Algae
Volume 17(2): 75-81, 2002

Phylogenetic Relationships between *Ulva conglobata* and *U. pertusa* from Jeju Island Inferred from nrDNA ITS 2 Sequences

Sae-Hoon Kang and Ki-Wan Lee

Faculty of Applied Marine Sciences, Cheju National University, Jeju 690-756, Korea

In this study, the length of ITS2 from four species of the Ulvaceae in Jeju Island varied between 167 and 203 bp. The results of this investigation showed that two genus, *Ulva* and *Enteromorpha* are grouped in a monophyletic assemblage with 100% bootstrap support in all phylogenetic trees. However, a thorough examination of these characters from representatives does not provided a way to identify any unique morphological features of clades in this tree. This study reveals that *Ulva conglobata* and *Ulva pertusa* belong to one clade in the phylogenetic tree with the samples from Jeju Island, Korea.

Key Words: *Enteromorpha*, ITS 2, Jeju (Cheju), phylogrnetic relationship, *Ulva*

INTRODUCTION

Ulva and *Enteromorpha* are two well-known marine green algal genera. In Korean waters, fourteen species and four genera have been listed already (Lee and Kang 1986; Lee *et al.* 1986). They are common inhabitants of the upper intertidal zone of the seashores and estuaries, and various artificial structures around the world. Characteristic tolerability of a wide range of salinities and water qualities, together with the production of large numbers of propagules causing green tides, contribute to the ecological success of these worldwide genera and to their significance as the most widespread and troublesome ship-fouling macroalga (Callow 1986).

The ITS sequences are located between the 18S and 28S ribosomal RNA genes, and this region includes the 5.8S rRNA gene and the spacers ITS1 and ITS2 (Baldwin and Johnson 1993). With analyzing of ITS sequence, Blomster *et al.* (1998) concluded that *Enteromorpha intestinalis* and *E. compressa* represent two distinct, genetically divergent, and reproductively isolated species that happen to be very difficult to distinguish from each other and could be regarded as cryptic species. Malta *et al.* (1999) proposed that three species of *Ulva* are all members of one highly polymorphic *Ulva* based on the ITS2 region. Tan *et al.* (1999) demonstrated that two genera, *Ulva* and *Enteromorpha* are not monophyletic and that the characteristic of *Ulva* and *Enteromorpha* morphologies had arisen independently several times throughout the evolutionary diversification of the group.

We will here describe the basic characteristics of the ITS2 sequences from *Enteromorpha intestinales*, *E. linza*, *Ulva conglobata* and *U. pertusa* in Jeju island to compare our results with the above previous studies.

MATERIALS AND METHODS

Ulva and *Enteromopha* thalli were collected from five sites in Jeju (formerly Cheju) (Fig. 1). Before DNA extraction, the tissue were rehydrated in distilled water and then cleaned immediately. Details of algal specimens used in this study are presented in Table 1.

Samples were identified on the basis of morphological characteristics such as habit and details of cell arrangement and organelles (Lee *et al.* 1986; Maggs and Ward 1996; Blomster *et al.* 1998). Details of cell morphology were observed in surface view using 100-400× microscopy.

Algal materials (50 mg) were ground with a ceramic mortar in liquid nitrogen for 2 min. and then DNA was extracted using a DNeasy® Plant Mini Kit (QIAGEN Inc.) according to the manufacturer's protocol. DNA extraction was directly used for PCR experiments.

The polymerase chain reaction (PCR) was used to amplify the nuclear ribosomal internal transcribed spac-

*Corresponding author (kiwanee@cheju.ac.kr)

Table 1. *Enteromorpha* and *Ulva* species used in the phylogenetic analyses

Species name	Code	Site	GenBank No.	Collection date or literature source
E. intestinalis	Eint-ham	Hamdeok		8 Apr. 2001
E. intestinalis	Eint-ojo	Ojo-ri		8 Apr. 2001
E. linza	Elin-ojo	Ojo-ri		8 Apr. 2001
E. linza	Elin-jung	Jungmun		7 Apr. 2001
U. conglobata	Ucon-jo	Jocheon		8 Apr. 2001
U. conglobata	Ucon-ojo	Ojo-ri		8 Apr. 2001
U. conglobata	Ucon-seong	Seongsan		8 Apr. 2001
U. pertusa	Uper-jo	Jocheon		8 Apr. 2001
U. pertusa	Uper-ojo	Ojo-ri		8 Apr. 2001
U. pertusa	Uper-seong	Seongsan		8 Apr. 2001
U. pertusa	Uper-jung	Jungmun		7 Apr. 2001
E. compressa	Ecom-AF202466		AF202466	Blomster *et al.* (2000)
E. compressa	Ecom-AJ234302		AJ234302	Tan *et al.* (1999)
E. intestinalis	Eint-AF202467		AF202467	Blomster *et al.* (2000)
E. intestinalis	Eint-AF202468		AF202468	Blomster *et al.* (2000)
E. prolifera	Epro-AF035354		AF035354	Blomster *et al.* (1998)
E. prolifera	Epro-AJ234304		AJ234304	Tan *et al.* (1999)
E. linza	Elin-AJ000204		AJ000204	Tan *et al.* (1999)
E. linza	Elin-AJ000203		AJ000203	Tan *et al.* (1999)
E. linza	Elin-AF153491		AF153491	Malta *et al.* (1999)
U. californica	Ucal-AJ234315		AJ234315	Tan *et al.* (1999)
U. fenestrata	Ufen-AJ234316		AJ234316	Tan *et al.* (1999)
U. lactuca	Ulac-AJ234311		AJ234311	Tan *et al.* (1999)
U. lactuca	Ulac-AJ000208		AJ000208	Tan *et al.* (1999)
U. pertusa	Uper-AJ234321		AJ234321	Tan *et al.* (1999)
U. pseudocurvata	Upse-AJ234312		AJ234312	Tan *et al.* (1999)
U. rigida	Urig-AF153490		AF153490	Malta *et al.* (1999)
Blidingia minima	Bmin-AJ000206		AJ000206	Tan *et al.* (1999)
Monostroma grevillei	Mgre-AJ000205		AJ000205	Tan & Sluiman (2000)

*Collection sites for GenBank datas refer to literature.

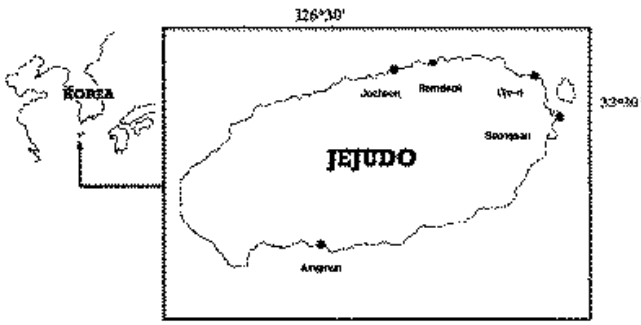

Fig. 1. Map of Jeju showing the sites where *Ulva* and *Enteromorpha* thalli were collected in 2001.

er 2 (ITS2). Details of primers for ITS2 region were described in Table 2. PCR amplification, Cloning of the PCR product and Sequencing were conducted as described by Kang (2001).

For phylogenetic analysis, unalignable sequence data were excluded from the full data set of sequences generated from individual primers. New sequences were aligned with published sequences (Table 1). Initial sequence alignments were constructed using the Clustral X program. Sequences were modified after inspection by eye of the profile of fluorescent peaks. Sequences from GenBank were aligned with those of this study for data analysis (Table 1).

Distance analyses were conducted by the tree-building algorithm of Neighbor-Joining (NJ) (Saitou and Nei 1987) and Minimum Evolution (ME) methods using Kimura' two parameters (Kimura 1980), Jukes-Cantor (Jukes and Cantor 1969) and Tamura and Nei's (Tamura and Nei 1993) distances within the program MEGA (version 2b3) (Kumar *et al.* 2001), respectively. In the parsimony analysis, the phylogenetic tree was constructed with using Maximum Parsimony (MP) (Fitch 1971) method with the program PAUP (version 4.0b8)

Table 2. Primers used for PCR amplification and sequencing in this study (primers designed by author)

Primer Name	Sequence	Target	Direction
5.8S-F1[a]	5'GTG AAT TGC AGA ATT CCG TC 3'	ITS2	Forward
26S[a] -R1	5'GCC TCA CCT GAA CTC AGG TC 3'	ITS2	Reverse
SK[b]	5'CGC TCT AGA ACT AGT GGA TC 3'		
T7[b]	5'GTA ATA CGA CTC ACT ATA GGG C 3'		

a: primers designed for PCR amplification

b: primers designed for the pBluescript phagemid vector based on STRATAGENE

(Swofford 1998). Base composition and patterns of substitution for pairwise comparisons were also analyzed with MEGA.

Bootstrappings of 1000 replications were performed to evaluate statistically the strength of support for each internal node in resulting trees (Felsenstein 1985). Bootstrap analyses were conducted with using MEGA for the NJ and ME methods and with PAUP for the MP method.

All trees were rooted with *Monostroma grevillei* and *Blidingia minima*, which were chosen as the outgroup because they represented a different genus and their sequences were alignable with, but more divergent than, all *Enteromorpha* and *Ulva* sequences.

RESULTS

In this study for the Ulvaceae, the length of ITS2 varied between 167 and 203 bp (data not shown). These lengths were not comparable to other genera in this research because the boundaries of the ITS regions are not at all coincident. Especially, the lengths of *Enteromorpha intestinalis* (AF202467) and *Ulva lactuca* (AJ000208) were shorter than others (Tan *et al.* 1999; Blomster *et al.* 2000).

The ITS2 sequence alignment used in this study is shown in Fig. 2. The result of alignment exhibits that G+C content values were higher than A+T ranging from 65.7 to 76.1%, excluding outgroups (G+C : 68.6 ± 2.4%, T : 16.6 ± 1.6%, A : 14.7 ± 1.4% on average).

Pairwise divergence in the ITS2 region using the Jukes and Cantor distance for samples of the Ulvaceae ranged from 0 to 26.2%. Considerable variability within individuals was detected in *Ulva pertusa*. There was a low level of sequence divergence between the *U. pertusa* and *U. conglobata* collected from Jeju (from 0 to 1.7%), while *U. pertusa* (AJ234321) showed a high level of divergence (from 12.5 to 14.4%). Also, the level of divergence in *Enteromorpha intestinalis* showed a high rate (6.0%). On the other hand, *E. linza* had a low level of divergence (from 0 to 0.8%). Significantly, the divergence between *U. pseudocurvata* and *E. compressa* showed a low level (0%) even though genus was different. And this group exhibited that the divergence was in excess of 17.5% compared with others. Although *E. intestinalis* and *E. compressa* was known to be similar with morphologically, their degree of divergence was very high (from 16.4 to 18.5%).

The phylogenetic trees obtained from all the analyses (Fig. 3 and Fig. 4), showed various clades of the Ulvaceae. Phylogenetic trees were constructed by the distance methods (NJ, ME) and the parsimony-based method (MP). MP was analyzed in weighted (Tv:Ts = 3:1) and unweighted. All phylogenetic analyses resulted in the monophyletic of a two genera *Ulva/Enteromorpha* assemblage with 100% bootstrap support, but the respective genera were not monophyletic (Figs 3, 4). Sequence divergence within the *Ulva/Enteromorpha* clade ranged from 0 to 26.2%. *Enteromorpha intestinalis* and *Ulva pseudocurvata*, which occupied a strongly supported sister group position to all other *Ulva* and *Enteromorpha* (Fig 3) exhibited a divergences in excess of 10% with all others. Several strongly supported interspecific and intergenetic clades were evident within where the sequence divergence was extremely low. The phylogenetic analyses clearly showed that the overall morphology of a sample was not correlated with its positon within the *Ulva/Enteromorpha* clade (Figs 3, 4). These results were comparable with those of Tan *et al.* (1999).

Ulva pertusa and *Ulva congolobata* assemblage collected in Jeju were strongly supported by bootstrap values (BP = 91%) as a monophyletic group (Fig. 4). Sequence divergence within this clade ranged from 0 to 1.7%, showing the same levels of sequence divergence found within other clearly monospecific groupings. So there was no evolutionary difference between them though *U. pertusa* samples had a perforated morphology, and *U. conglobata* samples had a fasciculate morphology. Also, *U. rigidia*

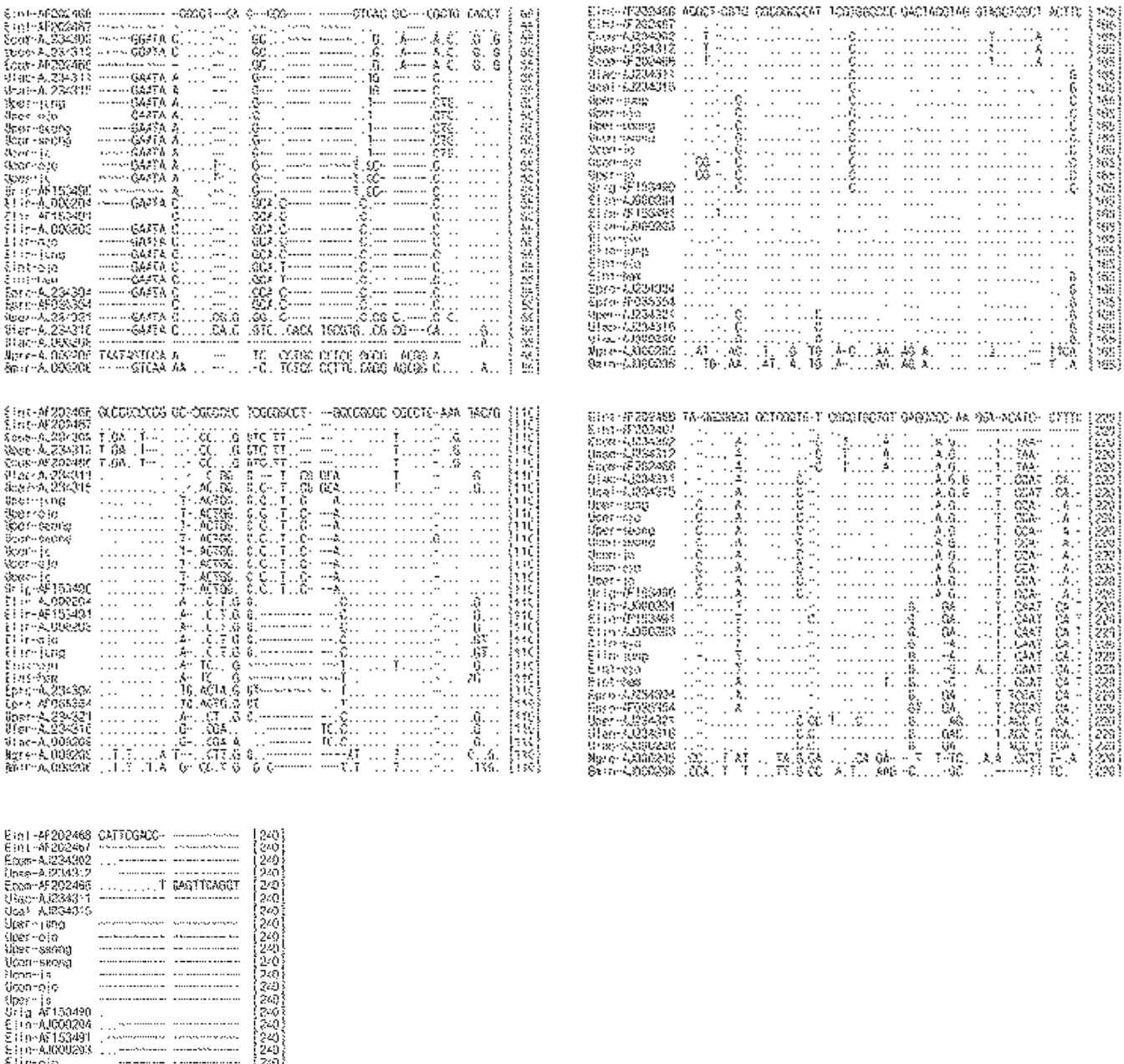

Fig. 2. Alignment of the ITS2 sequences of species of *Enteromorpha* and *Ulva*, and *Monostroma grevillei* and *Blidinggia minima* used as outgroup. Dots represent nucleotides identical to those of the first sequence, and dashes indicate gaps.

showed that sequence divergence between the members was low (from 0 to 1.7%). However, another sample (Uper-AJ234321) that was not grouped in the same clade, showed a low divergence (from 12.5 to 14.4%). Thus, those data indicate that there is no evolutionary distinction to be made between the members of these genera. This is not, however, suggesting that they are the same species. Other samples showed a high level of divergence and bootstrap support for mixed clades containing representatives of respective species ranged from 74 to 100% (Fig. 3). Therefore, their low values of interspecific divergence were found to be distinct phenomena in phylogenetic trees especially. All phylogenetic trees used *Blidingia nima* and *Monostroma grevillei* (Monostromataecae) as a outgroup. They were placed in a well-supported clade while sequence divergence had a high level (from 43.1 to 55.8%).

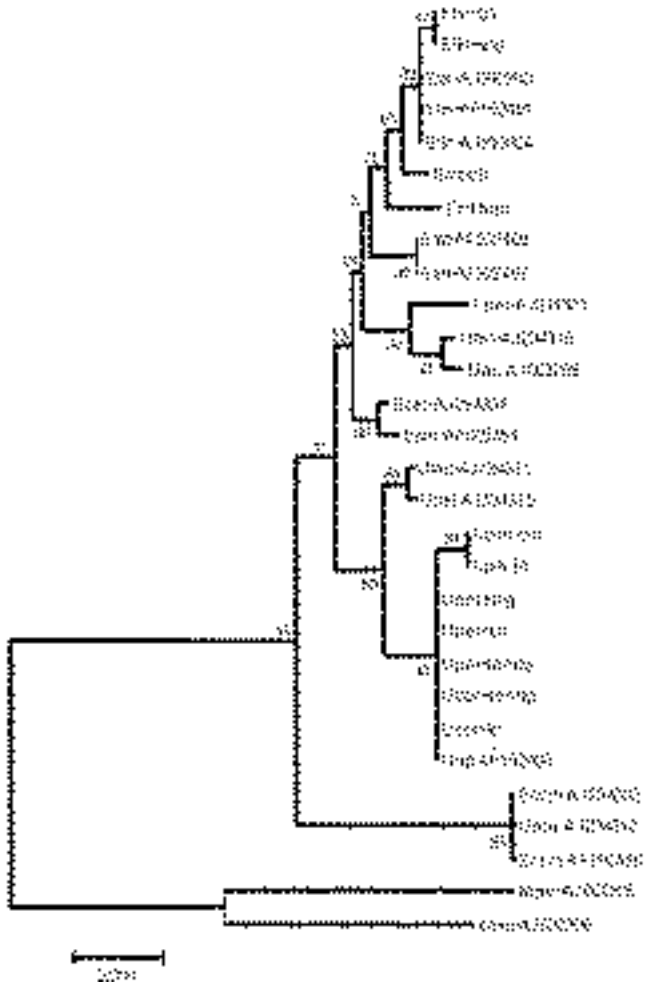

Fig. 3. Distance tree for ITS2 using the NJ method - Jukes and Cantor distance. Numbers on the nodes indicate bootstrap value (500 replicates). Branch lengths are proportional to the estimated mean number of substitutions site (see scale bar).

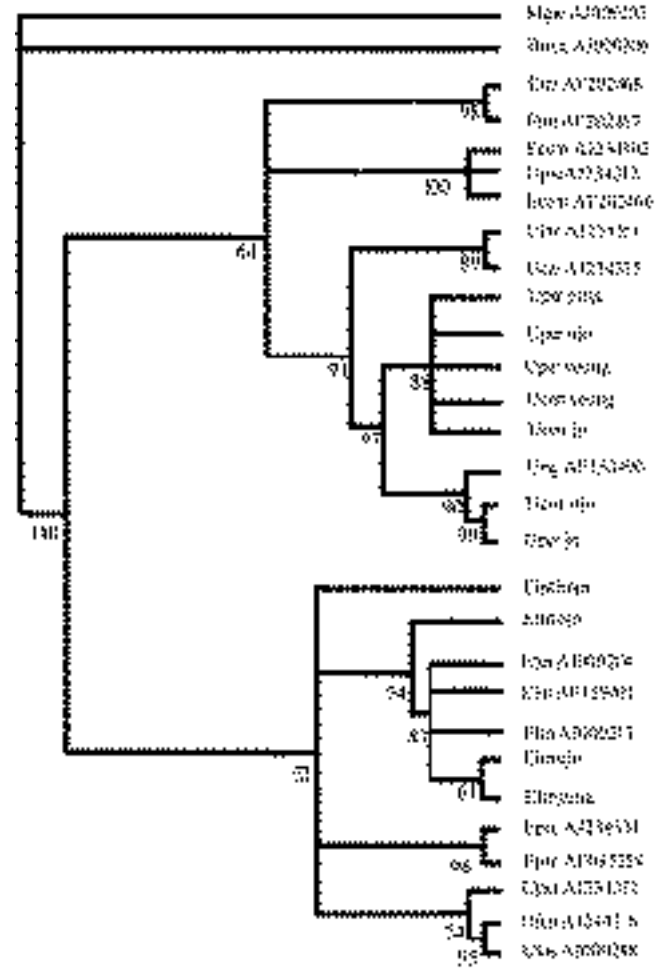

Fig. 4. MP tree for ITS2 (Tv:Ts = 3:1). Numbers below the nodes indicate bootstrap value (1000 replicates).

DISCUSSION

Recently, the ITS regions have been studied in several field especially molecular phylogenetic relationships (Leskinen and Pamilo 1997; Pillmann *et al*. 1997; Tan *et al.* 1999; Rousseau *et al*. 2000; Stiger *et al.* 2000) and morphological analyses in algae (Blomster *et al.* 1998, 1999; Coat *et al*. 1998; Malta *et al*. 1999; Fama *et al*. 2000; Woolcott *et al*. 2000; Bae and Lee 2001). Because they vary to different degrees between taxonomic species, and their alignments have been used for phylogenetic purposes (Coleman and Mai 1997). The general characteristics of the 18S - 28S intergenic region are similar to those of other organisms. The 5.8S rRNA gene sequence is conserved relative to the ITS sequences (Leskinen and Pamilo 1997). The ITS sequences of *Enteromorpha* and *Ulva* are short in comparison to those found in most green algae, being more similar in length to the homologous sequence in *Acrosiphonia arcta* and some angiosperms (Bakker *et al.* 1992).

In this study, the lengths of ITS2 were ranged from 167 to 203 bp. G+C content values were higher than A and T. These values were similar to those of other published works (Leskinen and Pamilo 1997).

Two genera, *Enteromorpha* and *Ulva* are widely regarded as easily distinguishable because of their dramatically different morphologies: *Ulva* species are flat, lettucelike blades with two cell layers in thickness, and *Enteromorpha* species form hollow liquid or gas-filled tubes with a one cell thickness, which may also be highly branched. However, cell walls do not merely provide rigidity. They are essential to cell growth and the developmental processes, such as axis formation in zygotes and branching in growing plants. When walls are too

weak, development may be impossible, as in a mutant form of *Ulva* that grew as an aggregate of undifferentiated cells, rather than first forming a filament and later a holdfast then forming a blade (Lobban and Harrison 1994). This flexibility of form among genetically homogeneous plants corroborates results from earlier culture studies that showed the development of both tubular and bladelike thalli from the same zoospore populations of several *Ulva* species (Gayral 1967; Bonneau *et al.* 1977; Provasoli and Pintner 1980). Gayral (1967) reported the occurrence of tubular thalli from both zoospore and gamete (parthenogenetic) cultures. The majority of both zoospores and parthenogenetic gametes developed into leafy thalli, whereas some developed into tubular ones. Culture study of Bonneau *et al.* (1997) showed progeny with some of the thalli were distromatic in some parts and they questioned the validity of maintaining *Ulva* and *Enteromorpha* as two separate genera. Provasoli and Pintner (1980) showed that *Ulva* cultures could form uniseriate filaments when axenic or *Enteromorpha* like-tubes if grown with a particular bacteria (Tan *et al.* 1999). Nakanishi *et al.* (1996) found that live bacteria are required for normal morphogenesis of *Ulva pertusa* in culture.

The results of this investigation showed that two genera *Ulva* and *Enteromorpha* are grouped in a monophyletic assemblage with 100% bootstrap support in all phylogenetic analyses. However, a thorough examination of these characters from representatives in this study does not provide data to identify any unique morphological features in all phylogenetic trees. Throughout analyzing ITS2 sequences in this study, it was proved that *U. conglobata* and *U. pertusa* assemblages were monophyletic groups even though morphological differences. It indicates that there is no evolutionary diversification to make them distinct. Phillps (1984) reported that *Ulva* has a morphological variation especially in *U. rigida.*

This study revealed that *U. conglobata* and *U. pertusa* belongs to one clade in the phylogenetic tree. Also, *Enteromorpha* and *Ulva* are not distinct evolutionary entities, and can result in a plant with either a blade or a tube morphology as proposed by Tan *et al.* (1999). In the future, this study should be accompanied with a developmental method and population translation relative with environmental factor to resolve many given questions. This data could be applied to interspecific and population variations, together with fouling research on green algae.

ACKNOWLEDGEMENTS

This work was mainly supported by a grant from the Academic Research Fund of the Cheju National University Development Foundation, and partially by Japan / Korea joint study (No.11695080).

REFERENCES

Bae E.H. and Lee I.K. 2001. *Umbraulva*, a new genus based on *Ulva japonica* (Holemes) Papenfuss (Ulvaceae, Chlorophyta). *Algae* **16:** 217-231.

Bakker F.T., Olsen J.L. and van den Hoek C. 1992. Nuclear ribosomal DNA internal transcribed spacer regions (ITS1 and ITS2) define discrete biogeographic groups in *Cladophora albida* (Chlorophyta). *J. Phycol.* **28:** 839-845.

Baldwin C.C. and Johnson G.D. 1993. Phylogeny of the Epinephelinae (Teleostei: Serranidae). *Bull. Mar. Sci.* **52:** 240-283.

Blomster J., Maggs C.A. and Stanhope M.J. 1998. Molecular and morphological analysis of *Enteromorpha intestinalis* and *E. compressa* (Chlorophyta) in the British Isles. *J. Phycol.* **34:** 319-340.

Blomster J., Maggs C.A. and Stanhope M.J. 1999. Extensive intraspecific morphological variation in *Enteromorpha muscoides* (Chlorophyta) revealed by molecular analysis. *J. Phycol.* **35:** 575-586.

Blomster J., Hoey E.M., Maggs C.A. and Stanhope M.J. 2000. Species-specific oligonucleotide probes for macroalgae: molecular discrimination of two marine fouling species of *Enteromopha* (Ulvophyceae). *Mol. Ecol.* **9:** 177-186.

Bonneau R.H., Brehm M.A. and Kern A.M. 1997. The impact of psychological stress on the efficacy of anti-viral adoptive immunotherapy in an immunocompromised host. *J. Neuroimmunol.* **78:** 19-33.

Callow M.E. 1986. Fouling algae from 'in-service' ships. *Bot. Mar.* **24:** 351-357

Coat G., Dion P., Noailles M.-C., Reviers B.De., Fontaine J.-M., Berger-Perrot Y. and Loiseaux-De Goer S. 1998. *Ulva armoricana* (Ulvales, Chlorophyta) from the coasts of Brittany (France). II. Nuclear rDNA ITS sequence analysis. *Eur. J. Phycol.* **33:** 81-86.

Coleman A.W. and Mai J.C. 1997. Ribosomal DNA ITS-1 and ITS-2 sequence comparisons as a tool for predicting genetic relatedness. *J. Mol. Evol.* **45:** 168-177.

Fama P., Olsen J.L., Stam T.W. and Procaccini G. 2000. High levels of intra- and iner-individual polymorphism in the rDNA ITS1 of *Caulerpa racemosa* (Chlorophyta). *Eur. J. Phycol.* **35:** 349-356.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783-91.

Fitch W.M. 1971. Toward defining the course of evolution: Minimum change for a specific tree topology. *Syst. Zool.* **20:** 406-416.

Gayral P. 1967. Mise au point sur les Ulvacées (Chlorophycées),

particulièrement sur les résultants de leur étude en laboratoire. *Botaniste* **50:** 205-251.

Jukes T.H. and Cantor C.R. 1969. Evolution of protein molecules. In: Munro H.N. (ed.), *Mammalian Protein Metabolism*. Academic Press, New York. pp. 21-132.

Kang G.Y. 2001. Phylogenetic Relationships among Groupers (Genus Epinephelus) Based on Mitochondrial Cytochrome b DNA Sequences. MS thesis, Cheju Nat. Univ.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*. **16:** 111-120.

Kumar S., Tamura K., Jakobsen I.B. and Nei M. 2001 MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics* **17:** 1244-1245.

Lee I.K. and Kang J.W. 1986. A check List of Marine Algae in Korea. *Kor. J. Phycol.* **1:** 311-325.

Lee I.K., Lee Y.P. and Ahn Y.S. 1986. Flora of Marine Algae in Cheju Island 1. Ulvaceae. *Kor. J. Phycol*. **1:** 157-167.

Leskinen E. and Pamilo P. 1997. Evolution of the ITS sequences of ribosomal DNA in *Enteromorpha* (Chlorophyceae). *Hereditas* **126:** 17-23.

Lobban C.S. and Harrison P.J. 1994. *Seaweed ecology and physiology*. 2nd ed. Cambridge University Press, Cambridge. 366 pp.

Maggs C.A. and Ward B.A. 1996. The genus *Pikea* (Dumontiaceae, Rhodophyta) in England and the North Pacific: comparative morphological, life history, and molecular studies. *J. Phycol*. **32:** 176-193.

Malta E.-J., Draisma S.G.A. and Kamermans P. 1999. Free-floating *Ulva* in the southwest Netherlands: species or morphotypes? A morphological, molecular and ecological comparison. *Eur. J. Phycol*. **34:** 443-454.

Nakanishi K., NishijimaI M., Nishumura M., Kuwano K. and Saga N. (1996). Bacteria that induce morphogenesis in *Ulva pertusa* (Chlorophyta) grown under axenic conditions. *J. Phycol*. **32:** 479-482.

Phillips J.A. 1984. The validity of morphological and anatomical characters in distinguishing species of *Ulva* in southern Australia. pp. 353-361. In: Irvine D.E.G. and John D.M. (eds), *Systematics of Green Algae.* Academic Press, London. 449 pp.

Pillman A., Woolcott G.W., Olsen J.L., Stam W.T. and King R.J. 1997. Inter- and intraspecific genetic variation in *Caulerpa* (Chlorophyta) based on nuclear rDNA ITS sequences. *Eur. J. Phycol.* **32:** 379-86.

Provasoli L. and Pintner I.J. 1980. Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). *J. Phycol.* **16:** 196-201.

Rousseau F., Reviers B.de., Leclerc M.-C., Asensi A. and Delepine R. 2000. Adenocystaceae fam. nov. (Phaeophyceae) based on morphological and molecular evidence. *Eur. J. Phycol*. **35:** 35-43.

Saitou N. and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4:** 406-425.

Stiger V., Horiguchi T., Yoshida T., Coleman A.W. and Masuda M. 2000. Phylogenetic relationships of *Sargassum* (Sargassaceae, Phaeophyceae) with reference to a taxonomic revision of the section *Phyllocystae* based on ITS-2 nrDNA sequences. *Phycol. Res.* **48:** 251-260.

Swofford D.L. 1998. *PAUP: phylogenetic analysis using parsimony*. Version 4. Sinauer Associates, Sunderland, MA.

Tamura K. and Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol*. **10:** 512-526.

Tan I.H. and Sluiman H.J. 2000. Molecular Phylogeny of selected Ulvales (Ulvophyceae, Chlorophyta). http://www.ncbi.nlm.nih.gov/entrez/.

Tan I.H., Blomster J., Hansen G., Leskinen E., Maggs C.A., Mann D.G., Sluiman H.J. and Stanhope M.J. 1999. Molecular phylogenetic evidence for a reversible morphogenetic switch controlling the gross morphology of two common genera of green seaweeds, *Ulva* and *Enteromorpha*. *Mol. Biol. Evol.* **16:** 1011-1018.

Woolcott G.W., Iima M. and King R.J. 2000. Speciation within *Blidingia minima* (Chlorophyta) in Japan: evidence from morphology, ontogeny, and analyses of nuclear rDNA ITS sequence. *J. phycol.* **36:** 227-236.

Accepted 17 April 2002