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Growth and RAPD Variation of *Enteromorpha prolifera* (Oeder) J. Agardh, (Ulvaceae, Chlorophyta) from Korea

Jang-Taek Yoon* and Gyu-Hwa Chung[1]

South Sea Fisheries Research Institute, NFRDI, Yosu 556-823, Korea
[1]Department of Biotechnology, Yosu National University, Yosu 550-250, Korea

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Enteromorpha prolifera of the isomorphic diploid sporophyte and the haploid gametophyte generations inhabit rocks, tidal flats and tidal pools in the middle parts of intertidal zones. In this experiment, their thalli were observed by bare eyes from October and experienced 74 ± 16.5 cm maximum growth the following March and April. The rate of occurrence of the thalli per month was highest in March, while their biomass peaked at 1,464 ± 41.5 g/m^2 in Jangheung in April. Genetic similarity was investigated samples of *E. prolifera* collected from Muan, Wando, Jangheung, Yosu and Jinhae, at the south coast of Korea. Random amplified polymorphic DNA (RAPD) markers were used. For the RAPD analysis, 3 ng of the DNA extracted from the thalli using he phenol/chloroform method was amplified by PCR with a 25 μL reaction solution, arbitrary primers and 36 cycles. Among the 60 primers used, 31 yielded products, most of which showed diverse electrophoresis patterns. Similarities among the groups compared ranged from 0.37 to 0.58. We conclude that the use of RAPD analysis is appropriate to characterize the genetic variability of this commercial species along its geographical distribution.

Key words: Growth, RAPD Variation, *Enteromorpha prolifera*

Introduction

The seaweed *Enteromorpha prolifera* J. Agardh belongs to Chlorophyta, Ulvales, and Ulvaceae (Lee and Kang, 1986). It is found in the Pacific Ocean, the Atlantic Ocean, the Red Sea, Africa, the Mediterranean Sea, the Adriatic Sea, and all parts of Korea (Kang, 1966).

It has been reported that plants belonging to the genus *Enteromorpha* have highly diverse growth rates and forms according to their habitat environment. They are significantly affected by water temperature, light intensity, and salinity (Kim et al., 1990; Kim et al., 1992a,b; Kim and Lee, 1992; Kim and Lee, 1993; Kim and Lee, 1994). *E. prolifera* in particular is significantly influenced by these factors due to its estuarine habitats (Kim et al., 1991).

E. prolifera has generally been subdivided according to morphology and internal structure into categories below the species level, e.g., *E. prolifera* var. *flexuosa* Wulf, *E. prolifera* var. *crinita* Roth, *E. prolifera* f. *pilifera* Kutz, *E. prolifera* var. *torta* (Mert.) comb. nev., etc. (Chapman, 1956). In Korea, however, classification below the species level has yet to be implemented. In Japan, *E. prolifera, E. compressa, E. intestinalis* and their processed goods are collectively referred to as "aonori", with *E. prolifera* being the major representative (Ohno and Miyanoue, 1980; JICA, 1993).

Although *E. prolifera* grows in all parts of Korea, large communities can be found mainly in the west and south coastal areas - particularly at the mouths of the Youngsan, Tamjin, Seomjin, and Nakdong Rivers where freshwater and broad tidal flats dominate. In these areas, natural *E. prolifera* is used for food, with approximately 50 tons (dry weight) exported to Japan yearly.

To make *E. prolifera* aquaculture breeding in Korea more cost-effective and the industry corres-

*Corresponding author: yoonjt@nfrdo/re/lr

pondingly more competitive, it is necessary to develop superior breeds. This basically requires an examination of the seaweeds characteristics. This study therefore aimed to describe the growth and ecological characteristics of *E. prolifera*. Likewise, the study sought to reveal *E. prolifera* genetic flexibility by examining similarities within the species using the RAPD (Random Amplified Polymorphic DNAs) (RAPD) analysis (Cho et al., 1997).

Materials and Methods

1. Ecological characteristics

The ecology of *E. prolifera* was investigated at low tide from January to December 1998 in three different sea areas - the Unnam-myon, Muan-gun, Jeollanamdo, the Hoijin-myon, Jangheung-gun, Jeollanamdo, and the Yongwon-dong, Jinhae-city, Kyongsangnamdo. To determine the occurrence rate of seaweeds, eight 50×50 cm quadrats divided into 10 ×10 cm squares were randomly placed at representative regions. The resulting average frequency and cover were then determined. To measure the algal growth rates, the thalli inside the quadrats were collected and stored in an icebox at 10℃. The algae samples carried to the laboratory were then washed more than three times in filtered sea water. Thirty individual seaweeds were arbitrarily chosen for length measurement. Biomass was measured as wet weight per quadrat. To examine the *E. prolifera* morphogenesis, mature parent algae were selected and dried for 24 hrs in a shade condition inside the laboratory, at above 15℃. About 1 kg (wet weight) of the dried algae was then put into a 20 L circular water tank to induce the release of gametes at 20℃ water temperature and 4,000 lux light intensity. To help the released gametes fertilize more easily and induce adhesion onto substrates, they were subjected to dark treatment, i.e., the tank was covered with black vinyl sheet for 24 hrs. The zygotes that adhered to 10×20 cm plastic panels were cultured in a low-temperature incubator (EYELA, MTI-202) at 10℃.

The culture medium was prepared by filtering sea water through a 47 mm in diameter, 0.45 μm mesh size-membrane filter (Whatman, E298) prior to autoclaving at 100℃ for 10 minutes. This culture medium was renewed every five days during the observation of the alga's developmental process. The temperature and salt concentration of seawater at *E. proliferas* natural habitat i.e., the field where the plants were collected every month, were directly measured using a thermo-salinometer at the time when the samples were gathered. The concentration of nutrients were analyzed from 1 L of water collected in a plastic bottle from the Fisheries Resources and Environment Division at South Sea Fisheries Research Institute and National Fisheries Research and Development Institute.

2. RAPD analysis

For the RAPD analysis, wild *E. prolifera* samples were collected from five regions around the south coast of Korea (Muan, Wando, Jangheung, Yosu, and Jinhae) in February 1999 (Fig. 1). The collected samples were immediately carried to the laboratory and washed with filtered water prior to examining their morphological characteristics. Then they were dried and stored in the deep freezer at −80℃. The stored samples were washed three times with brushes in 15~20℃ filtered and autoclaved sea water to remove foreign substances. About 100 mg wet weight of sample from each region was used as samples for the RAPD analysis (Table 1). RAPD analysis involved DNA preparation, primer design, determining PCR reaction conditions, electrophoresis, and statistical analysis were conducted in the way described by Park et al. (1998).

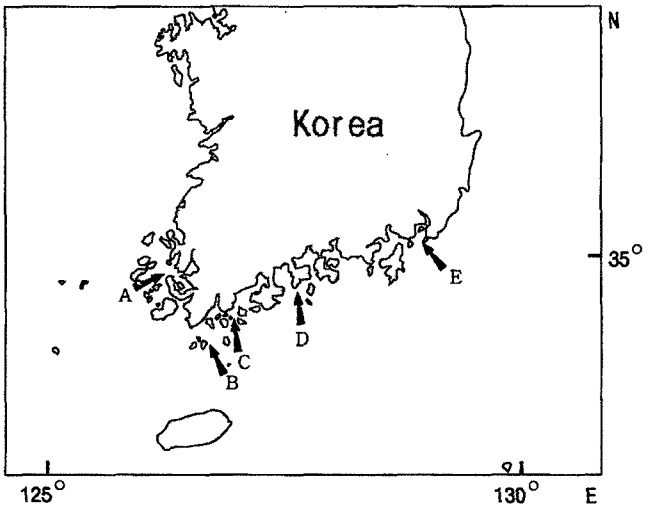


Fig. 1. A map showing the sampling sites of *E. prolifera* (A. Muan; B, Wando; C, Jangheung; D, Yosu; E, Jinhae).

Table 1. Collection and characteristics of *E. prolifera* samples examined

Sample number	Collection sites	Collected dates	Characteristics		
			Color	Width	Branch
S1	Muan	Feb. 11	Yellowish green	1.0 mm	thorny
S2	Wando	Feb. 4	Bright green	1.2 mm	flatten
S3	Jangheung	Feb. 23	Green	1.0 mm	flatten
S4	Yosu	Feb. 1	Dark green	0.8 mm	filamentous
S5	Jinhae	Feb. 19	Dark green	1.0 mm	filamentous

Results

1. Habitat environment

The sea area of Unnam-myon, Muan-gun, Jeollanamdo is surrounded by several islands, with spring water flowing along the shore. The upper part of the intertidal zone consists of sand. On the other hand, the middle and lower parts consist of mud. The *E. prolifera* habitat tilts during ebb tide, thereby exposing the fronds to the air except those in the waterway. Due to their repeated exposure and deposition, parts of the *E. prolifera* thalli are often buried in the bottom. The *E. prolifera* habitat was formed along the tidal flat. During the withering period of April and May, thalli that were detached from the substrates came afloat and accumulated in the upper tidal zone (Fig. 2A).

The sea area of Hoijin-myon, Jangheung-gun, Jeollanamdo is an indented bay, thus it rarely meets waves driven by wind or tide. At the upper part of the area is a reservoir, which has led to the formation of a waterway in the middle of the intertidal zone. Thus, fresh water flows in freely. An oyster nursery shares the *E. prolifera* habitat, thus, oysters are sometimes covered with blooming *E. prolifera*. The bottom material is composed of fine mud, which is presumed to have been carried from upstream by the inflow of river water over a long period of time. At the ebb tide, the *E. prolifera* habitat is still covered with sea water at a level lower than 5 cm., instead of being exposed to air. The water temperature is high all throughout in the summer, and very low in winter (Fig. 2B).

The inspected sea area in Yongwon-dong, Jinhae-city, Kyongsangnamdo faces Gadeok island in Busan-city, with a tributary to the Nakdong River passing through it. Provided with freshwater throughout the year, the area has therefore undergone extreme changes in salinity and registered a high turbidity. Additionally, new harbor construction and reclamation have caused the permanent disappearance of the *E. prolifera* habitat. Nevertheless, intermediate rearing facilities for oysters was equipped within the examined sea area, and some *E. prolifera* still adhere to the pillars with other seaweeds. The bottom consists of a wide sand flat. Fishermen breed short-necked clams nearby, called Bajirak in Korean (Fig. 2C).

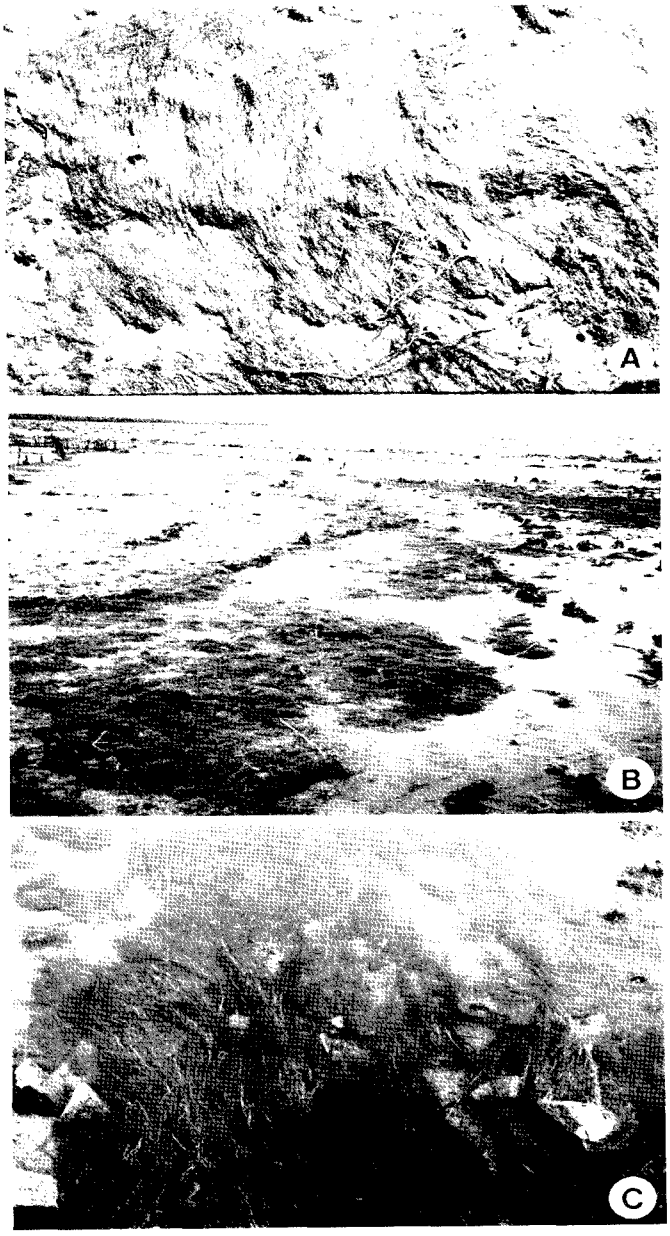


Fig. 2. Community of *E. prolifera* at Muan (A), Jangheung (B) and Jinhae (C).

Changes in water temperature and salinity in each *E. prolifera* habitat examined are shown in Fig. 3. Water temperature was lowest in the Muan sea area in January and February, and highest in August and September. The water temperature in the Jangheung sea area changed moderately with changes in season, while the water temperature in the Jinhae sea area was highly irregular. Jangheung had consistently high salinity, while Muan exhibited relatively low salinities of 19.7‰ and 21.8‰ in August and October, respectively. Jinhae exhibited generally low salinity. Its salinity of less than 10‰ which was observed between June and August was due to the inundation of freshwater around the examined area. Such freshwater inundation was caused by heavy seasonal rainfall.

As one of the nutrient salts in the *E. prolifera* habitats, phosphate (PO_4-P) was examined and the detected amount was highest in Jinhae and Muan, particularly in March and June (3.75 mg-at/L). In Jangheung, it was observed to be constant throughout the year. Phosphate analysis according to season showed generally high values in fall and summer, except for Jinhae which registered high values in winter (Fig. 4A). The total amount of dissolved inorganic nitrogen (DIN) gradually increased from January to May in Jinhae, peaked at 100.05 mg-at/L in June in Muan, and dropped to at 75.84 mg-at/L in July in Jangheung. The annual trend of the total nitrogen amount in the *E. prolifera* habitats was similar to that of the phosphate salts (Fig. 4B).

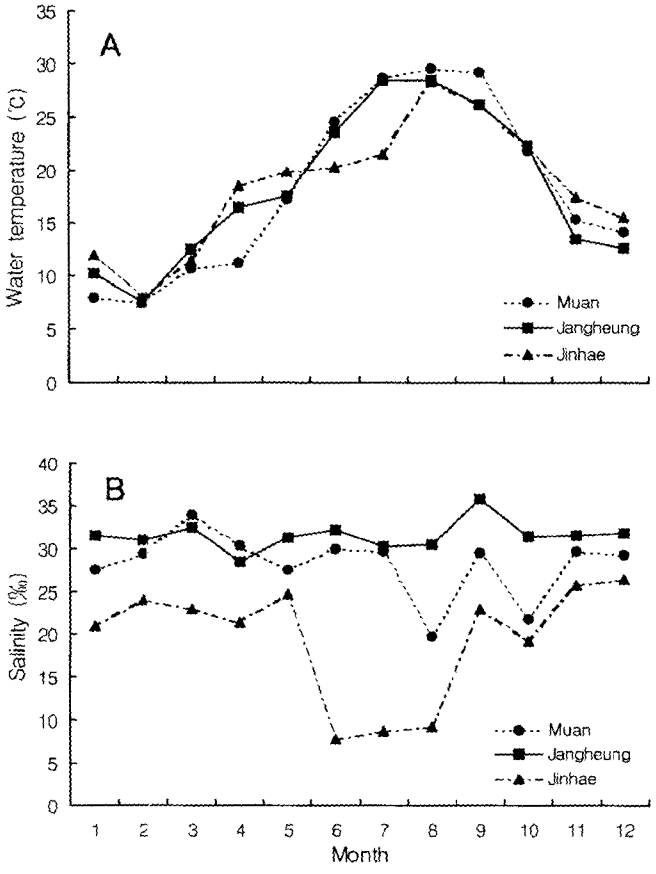


Fig. 3. Monthly variation of water temperature and salinity in the sampling sites of *E. prolifera*.

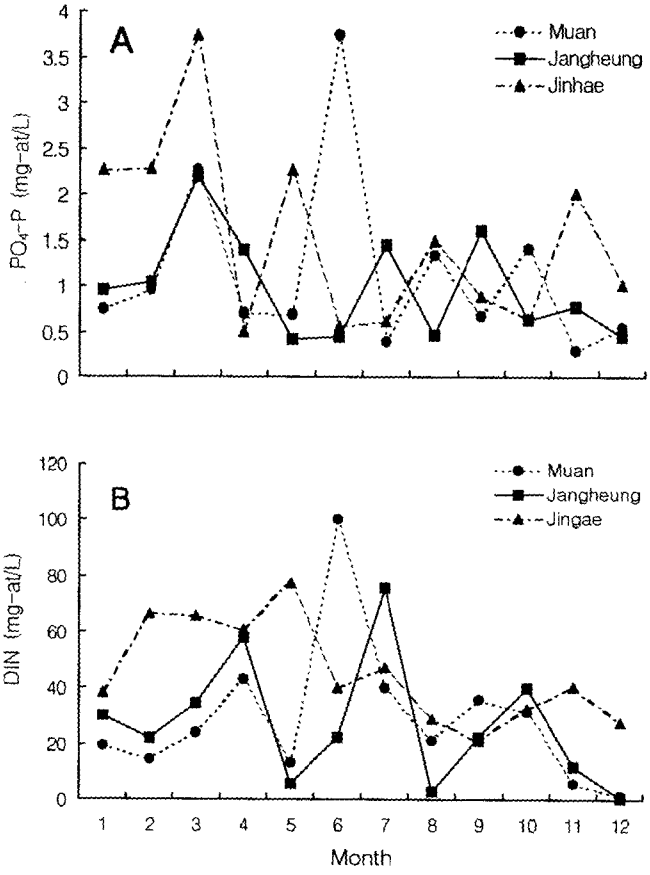


Fig. 4. Monthly variations of PO_4-P and DIN in the sampling sites of *E. prolifera*.

2. Growth

E. prolifera diploid sporophyte generation and haploid gametophyte generation were isomorphic. The gametophytes that were formed through parthenogenesis were also isomorphic and repeatedly generated in a natural state. Thirty minutes after inducing gamete release from mature *E. prolifera* thalli, the number of gametes per 100x view (1 mL) was 83~137. The gametes were highly motile. The

size of a gamete was 6~8 μm in major diameter and 2~3 μm in minor diameter. Eighty minutes after gamete release, about 30% of the gametes formed zygotes. The size of the zygotes was 4~6 μm, while the size of the formed gametes of through parthenogenesis was 2~3 μm. Twenty four hours after dark treatment, most of the zygotes adhered to a 10×20 cm plastic panel. The zygotes that were fixed on the substrate were elongated. As cell division progressed, their elongated upper part gradually grew, and their basal part also turned colorless. Lateral branches did not develop until the young thalli grew 2 mm long. The thalli grew more than 23 cm in 20 days, although the degree of growth varied with environmental conditions.

On the other hand, the upper parts of the thalli matured to form zoosporangia at high water temperature and long light period. Zoospores were later released from the thalli. The zoospores were relatively bigger than the gametes at 8~10 μm in major diameter and 2~4 μm in minor diameter. With four flagella, they swam in the water for about two hours until they made contact with and adhered to the substrate, and accordingly began to grow. Parthenogenesis of *E. prolifera* occurred throughout at natural conditions of above 15℃ water temperature and long light period. In the regions without exposure, however, maturation lagged, only growth was sustained.

3. Analysis of vegetation

The rates of occurrence of *E. prolifera* by month and region are shown in Table 2. The rate of occurrence of *E. prolifera* by month was highest in March. By region, *E. prolifera*'s rate of occurrence was highest in Jangheung, particularly in March and April. In Muan, Jeollanamdo, the *E. prolifera* thalli were exposed to every ebb tide, thus no damage was caused by inundation of freshwater. Likewise, ecological changes were maintained throughout the year. In Jangheung, the sudden decrease in the rate of occurrence of *E. prolifera* in May was a result of the rise in water and air temperatures when the sea water stayed up to about 5 cm, thereby causing the thalli to mature rapidly. The thalli quickly released gametes or zoospores, and then disappeared. Since the *E. prolifera* habitat in Jinhae is small, it was frequently inundated by the river. Thus, thalli were easily detached. Since it was always submerged in fresh or marine water, however, continuous development and reproduction of thalli took place throughout the year.

Table 2. Monthly appearence rate of *E. prolifera* at different sampling sites

Sites	Muan (%)		Jangheung (%)		Jinhae (%)	
Month	Frequency	Coverage	Frequency	Coverage	Frequency	Coverage
1	82	65	64	48	24	18
2	84	57	72	52	16	5
3	92	63	96	67	48	27
4	68	39	98	82	32	21
5	24	18	8	4	+	+
6	12	6	−	−	−	−
7	+	+	−	−	−	−
8	+	+	−	−	−	−
9	+	+	−	−	7	+
10	14	5	+	+	12	5
11	18	5	+	+	15	8
12	56	34	48	27	20	12

+, below 5% appearence rate
−, non appearence.

The *E. prolifera* thalli collected from the Muan sea area had a pale-green color and rough texture. On the other hand, the samples from Jinhae were dark-green and smooth throughout the year. The Jangheung samples were light-green and smooth. They usually form communities in winter and spring, rarely appearing in summer. The differences in body color and frond texture occurred probably because the thalli from Jinhae were hardly affected by sapropel, due to the Nakdong freshwater and sandy mud. On the other hand, the samples from Muan were highly affected by sapropel due to the areas turbid sea water and mud. The samples from Jangheung were relatively less influenced by mud, since they inhabited tidal pools filled with sea water even at ebb tides.

4. Length and biomass

For natural *E. prolifera* growth in length, the growth curve remained constant from December to March, and began to decline in April (Fig. 5A). For the average size of thalli, samples from Jangheung were largest at 63 ± 13.8 cm and 74 ± 16.5 cm in March and April, respectively. Muan samples followed with 58 ± 8.2 cm in March. These were much shorter in April than March because of their early exposure to abundant sunshine and long light

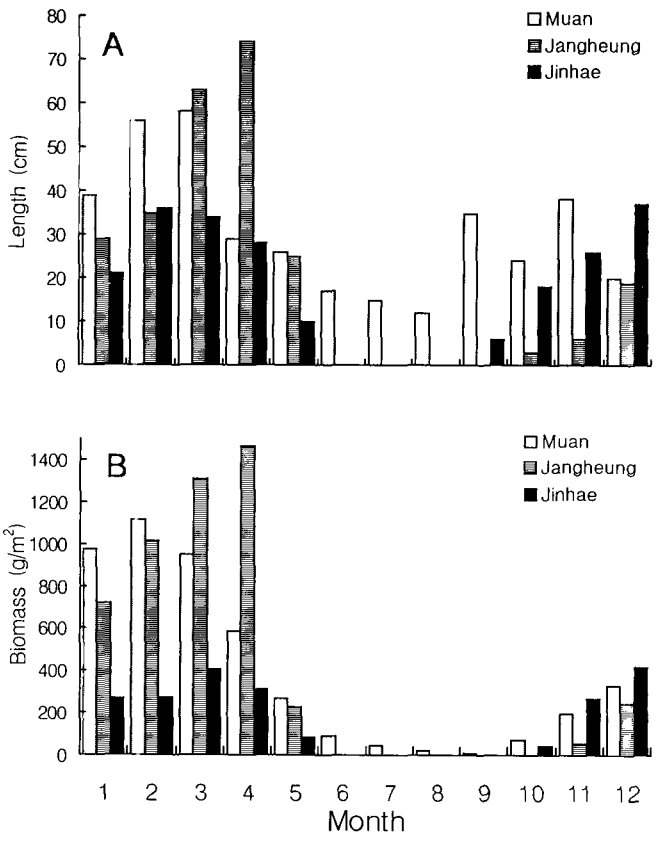


Fig. 5. Monthly variations of length (A) and biomass (B) in the sampling sites of *E. prolifera*.

period, caused by their frequent exposure to every ebb tide, stimulated their maturation. In the Muan sea area, thalli longer than 10 cm were observed even after June, while some thalli grew only branches, since they were buried in mud so that only the branches were observable. The *E. prolifera* that were repeating growth and maturation in summer rapidly grew in September. One cm young thalli then began to emerge in October. After November, their growth accelerated. On the other hand, thalli from Jangheung could be observed from October, with growth peaking in April. Samples from Jinhae began to develop in September in the area where fresh water flowed in, then gradually increased. The thalli collected in January, however, were rather small. In general the thalli from Jinhae were small.

The biomass of *E. prolifera* was almost proportional to the growth in length in all sample. The highest biomass values ($1,208 \pm 23.5$ g/m^2 and $1,464 \pm 41.5$ g/m^2) in March and April were obtained respectively in Jangheung (Fig. 5B). The next highest biomass values ($1,113 \pm 16$ g/m^2) in February, gradually decreasing in March were obtained in Muan. The *E. prolifera* from Jinhae exhibited the lowest biomass, with its highest value (405 ± 12.0 g/m^2) obtained in March. The growth of thalli and changes in biomass of *E. prolifera* differed according to the sea area, while growth curves were consistent.

5. Genetic variation

Using the phenol/chloroform method, DNA extraction from the *E. prolifera* samples from each region yielded 48~73 μg of DNA per 100 mg wet weight sample. In S1, in particular, a relatively small amount of DNA was extracted (Table 3). In addition, the $A_{260/280}$ values of the extracted DNAs ranged from 1.41 to 1.65. This indicates that their protein content was relatively low. The DNAs extracted by this method could thus be used as PCR templates, since only a minute amount of template DNA is required for PCR reaction. The results from the electrophoresis of the DNA extracted from each region on a 0.5% agarose gel are shown in Fig. 6.

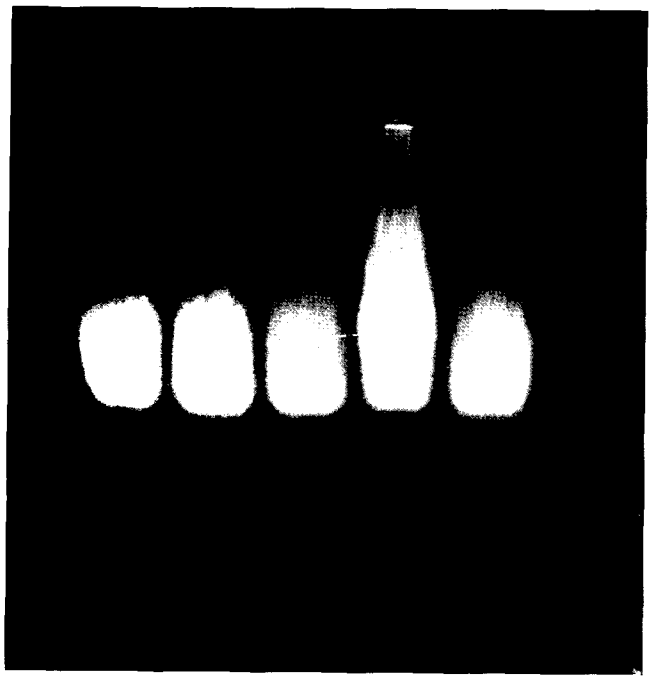
Table 3. Yield and purity of nucleic acids from *E. prolifera* thalli using phenol/chloroform extraction methods

	S1	S2	S3	S4	S5
DNA*	48.0	72.0	72.9	63.3	72.3
$A_{260/280}$	1.56	1.65	1.65	1.41	1.43

* μL per 100 mg wet weight thalli.

To find the optimum PCR condition for the RAPD analysis, varying conditions of annealing temperature, number of cycles, amount of Taq polymerase, amount of template DNA, and primer concentration were investigated. Results showed that as annealing temperature increased to 38℃, extra bands were reduced. Product confirmation was not available with 26 cycles, while clear bands were formed either with 36 or 46 cycles with little difference.

Comparing 0.1 μL, 0.15 μL, and 0.2 μL of Taq polymerase, the number of extra bands increased as the amount of polymerase increased. Moreover, 0.1 μL was appropriate for fewer extra bands and pattern identification, despite the somewhat reduced clarity of bands. Among the 1.5 ng, 3 ng and 6 ng of template DNA, the clearest bands were seen at 3 ng. On the other hand, higher concentrations yielded more extra bands among primer concentrations of 5 pM,



S1 S2 S3 S4 S5

Fig. 6. Genomic DNA concentration of five *E. prolifera* samples (as described in Table 1).

10 pM and 15 pM.

Among the 60 arbitrary primers from the Operon company used in the PCR, 31 exhibited products. The products appeared as 1~7 bands, ranging from 750 to 1,500 bp in size according to primers. Most primers yielded 1 to 4 main bands and diverse band patterns. Fig. 7 shows the polymorphic pattern in OPA02 and the monomorphic pattern in OPA15, which resulted from the electrophores is of the PCR products.

The numbers of bands amplified from each regional sample were as follows: 72 in S1; 78 in S2; 58 in S3; 55 in S4; and 78 in S5. The similarity among the regional groups as obtained from the DNA patterns, ranged from 0.37 to 0.58 (Table 4). S1 and S2 showed the highest value of 0.58, while they exhibited values of 0.37 and 0.38, respectively with S5. Other pairs showed intermediate values ranging from 0.43 to 0.50. Thus, allowing for distances between the collection regions, there is low geographical diversity among these five groups and no distinct phylogenetic group was formed.

Table 4. The similarity matrix based on Jaccard's equation (The total number of amplified products revealed in each sample is shown in the diagonal)

Samples	S1	S2	S3	S4	S5
S1	72				
S2	0.58	78			
S3	0.43	0.45	58		
S4	0.50	0.46	0.49	55	
S5	0.37	0.38	0.48	0.48	78

Discussion

The water temperature of Muan reached the lowest level in January and February and the highest level in August and September. It is inferred that these were the direct effects of changes in air temperature on the Muan sea area, caused by low water depth and exposure to air during ebb tides. In terms of salinity, Jangheung maintained a high salinity throughout the year, while Jinhae had a low salinity that went below 10‰ in summer. The lowering of salinity in the Jinhae sea area was probably caused by the irregular changes in water temperature and the inflow of the Nakdong River. On the other hand, nutrient exhibited a similar tendency by region and by month. The peak in the amount of phosphate and nitrogen in the Muan sea area in June was probably due to the fertilizer components that flowed in from the surrounding farms during heavy rainfall. *E. prolifera* habitats, however, are in intertidal areas with 2~3 hours of exposure, and all three areas were under the influence of freshwater.

The gametophytes and sporophytes of *E. prolifera* underwent isomorphic alternation of generation with the same forms and sizes. Thus, it is difficult to distinguish each generation without observing gametes and zygotes after inducing the spore release. However, *E. prolifera* always releases spores at a water temperature above 15℃ and within a long light period (16L:8D). Such conditions can therefore be effectively used in propagation and breeding.

The life cycle of *E. prolifera* was reported to take 7 months with culturing (Kim et al., 1991), but this was the result of the environmental condition maintained by culturing. At conditions of actively changing environmental factors, e.g., natural state, much shorter time is required for alternation of generation. In this study, thalli more or less 1 cm which

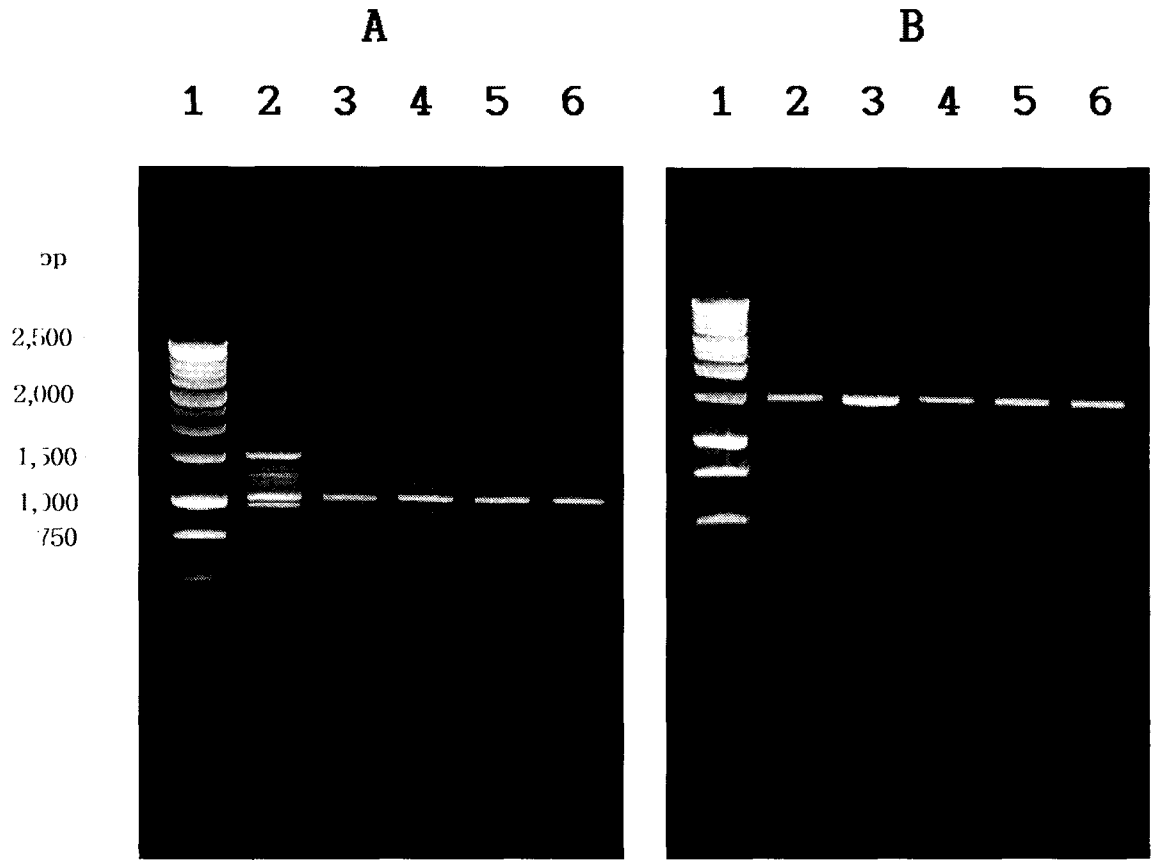


Fig. 7. Examples of RAPD amplification of *E. prolifera* genomic DNA showing polymorphic (A) and monomorphic (B) banding patterns. Reactions were performed with OPA02 and OPA15 primer each (Lane 1, 1 kb DNA lader from Promega; Lane 2, S1; Lane 3, S2; Lane 4, S3; Lane 5, S4; Lane 6, S5).

was grown from gametes either through parthenogenesis or by zygotes, matured and were released new gametes at a high water temperature and within a long light period. This indicates that the life cycle of *E. prolifera* differs according to its environment.

The rate of occurrence of *E. prolifera* in natural state varied according to region, with the highest frequencies and covers occurring in February and March. The plants growth rate (i.e., increase in length and biomass) was highest in Jangheung, probably due to the environmental characteristics peculiar to the Jangheung sea area. The biomass of the thalli that grew wild in the Jinhae sea area was less than that of the other sea areas, because its frequent inundation by the Nakdong River resulted in the detachment of a large amount of thalli. The pale-green color of the body and rough texture of the thalli from Muan were probably caused by the continuous exposure of the sea area to the air at every ebb tide. Likewise, the green or dark-green color and smooth texture of the thalli from Jangheung and Jinhae were probably caused by their non-susceptibility to external factors due to their continuous submersion in sea water. It was often observed that *E. prolifera* thalli rapidly grew when the water temperature went down in November and December or when rains fell in February and March. On the other hand, sudden discoloration followed by melting of *E. prolifera* thalli occurred at high temperatures or with much sunshine. These rapid changes in growth indicate that the plants are very sensitive to the external environment. Kim et al. (1991) reported that the growth rate of the germlings was highest within a long light period (16L :8D), while the growth of the germlings accelerated at 30~125 $\mu E \cdot m^{-2} \cdot S^{-1}$ light intensity, 5~15℃ water temperature, and 16~32‰ salinity. The results of this study were consistent with Kim et als study.

Extraction of DNA through the phenol/chloro-

form method, the *E. prolifera* samples collected in five sea areas of the south coast, yielded 48~73 μg DNA per 100 mg sample. A relatively small amount of DNA was detected in the sample (S1) from the Muan sea area compared to that of the samples from the other sea areas. This was probably due to peculiarities in the *E. prolifera* habitat environment in the Muan sea area. The $A_{260/280}$ values of these extracted DNAs ranged from 1.41 to 1.65, indicating their relatively low protein content. As such, they can serve as PCR templates for RAPD analysis. Similarities among the collected groups ranged from 0.37 to 0.58, as indicated by the DNA banding patterns in each sea area. The samples from Muan (S1) and Wando (S2) in Jeollanamdo exhibited the highest value of 0.58. They had a low similarity (0.37 and 0.38, respectively) with the samples from Jinhae (S5). Similarities among other groups ranged from 0.43 to 0.50, indicating low geographical diversity among these groups and no distinct phylogenetic group formed among them.

In Korea and Japan, *E. prolifera* is used for food with other *Enteromorpha* species. Ohno and Miyanoue (1980) reported that the volume of production of *E. prolifera* is dependent on temperature and precipitation in fall and winter, i.e., production volume increases as precipitation and temperature decrease. They added that turbidity considerably affects *E. prolifera* growth and quality, while; production of high-quality *E. prolifera* requires 1.5~2.9 ppm. They emphasized the need to prevent river pollution.

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