*A*	*Ae*	*H*	H_O
P. tenera	22	22.68	1.227	1.165	0.094	0.137
P. yezoensis	32	32.99	1.330	1.214	0.129	0.191
P. seriata	37	38.14	1.381	1.241	0.147	0.219
P. suborbiculata	21	21.65	1.217	1.138	0.084	0.124
P. dentata	15	15.46	1.155	1.097	0.059	0.088
Mean	25.4	26.18	1.262	1.171	0.103	0.151
t-test	*	*	*ns*	*	**	**

ns: Not significant; * = $p < 0.05$; ** = $p < 0.01$.

congeneric species (Ham-rick et al., 1992). However, this high value is not especially surprising if we consider that the *Porphyra* collections were made over a narrow geographic area. Mean genetic identity of the *Porphyra* species is slightly higher than expected. It is probable that directional movement toward genetic similarity in a relatively homogeneous habitat operates in the populations of *Porphyra*.

An indirect estimate of gene flow based on G_{ST} was 0.926. It is similar to or lower than that in eelgrass, *Zostera marina*, where it was shown to be 1.1-2.8 (Ruckelshaus, 1998). *Z. marina* is a perennial angiosperm inhabiting soft-bottom marine habitats, ranging from the intertidal zone to depths of approximately 15 m in temperate latitudes (Den Hartog, 1970). An *Nm* value greater than 1.0 is considered necessary to prevent divergence resulting from genetic drift (Wright, 1951). Although the level of gene flow in *Porphyra* is not high enough to counter-balance genetic drift, these values were lower than those obtained for other species (Hamrick et al., 1992).

Discussion

Genetic diversity and population structure

Estimates of genetic diversity in *Porphyra* obtained from RAPD analysis can be compared with those obtained from allozyme analysis. One may also try to compare genetic diversity in *Porphyra* and in other marine algae, though differences between investigations (in the number of loci, populations sampled, and the enzyme systems studied) may preclude meaningful direct comparisons. The percentage of polymorphic loci (P_P) and mean numbers of alleles per locus (*A*) obtained for *Porphyra* in the present investigation are 26.2% and 1.3, respectively. Hwang with coauthors analyzed eleven taxa of *Porphyra* by starch gel electrophoresis. In that study, P_P and *A* were 21.7 and 1.4, respectively (Hwang et al., 1998). Estimates of polymorphism of both RAPD and isozyme markers are, therefore, very similar for the genus. It can be concluded that RAPD and isozymes reveal similar patterns of genetic diversity. These markers in *Porphyra* probably experience similar evolutionary forces.

A striking feature revealed in this study is a high intra-species variation (64%). Taking into account the sexual reproduction and outcrossed mating systems of *Porphyra*, the mean identity value of 0.664 shown for the five species is higher than it could be expected for

Table 4. Total genetic diversity (H_T), intra-species genetic diversity (H_S), inter-species genetic diversity (G_{ST}) and gene flow (*Nm*) between the pairs of species.

Locus	H_T	H_S	G_{ST}	*Nm*
P. dentata	0.066	0.035	0.474	0.556
P. yezoensis	0.022	0.015	0.333	1.000
P. seriata	0.226	0.151	0.333	1.000
P. suborbiculata	0.059	0.042	0.290	1.222
P. tenera	0.172	0.109	0.370	0.852
Mean	0.109	0.070	0.360	0.926
t-test	**	**	*ns*	*

ns: Not significant; * = $p < 0.05$; ** = $p < 0.01$.

Phylogenetic relationships within *Porphyra*

In the generated phylogeny, *P. dentata* and *P. tenera* clades are separated from each other, these two species being also distinguishable from each other in blades. The position of *P. dentata, P. suborbiculata*, and *P. seriata* on the phylogenetic tree matches very well their classification according to morphological characters. However, our results show *P. dentata* and *P. yezoensis* to be closely related, while previous morphological taxonomic studies did not consider these two species as very close relatives. *P. dentate* is an androdioeious species, whereas *P. yezoensis* is a monoecious one (Miura, 1988).

Since species of *Porphyra* show a wide range of morphological and geographical variation, it is difficult

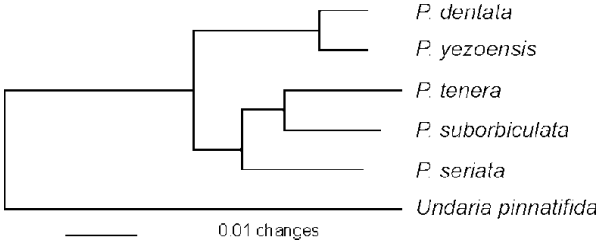

Fig. 1. A phylogenetic tree of five *Porphyra* species based on RAPD analysis.

Table 5. Nei's unbiased genetic identity values of among *Porphyra* species (above diagonal) and genetic distances among species (below diagonal).

Locus	*P. dentata*	*P. yezoensis*	*P. seriata*	*P. suborbiculata*	*P. tenera*
P. dentata	-	0.737	0.698	0.667	0.556
P. yezoensis	0.305	-	0.652	0.572	0.966
P. seriata	0.572	0.560	-	0.593	0.490
P. suborbiculata	0.360	0.428	0.522	-	0.696
P. tenera	0.384	0.035	0.713	0.362	-

to elucidate phylogenetic relationships using morphological characteristics (Hwang and Lee, 2002). Molecular approaches could be applied for this purpose. At present there is good agreement about the relationships of *Porphyra* species derived from allozyme analysis (Hwang et al., 1998). Though a small number of species in our study does not allow a detailed discussion of relationships within the *Porphyra* complex, it shows that further RAPD analysis will certainly provide an enhanced view on the phylogeny of this genus. Additional molecular experiments such as AFLP, SSR, and ITS would be also useful.

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