

Phylogeny Study of Genus *Pelvetia* in Korea by Internal Transcribed Spacer Sequence (ITS)

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The brown algae, or phaeophytes, are a large group of multicellular algae, including many notable types of seaweed. We analysed intra- and interspecific phylogenetic studies within the genus *Pelvetia* in Korea and compared them with results of both same and different species in GenBank. The sequences for *P. babingtonii* in Korea were generally similar to those for *P. babingtonii* AF102957, and the sequences of *P. siliquosa* in Korea were also similar to those of *P. siliquosa* AF102958. Sequence variation within the *Pelvetia* is mostly due to nucleotide substitutions, although several small indels and some large indels can be found. Another source of sequence divergence is length variation due to stretches of short repeats that occur at the ITS1 or ITS2 in all the *Pelvetia*. NJ analysis consists mainly of two clades. One of them contains *P. canaliculata* and *P. limitata*, and is sister to the rest of the genus (*P. siliquosa*, *P. compressa*, and *P. babingtonii*). *P. babingtonii* is not grouped one clade. In the MP analysis, ten accessions or populations were fully resolved and all accessions from the same species formed with 99% or 100% bootstrap supports.

Key words : ITS, phylogenetic analyses, *Pelvetia babingtonii*, *P. siliquosa*

Introduction

Algae encompass several different groups of usually relatively simple living organisms that capture light energy through photosynthesis, converting inorganic substances into simple sugars using the captured energy [1].

The brown algae or phaeophytes are a large group of multicellular algae, including many notable seaweeds [2]. They play an important role in marine environments. For instance *Macrocystis* kelp, which may reach 60 m in length, form prominent underwater forests [3]. Many brown algae, such as *Fucus*, of which bladder wrack is one species, are found along the seashore and some are used as food. Brown algae belong to a very large group called the heterokonts, most of which are colored flagellates [4].

Brown algae belong to a very large group called the heterokonts, most of which are colored flagellates. Most contain the pigment fucoxanthin, which is responsible for the distinctive greenish-brown color that gives brown algae their name. Brown algae are unique among heterokonts in developing into multicellular forms with differentiated tissues, but they reproduce by means of flagellate spore, which

closely resemble other heterokont cells. Genetic studies show their closest relatives are the yellow-green algae.

Any species with a highly structured variation pattern must show different distributions of phenotypic characteristics at different locations. Data drawn from such a population would strongly distort intra-generic taxonomic conclusions, unless allowance is made for the structure [15]. In a taxonomic study, Rice and Chapman [15] showed that they are discrete, and presented some intraspecific variation information to support this conclusion. The form of this variation is examined here in detail, and possible causes of the observed structures are considered.

Brown algae of the Fucaeeae dominate the biomass. Many macroalgal species are noted for showing great intraspecific phenotypic variability [13,17]. The factors controlling this variation have often been studied, frequently with regard to the relative importance of genetic and environmental influences [17]. However, the structural form of the variability and its expression in geographic space have rarely been considered.

Pelvetia is a brown algae a genus of seaweed that lives in the intertidal zones of rocky shores, found on the Atlantic coasts of Europe and North America [1,5].

In this paper, analyses of the intraspecific molecular variation patterns of *Pelvetia babingtonii* Korea is first presented. This species is an intertidal brown seaweed of North Pacific rocky shores. It is suppose that this species have been in-

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troduced from Japan by sea current or entry of ships. We analysed intra- and interspecific phylogenetic relationships within genus *Pelvetia* in Korea and to compare with the results of same and other species in GenBank.

Materials and Methods

Sample materials

The genus *Pelvetia* consists of six species and two varieties; *Pelvetia babingtonii*, *Pelvetia calaliculata* var. *acutiloba*, *Pelvetia canaliculata*, *Pelvetia compressa* (formerly *Pelvetia fastigiata*), *Pelvetia galapagensis*, *Pelvetia siliquosa*, *Pelvetia wrightii*, *Pelvetia wrightii* f. *babingtonii*. Most species of *Pelvetia* occur in the Pacific except *P. canaliculata*. *P. canaliculata* occurs exclusively in the Atlantic.

The ITS sequences included in this study were obtained from GenBank. In addition, the ITS sequences of all Korean occurred species were used in this study to compare with the results of same species in the list of GenBank. Table 1 lists the genes, taxa, and GenBank accession numbers. The data and alignments are available on request from the author.

P. babingtonii and *P. siliquosa* were collected from two populations in Korea (Table 1). Fifteen plants were randomly collected from the each population. The use of exclusively mature plants reduces the effects of age on morphology in the data set, since two species mature when two-years old, following the definitions given by Rice and Chapman [15].

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

ITS analysis

Primer sets of about 20 bases in length were used for PCR

analysis. These primers were based on well-characteristic DNA sequences and were designed making use of conserved regions of the 18S and 28S rRNA genes to amplify the noncoding regions between the ITS1 and ITS2 and 5.8S rRNA gene [21]). The ITS region of each sample was symmetrically amplified by PCR either with the primers ITS5 and ITS4 [21] (Table 2) or with F5 and F4, which we specifically designed for *Fucus* from a sequence for the small subunit (SSU) of rDNA available for *F. gardneri* at GenBank (X53987) and on our own sequences for the 59 end of the LSU of *Fucus*.

PCR materials (50 μ l volume) included 50 ng of genomic DNA, 100 μ M of each dNTP, 0.2 μ M of each primer, 1x enzyme buffer, and 2 unit of Taq polymerase. The amplification profile was 28 cycles of 94°C for 30 sec, 42°C for 60 sec, 72°C for 60 sec, preceded by an initial denaturation at 94°C for 90 sec and followed by a final extension at 72°C for 5 min.

PCR products were separated on 1.5% agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN). The amplified fragments were cloned into a bluescript vector and sequenced using ABI Prism 377 Sequencer (Applied Biosystem, USA). At least seven individuals' clones of each population were analyzed.

Alignment and phylogenetic analysis

The chromatogram output for each sample was edited using the software Sequence Navigator 1.0.1 (Applied Biosystems Inc.), and the sequences were manually aligned. To determine the ITS boundaries for the Fucaceae, the flanking sequences of ITS-1 and ITS-2 were aligned with all ITS sequences listed in GenBank that include the flanking sequence for the SSU, the 5.8S, and the LSU genes [19]. The limits of ITS-1 and ITS-2 varied among all these sequences; thus, the positions at which a sharp change occurs from a

Table 1. Accessions used in the molecular study of the genus *Pelvetia babingtonii* including population locations and GenBank accession numbers

Taxon	Code	Geographic origin	GenBank accession number
<i>P. canaliculata</i>	<i>P. canaliculata</i> 953	Sotra, near Bergen, southern Norway	AF102953
	<i>P. canaliculata</i> 955	Port St. Mary, Chapel Bay, Isle of Man, USA	AF102955
<i>P. limitata</i>	<i>P. limitata</i> 951	California, Pigeon Point, San Mateo, USA	AF102951
	<i>P. limitata</i> 952	Oregon, Boiler Bay, USA	AF102952
<i>P. compressa</i>	<i>P. compressa</i> 956	California, Pigeon Point, San Mateo, USA	AF102956
<i>P. babingtonii</i>	<i>P. babingtonii</i> 957	Muroran, Hokkaido, Japan	AF102957
	<i>P. babingtonii</i> Korea	Gijang-gun, Busan, Korea	This study
<i>P. siliquosa</i>	<i>P. siliquosa</i> 958	Shadong Peninsula, China	AF102958
	<i>P. siliquosa</i> Korea	Namhae-gun, Gyeongsangnam-do, Korea	This study

Table 2. Synthetic primer sequencing (5'-3') used for the ITS analysis in this study

No.	Primer	Reference
ITS1F	5'-TCCGTAGGTGAACCTGCGG-3'	White et al. (1990)
ITS2	5'-GCTGCGTTCTTCATCGATGC-3'	White et al. (1990)
ITS3	5'-GCATCGATGAAGAACGCAGC-3'	White et al. (1990)
ITS4R	5'-TCCTCCGCTTATTGATATGC-3'	White et al. (1990)
F1	5'-CGCACCTACCGATTGAATGT-3'	Serrato et al. (1999)
F2	5'-GCGACTTGCAGAATCCAGTG-3'	Serrato et al. (1999)
F3	5'-ACAGACTCCGACAAGCAT-3'	Serrato et al. (1999)
F4	5'-CTCTTGCTTGATCGGAGGTC-3'	Serrato et al. (1999)

sequence that is conserved across all taxa to a sequence that becomes variable (and vice versa for the 39 ends of the ITS regions) are those most likely to be the real boundaries between the rRNA genes and the spacers, and these were considered as such in this study.

A pairwise alignment was calculated using the ClustalX program. Phylogenetic relationship were estimated by MEGA version 4.1 [20] treating all alignment gaps as missing. A maximum parsimony tree (MP) was inferred using heuristic search, branch-swapping options and tree bisection-reconnection. Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated sampling of the data. In addition, a phylogenetic tree was constructed by the neighbor-joining (NJ) method [18] using the NEIGHBOR program in PHYLIP version 3.57 [7].

Results

The complete ITS1, 5.8S rRNA coding region, ITS2, and stretch of the 5' end of the large subunit rRNA coding region were PCR amplified, cloned, and sequenced for the both Korean distributed species, *P. babingtonii* and *P. siliquosa*. The aligned data sets for *P. babingtonii* and *P. siliquosa* are 949

nucleotides (bp), 933 bp, and 995 bp, respectively (Table 3). The ITS sequences of same and other species in same genus included in this study were obtained from GenBank.

The sequences for *P. babingtonii* Korea were generally similar to those for *P. babingtonii* AF102957 and the sequences of *P. siliquosa* in Korea were also similar to those for *P. siliquosa* AF102958.

Aligned nucleotide sequences of the length of ITS1 were varied within genus *Pelvetia* varying from 401 in *P. babingtonii* and *P. siliquosa* to 486 in *P. limitata*. Especially, *P. siliquosa* included 14-72 bp more in the ITS1 than those *P. canaliculata*. The 5.8S subunit of all taxa of *Pelvetia* was found to constant of 152-153 bp nucleotides. Aligned nucleotide sequences of the length of ITS2 varied from 271 bp in *P. canaliculata* AF102955 to 409 bp in *P. babingtonii* AF102957. *P. canaliculata* and showed the highest number of different bases between all the sequences studied.

Total alignment length is 1161 positions, of which 273 are parsimony-informative, 51 variable but parsimony-uninformative, and 122 constant characters. The base furtherance of G+C content was showed 47%-53%, and it is 56%-60% in ITS-2 (Table 3). The base furtherance showed the difference to the by a total taxon (Table 4). A distances matrix as the fraction of sites between two sequences in a multiple alignment among species was used to evaluate relatedness among species (Table 4). The distances between same species ranged from a minimum value of 0.001 between *P. siliquosa* 958 and *P. siliquosa* Korea and the maximum value of 0.005 between *P. limitata* 951 and *P. limitata* 952.

Alignment of the DNA sequences required adding numerous gaps. Sequence variation within the *Pelvetia* is mostly due to nucleotide substitutions, although several small indels and some large indels can be found. Another source of sequence divergence is length variation due to stretches of short re-

Table 3. Sizes and GC contents of ITS-1, 5.8S and ITS-2 regions of genus *Pelvetia*

Taxon	ITS-1		5.8S		ITS-2		Total
	Length (bp)	GC (%)	Length (bp)	GC (%)	Length (bp)	GC (%)	Length (bp)
<i>P. canaliculata</i> 953	414	53	153	52	383	58	950
<i>P. canaliculata</i> 955	413	53	153	52	271	59	837
<i>P. limitata</i> 951	486	51	152	51	387	56	1025
<i>P. limitata</i> 952	456	52	152	51	387	56	995
<i>P. compressa</i> 956	404	48	153	51	398	60	955
<i>P. babingtonii</i> 957	401	47	153	52	409	60	963
<i>P. babingtonii</i> Korea	402	49	153	52	388	58	933
<i>P. siliquosa</i> 958	401	47	153	52	385	60	939
<i>P. siliquosa</i> Korea	401	47	153	52	386	59	940

The codes of taxon are the same as Table 1.

Table 4. The distances as the fraction of sites between two sequences in a multiple alignment among species of genus *Pelvetia* using ITS analysis

Taxon	can 953	can 955	lim 951	lim 952	com 956	bab 957	bab Kor	sil 958	sil Kor
<i>P. canaliculata</i> 953	-								
<i>P. canaliculata</i> 955	0.002	-							
<i>P. limitata</i> 951	1.258	1.267	-						
<i>P. limitata</i> 952	1.267	1.276	0.005	-					
<i>P. compressa</i> 956	2.655	2.666	2.396	2.356	-				
<i>P. babingtonii</i> 957	2.283	2.289	2.079	2.074	0.354	-			
<i>P. babingtonii</i> Korea	2.300	2.306	2.093	2.088	0.355	0.002	-		
<i>P. siliquosa</i> 958	2.538	2.546	2.480	2.486	0.437	0.141	0.141	-	
<i>P. siliquosa</i> Korea	2.516	2.524	2.459	2.465	2.434	0.139	0.139	0.001	-

The taxon codes of first line are the same as those of first column.

peats that occur at the ITS1 or ITS2 in all the *Pelvetia*.

Within *Pelvetia*, *P. siliquosa* is strikingly different from the others (Table 3). *P. canaliculata* is occurs the Atlantic was also divergence from the Pacific species of *Pelvetia*, whereas these differ from each other by only 0.1%-1.2% in their ITS regions. In *Pelvetia*, the divergence between ITS sequences from different geographic regions is in many cases in the same order of magnitude as the divergence between different species (Table 4). Considerable within-individual variability was detected in *P. canaliculata* and *P. limitata*, the species of *P. babingtonii* for which more within-individual sequences were obtained, from several individuals from different geographic regions. In *P. babingtonii* in Korea, most ITS sequences differed by about 0.1% from the other ITS sequences determined from the same species in America.

NJ analysis consists mainly of two clades (Fig. 1). One of them contains *P. canaliculata* and *P. limitata*, and is sister to the rest of the genus (*P. siliquosa*, *P. compressa*, and *P. babingtonii*). *P. babingtonii* is not grouped one clade, with the exception of the weakly supported *P. babingtonii* in Korea (47%) and section *P. compressa* (57%). In the MP analysis,

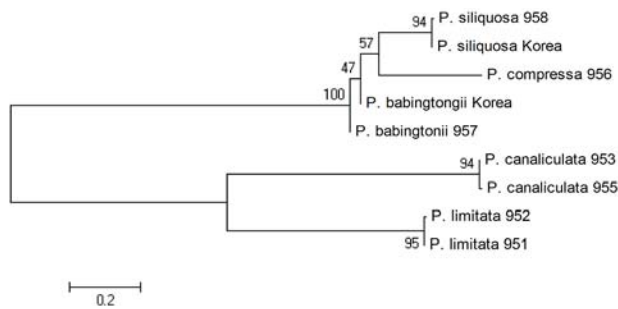


Fig. 1. Phenogram constructed by the Neighbor-joining method. Numbers on the branches indicate bootstrap proportions (1000 replicates). The codes of taxon are the same as Table 1.

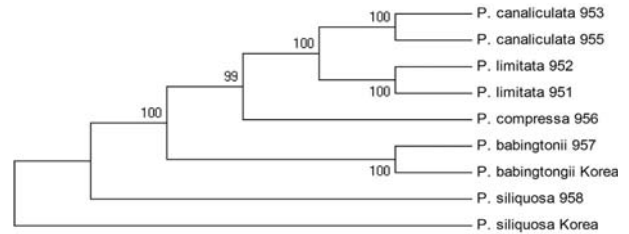


Fig. 2. The most parsimonious tree (using PAUP 4b10, exhaustive search, unweighted parsimony analysis, gaps=fifth state) from the 1161 aligned positions of the initial matrix).

ten accessions or populations were most fully resolved and all populations (accessions) from same species formed with 99% or 100% bootstrap support (Fig. 2).

Discussion

The rubisco spacer sequences give the good resolution at the genus *Pelvetia* and species level [10,11]. Lee [10] worked on the studied rubisco spacer for examining generic relationships with in the *Pelvetia* (*P. babingtonii*, *P. canaliculata*, *P. fastgiata*, and *P. siliquosa*) and *Pelvetopsis* (*P. limitata*). In its trees, *P. siliquosa* in Korea and *P. babingtonii* in Japan were grouped into intermost clade and consistent with our results. *P. limitata* was situated between north Pacific species and European *P. canaliculata* [10]. However, in this study, *P. canaliculata* was close to *P. limitata* (Figs. 1 and 2) Bootstrap analysis strongly supported (100% for MP) clade of the *P. canaliculata* and *P. limitata*. Serrato et al [19] also reported that *P. canaliculata* was positioned as the sister group the *P. limitata*. Lee et al [9] studied phylogenetic relationships of *Pelvetia* and *Pelvetiopsis* (Phaeophyceae) based on small subunit ribosomal DNA sequences. They suggested that the genus *Pelvetia* was not monophyetic and the genus

Pelvetiopsis was not closely related to *Pelvetia*. However the results were included small subunit (SSU) rDNA sequence data which were varied according to individuals of same species. If the SSU data were excluded from they data, it is very similar to some part of our results (e.g. *P. canaliculata* is sister to *P. limitata*).

The results for two *P. babingtonii* and *P. siliquosa* in Korea using ITS were generally similar to those for *P. babingtonii* and *P. siliquosa* in north America. The relationships are illustrated in Figs. 1 and 2. However, *P. babingtonii* AF102957 and *P. babingtonii* Korea differed from each other by only the four nucleotides. This low number of variable sites explains their grouping in the distance analysis and very recent radiation [8]. In Fucaceae, examination of the variance components reveals that about half of the variance was due to within site, individual variation [15]. In addition, the morphology of *Fucus* responds to latitude, longitude and exposure, but that these variables can only account for a small part of the variation in the data set. Much of this relates to exposure, which is widely supposed to have a major influence on seaweed phenotypes [5,16]. The remainder may be controlled, via latitude and longitude, by clines in sunlight intensity, photoperiod and temperature and by geographic distance leading to restricted gene flow [17].

If widespread extinction of some species (e.g. *Fucus spiralis*) of *Fucus* occurred during the LGM, its modern distribution may result from processes similar to that reported for the intertidal gastropod *Nucella* in the northeastern Pacific [12]. The patterns are consistent with the hypothesis that high intertidal dwelling *Fucus* recently colonized the northeastern Pacific following widespread population loss during the last glacial maximum (LGM) due to more exposure to cold temperature stress in air during low tides, a stress that *Fucus* was able to minimize because of its lower position in the intertidal and which is reflected in higher population structure and diversity.

Recently, Coyer et al [4] reported intra- and inter-specific phylogenetic relationships within the genus *Fucus* (275 individuals representing 16 taxa) using two regions of the mitochondrion and species of *Fucus* are considered as the rapidly evolving genus. For example, *F. spiralis* typically is confined to the high intertidal throughout much of its extant range, it is conceivable that most populations in the North Pacific could not have survived the stress of cold air temperatures during the LGM and subsequent post-LGM recolonization from the few surviving populations in the southern refugia was retarded because of competition with other in-

tertidal Fucales (e.g., genera *Pelvetia*, *Hesperophycus*, *Pelvetiopsis*, and *Silvetia*) and the Gigartinales (intertidal red algae). On the other hand, the mid-to-low intertidal *F. distichus* was afforded a refuge in depth during the LGM and their persistent existence is revealed by extant high levels of haplotype and nucleotide diversity. This scenario assumes that colonization of the North Atlantic by the *F. spiralis* ancestor and *F. distichus* ancestor occurred before the LGM.

We observed a substantial genetic differentiation between morphotype among genus *Pelvetia* using Lee's study [10]. Furthermore, populations of the same species revealed a coherent pattern of genetic variation, although widely dispersed populations of common species were influenced by isolation-by-distance effects [14]. Given the rapid fertilization of released oogonia in these algae, varying heights of plants on the shore (and hence times of immersion on a rising tide) may impose considerably greater barriers to interbreeding than their geographic proximity would suggest. Once a zygote is formed, it can probably only be dispersed over limited distances. Nothing is known of the extent of dispersion in *Pelvetia* spp., but estimates for other macroalgae range from a maximum of three meters (*Postelsia palmaeformis*) [6] and a mean of five meters (*Macrocystis pyrifera*) [1]. Thus, the plants appear to form a morphological patchwork or "mosaic"; those within any one "cell" of which are relatively homogeneous. Alternatively, it is possible that the phenotypic variation pattern takes the form of a complex of "micro-clines" in geographic space, including those relating to exposure, or some intermediate involving clinal boundaries to mosaic cells [15]. Thus, it appears probable that sexual reproduction in these species involves a high degree of self-fertilization. The timing of gamete release may further limit gene exchange, albeit not as much as self-fertilization does. Sequentially species of *Pelvetia* are accompany general modes of natural speciation, based on the extent to which speciation and populations are geographically isolated from one another.

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초록 : ITS에 의한 한국 내 *Pelvetia*속 분류군의 계통학적 연구

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갈조류 모자반과 *Pelvetia*속은 다세포 조류로 많은 해조류를 포함하고 있으며 북반구 태평양과 대서양에 분포되어 있다. *Pelvetia*속에 속하는 우리나라의 동종 및 근연한 같은 속내 종에 대해 유전자은행을 통해 밝혀진 ITS에 의한 서열을 이용하여 계통관계를 조사하였다. 한국의 *P. babingtonii*은 북미의 *P. babingtonii* AF102957과 유사한 핵산서열로 계통도 분석에서 같은 분지군을 형성하였다. 한국의 *P. siliquosa*은 역시 북미의 *P. siliquosa* AF102958과 유사한 핵산서열로 계통도 분석에서 같은 분지군을 형성하였다. *Pelvetia* 속 내 여러 종간은 결실이나 삽입에 의한 indel이 많은 반면 같은 종내 계통은 치환에 의한 차이가 현저하였다. 이 속은 NJ분석에서 크게 두 분지군으로 나뉘는데 한 그룹은 *P. canaliculata*와 *P. limitata*이며 나머지 그룹은 *P. siliquosa*, *P. compressa*, *P. babingtonii*을 포함하고 있었다. ITS 서열로 한국 내 분류군과 북미 간 분류군이 잘 구분되었다. MP 분석에서도 높은 지지도로 잘 구분되었다. 따라서 ITS 서열로 종 동정에 이용할 수 있었으며, 종의 보전이나 생식질 보전에 기초로 이용될 수 있을 것으로 사료된다.