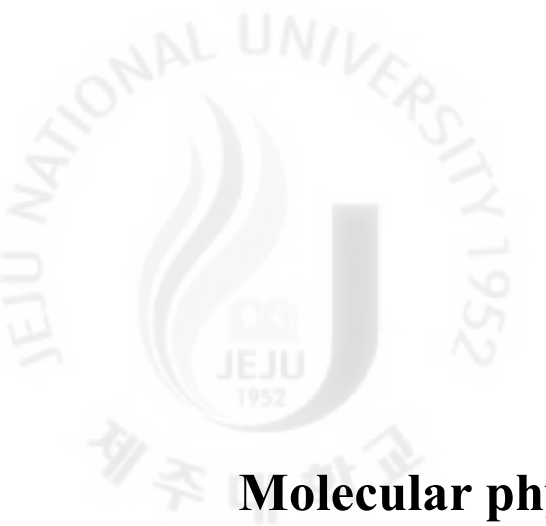

Master's Thesis

Molecular phylogeny and DNA barcoding of Gracilariaceae (Gracilariales, Rhodophyta) from Asia-Pacific region

Mi Yeon Yang

Department of Biology

GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

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Molecular phylogeny and DNA barcoding of Gracilariaceae (Gracilariales, Rhodophyta) from Asia-Pacific region

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(Supervised by Professor Myung Sook Kim)

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This thesis has been examined and approved.

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Chairperson of the Committee

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Date

Department of Biology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

아시아-태평양산 홍조류 꼬시래기과의 분자계통 및 DNA barcoding

지도교수 김 명 숙

양 미 연

이 논문을 이학 석사학위 논문으로 제출함

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CONTENTS

ABSTRACT

The family Gracilariaceae has been well known as the economic agarophytes. Although they have been extensively investigated and well defined from both an anatomical and a molecular point of view, the species boundary and classifications are very difficult because of the high morphological plasticity, the lack of diagnostic characteristics and great species diversity. The aim of the present study is to acquire a better understanding of the phylogenetic relationships, species diversity and species boundaries of the Gracilariaceae. To clarify the phylogenetic relationships and species boundary, we performed molecular analyses for 115 specimens collected from Asia-Pacific region. A total of 22 species including five cryptic species from Asia-Pacific region has been identified using plastid *rbc*L and mitochondrial COI genes. The results of phylogenetic relationships inferred from *rbc*L gene have confirmed the monophyly of the genera, *Gracilaria, Gracilariopsis* and *Hydropuntia*. The phylogenetic trees estimated by ML and MP have been divided into two large clades; one is *Gracilaria sensu lato* including *Hydropuntia* and *G. vermiculophylla* clade, and the other is *Gracilariopsis*. The *rbc*L genetic divergence among genera in Gracilariaceae ranged from 1.1% to 14.6%. The DNA barcoding data based on COI gene permitted us to verify 7 *Gracilaria*, 2 *Hydropuntia* and 2 *Gracilariopsis* species from Asia-Pacific region. The intraspecific divergences of COI ranged from 0% to 1.5%, and interspecific divergences ranged from 0% to 14.7%. All species included in this study formed strong clades independently, indicating that DNA barcoding can effectively identify Gracilariaceae species. Our wide geographic sampling allowed us to extend the geographic range of the number of the species. *Gracilaria perplexa,* previously known from Australia as type locality, is here reported for the first time from Okinawa, Japan. *Hydropuntia fisheri,*

previously known from Thailand and Vietnam, is reported for the first time from Malaysia. *Gracilariopsis heteroclada,* previously known from Hainan in China and the Philippines, is reported for the first time from the Malaysia. *Gracilaria* sp.1 and sp.3 have potential to be new species with distinct morphological characteristics.

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Ⅰ. INTRODUCTION

The family Gracilariaceae Nägeli (1847) belongs to Gracilariales, an order of marine red algae described by Fredericq and Hommersand (1989). The Gracilairaceae is characterized as follows: 1) a female reproductive structure consisting of a two-celled carpogonial branches, 2) a supporting cell producing other two to three two-celled sterile filaments, 3) the fertilized carpogonium fusing with its supporting cell to form the fusion cell, 4) the cells of the sterile filaments fusing into the fusion cell, and 5) the gonimoblasts developing directly and primarily outward from the fusion cell (Fredericq and Hommersand, 1989; 1990b). The gracilarioid algae include some of the most valuable marine plants. They have been extensively investigated over the last 30 years, and many studies have provided comprehensive information on their life history, cultivation, taxonomy, and utilization (Bellorin *et al*., 2002; Rueness, 2005). However, high morphological plasticity, the lack of diagnostic characters and great species diversity in the Gracilariaceae, have made it difficult to define the species boundary of this group.

The Gracilariaceae is well defined from both an anatomical and a molecular point of view within Rhodophyta, but the intergeneric taxonomy had a somewhat more complex history (Bird 1995; Bellorin *et al*., 2002). Currently, the Gracilariaceae is composed of up to seven genera (Fredericq and Hommersand, 1990b), namely *Gracilaria* Greville (1830), *Gracilariopsis* Dawson (1949), *Hydropuntia* Montagne (1842), *Melanthalia* Montagne (1843), *Curdiea* Harvey (1855), *Gracilariophila* Setchell et Wilson in Wilson (1910), and *Congracilaria* Yamamoto (1986). Studies on the phylogenetic relationships inferred from molecular data have produced stable clades at the genus level, such as *Gracilaria sensu stricto*, *Gracilariopsis*, *Hydropuntia, Curdiea/Melanthalia*, and confirmed the monophyly of

the family (Bellorin *et al*., 2002; Gurgel and Fredericq, 2004). *Gracilaria* and *Gracilariopsis* have been confirmed to be monophyletic groups whereas *Hydropuntia* has received less support. The distribution of *Curdiea* and *Melanthalia*, are limited to around southern Australia, Tasmania, and New Zealand. These previous studies approached to the phylogeny of the Gracilariaceae on a global scale or focused on a regional scale, such as southern Africa (Iyer *et al*., 2005). On the other hand, the phylogeny and species diversity of the Gracilariaceae occurring in Asia-Pacific region still remained as a gap in our knowledge. Kim *et al*. (2008a) replaced *G. parvispora* instead of *G. bursa-pastoris* from Korea and Japan using *rbc*L sequence data, and Kim *et al*. (2006) determined phylogenetic affinities of flattened *Gracilaria* species from Korea. Lin (2008) and Hau and Lin (2006) described a new species of *Gracilariopsis* from Taiwan and Vietnam, respectively. These show that comprehensive study of Asia-Pacific region is needed for phylogenetic relationships of Gracilariaceae.

DNA sequence analysis is the most widely used molecular technique for inferring phylogenetic relationships at the species level within the Gracilariaceae (Iyer *et al*., 2005; Kim *et al*., 2008b; Bellorin *et al*., 2008). It is a useful taxonomic tool for distinguishing between organisms that are difficult to distinguish based on morphological characteristics alone. Sequence analyses from different DNA regions have been used for many studies: chloroplast-encoded *rbc*L gene (Gurgel and Fredericq, 2004; Gargiulo *et al*., 2006), *rbc*L-*rbc*S intergenic spacer region (i.e. Rubisco spacer; Goff *et al*., 1994; Iyer *et al*., 2005; Rueness, 2005), the mitochondrial *cox*2-*cox*3 spacer (Cohen *et al*., 2004; Terada and Shimada, 2005), the *cox*1 gene (Robba *et al*., 2006, Yang *et al*., 2008), and the nuclear internal transcribed spacers of the ribosomal cistron (i.e. ITS; Goff *et al*., 1994; Bellorin *et al*., 2002). Although many genes have been used for phylogenetic inference, the chloroplast-encoded *rbc*L gene are widely used, and considered to provide optimal resolution for

inferring species level phylogenetic relationships within the Gracilariaceae (Gurgel and Fredericq, 2004; Kim *et al*., 2006; Kim *et al*., 2008b). However, this concern for molecular identification tools among algal systematics has the lack of a standard marker for rapid identification of specimen.

DNA barcoding has become an important tool for the identification of gracilarioid species (Saunders, 2005; Kim *et al*., 2010). DNA barcoding makes possible to have a comprehensive species-specific sequence library for eukaryotes (Marshall, 2005), offering the opportunity for a standardized system of species identification based on the analysis of small fragments of DNA (Lara *et al*., 2010). A DNA barcode is easily sequenced due to the short, standardized DNA region used, and provides a rapid and efficient tool for taxonomic and biodiversity research (Hajibabaei *et al*., 2007). To solve the difficulty of distinguishing among Gracilariaceae species based on morphology, the sequencing of a short, standard region of DNA barcoding, was proposed. Mitochondrial-encoded COI, a 660-base fragment at the 5' end of *cox*1, is a fast-evolving gene that has recently proven valuable for barcoding red algae species (Clarkston and Saunders, 2010; MS Kim *et al*., 2010). Use of COI may therefore allow a better understanding of the species delimitation among marine red algae.

Molecular data is expected to develop a classification and phylogeny of the Gracilariaceae as important criterion. The aim of the present study is to acquire a better understanding of the phylogenetic relationships, species diversity and species boundaries of the Gracilariaceae. Based on two molecular markers as *rbc*L and COI, 22 species from Asia-Pacific region are ascertained with clarifying their taxonomic identities, and we provide genetic diversity within and among the members of the family Gracilariaceae.

Ⅱ. MATERIALS AND METHODS

1. Taxon sampling

115 specimens corresponding to the family Gracilariaceae were collected or sent by collaborators from the Korea, Japan, China, Malaysia, Taiwan, Thailand, the Philippines and Australia. Specimens, collection sites, and GenBank accession numbers are listed in Table 1. We removed other small organisms attached from the thalli under a dissecting microscope. The cleaned thalli were air-dried and stored in silica gel dessicant for molecular analyses, and housed at the herbarium of Jeju National University (JNUB), Jeju, Korea.

2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from dried thalli in liquid nitrogen using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). All the isolation steps were carried out according to the instructions of the manufacturer with minor modification by incubating the disrupted samples with buffer AP1 for minimum 1 hour at 63℃. The extracted DNA was stored at -20°C and used to amplify *rbc*L and COI.

PCR amplification was performed on a total volume of 20 µl using AccuPower PCR Premix (Bioneer, Daejeon, Korea), and 1-10 ng of template DNA. For amplification and sequencing of the *rbc*L gene, the following specific primer pairs were used: *rbc*LF7-*rbc*LR753 and *rbc*LF645-*rbc*S start (Gavio and Fredericq, 2002). PCR reaction of *rbc*L gene was carried out with an initial denaturation at 96°C for 4 min, followed by 35 cycles of amplification (denaturation at 94°C for 1 min, annealing at 50°C for 1min and extension at 72°C for 2 min) with a final extension at 72°C for 7min. The COI region was amplified using the following primer pairs: GazF1-GazR1 (Saunders, 2005) and GHalF-COX1R1

(Saunders, 2008). PCR reaction of COI was carried out with an initial denaturation at 96°C for 4 min, followed by 40 cycles of amplification (denaturation at 94°C for 30s, annealing at 45°C for 30s and extension at 72°C for 1min) with a final extension 72°C for 7min. All PCR amplifications were carried out using Swift MaxPro thermal cyclers (ESCO, Singapore). The PCR products were purified using the AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea) and then sequenced commercially (Macrogen, Seoul, Korea).

3. Alignments and molecular analyses

Analyses of *rbc*L and COI region sequences of the forward and reverse strands were determined for all PCR products using an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Both electropherogram outputs from each sample were edited using Chromas version 1.45 (Queensland, Australia). Total *rbc*L and COI sequences were organized using the multiple-sequence editing program BioEdit (Hall, 1999) and aligned visually. To assess the level of variation in the sequences of *rbc*L and COI, uncorrected (p) pair-wise genetic distances were estimated with PAUP* v4.0b10 (Swofford, 2002).

To confirm the taxonomic position of Gracilariaceae, Maximum likelihood (ML) and Maximum parsimony (MP) analysis were performed using PAUP 4.0 (Swofford, 2002). Total *rbc*L sequences including 51 sequences from GenBank were aligned with other Gracilariaceae species, and two genera, *Curdiea crassa* [AY049427] and *Melanthalia obtusata* [AY046431], were used as an outgroup.

For ML tree, we performed a likelihood ratio test using MODELTEST (ver. 3.7; Posada and Crandall 1988) to determine the best available model for our sequence data. We selected general time reversible (GTR) model with gamma correction for among-site variation (Γ) and the proportion of invariable sites (I). ML tree were estimated using a heuristic search

with 100 random-additional sequence replicates and TBR branch swapping. To test node stability, ML bootstrap analyses were performed with 100 replicates. MP tree were constructed using heuristic search algorithm with the following settings: 1000 random sequence additions, tree bisection-reconnection (TBR) branch swapping, MulTrees, all characters unordered and unweighted, and branches with a maximum length of zero collapsed to yield polytomies. Bootstrap values for the resulting nodes were assessed using 1000 bootstrapping replicates with ten random sequence additions, TBR and MulTrees.

Clustering trees on COI were performed in MEGA 4.0 (Tamura *et al*., 2007) using the Neighbor-joining (NJ) algorithm based on Kimura -2-parameter (K2P) distance method. To compare other data, we contained 13 COI sequences from GenBank. NJ tree was used to provide a visual display of COI variation within and between species.

Table 1. List of included specimens, collection localities and GenBank accession numbers

Species & voucher	Location	Date	Collector	GenBank	
				COI	*rbc*L
***Gracilaria arcuata* Zanardini**					
YG008	Ikei island: Uruma: Okinawa: Japan	03/03/10	M.S. Kim and M.Y. Yang	JQ026057	JQ026031
***Gracilaria articulata* C.F. Chang & B.M. Xia**					
YG007	Howaito beach: Uruma: Okinawa: Japan	03/03/10	M.S. Kim and M.Y. Yang	JQ026054	JQ026032
***Gracilaria blodgettii* Harvey**					
G058	Matou: Sanya: Hainan: China	01/29/10	M.S. Kim	JQ026055	JN605794
***Gracilaria incurvata* Okamura**					
JD100404-14	Jongdal: Jeju: Korea	04/04/10	E.G. Han	HQ322060	
MI02	Misaki: Miura: Kanagawa: Japan	04/29/10	J.C. Kang and H.W. Lee	HQ322023	
MI03	Misaki: Miura: Kanagawa: Japan	04/29/10	J.C. Kang and H.W. Lee	HQ322017	
MI56	Misaki: Miura: Kanagawa: Japan	04/30/10	J.C. Kang and H.W. Lee	HQ322026	JQ026024
CN-G353	Hansuri: Jeju: Korea	05/05/04	M.S. Kim	HQ322020	
G134	Hansuri: Jeju: Korea	06/26/10	M.Y. Yang and E.G. Han	HQ322019	
CN-G352	Hansuri: Jeju: Korea	05/05/04	M.S. Kim	HQ322021	
HG100404-5	Hagwi: Jeju: Korea	04/14/10	J.C. Kang	HQ322018	
***Gracilaria parvispora* I.A.Abbott**					
G153	Hado: Jeju: Korea	08/08/10	M.S. Kim	HQ322033	
G154	Sinyang: Jeju: Korea	03/27/10	M.S. Kim	HQ322032	
GM51	Dukchonri: Geomundo: Yeosu: Korea	06/12/10	M.Y. Yang and E.G. Han	HQ322030	
G074	Hamduck: Jeju: Korea	07/21/10	M.Y. Yang	HQ322034	

JD100404-20	Jongdal: Jeju: Korea	04/04/10	E.G. Han	HQ322038	
GB0601	Daejung: Jeju: Korea	06/06/05	M.S. Kim	HG322047	JQ026040
MI59	Misaki: Miura: Kanagawa: Japan	04/30/10	J.C. Kang and H.W. Lee	HQ322037	
MI61	Misaki: Miura: Kanagawa: Japan	04/30/10	J.C. Kang and H.W. Lee	HQ322035	
MI60	Misaki: Miura: Kanagawa: Japan	04/30/10	J.C. Kang and H.W. Lee	HQ322036	
***Gracilaria salicornia* (C.Agardh) E.Y.Dawson**					
G056	Xiaodonghai Bay: Sanya: Hainan: China	01/30/10	M.S. Kim	JN790237	JN605796
G169	Morib: Malaysia	11/23/07	M.S. Kim	JN790224	
G0606	Samae Say villar Chouburi: Thailand	07/26/09	Jutarat W.	JN790235	
G182	Phuket: Tailand	05/05/08	M.S. Kim	JN790220	
G175	Phuket: Tailand	05/05/08	M.S. Kim	JN790221	
G410	Barangay Bulo: Zamboanga City: Philippines	04/10/10	P.J.L. Geraldino	JN790231	
G404	Sangali Bay: Zamboanga Ciry: Philippines	04/10/10	P.J.L. Geraldino	JN790197	
G089	Rizal Beach: Gubat Bay: Philippines	02/02/10	P.J.L. Geraldino	JN790238	
G090	Bulusan: Sorsogon: Philippines	02/02/10	P.J.L. Geraldino	JN790239	
YG010	Ikei Island: Uruma: Okinawa: Japan	03/03/10	M.S. Kim and M.Y. Yang	JN790236	
G361	Ikei Island: Uruma: Okinawa: Japan	03/03/10	M.S. Kim and M.Y. Yang	JN790234	JQ026045
***Gracilaria tenuistipitata* C.F.Chang and B.M.Xia**					
G452	Baan Aow Saay: Kok Tay yoaw: Songkla: Thailand	07/17/09	Jutarat W.	JQ026074	
GF0601	Baan Aow Saay: Kok Tay yoaw: Songkla: Thailand	07/17/09	Jutarat W.	JQ026069	
G449	Baan Aow Saay: Kok Tay yoaw: Songkla: Thailand	07/17/09	Jutarat W.	JQ026073	
G200	Baan Aow Saay: Kok Tay yoaw: Songkla: Thailand	07/17/09	Jutarat W.	JQ026072	
GC0508	Phuket: Tailand	05/05/08	M.S. Kim	JQ026071	

G460	Middle Banks: Penang: Malaysia	12/10/07	E. Phiak	JQ026075	
G3019	Big Korean Island: Penang: Malaysia	12/10/07	E. Phiak	JQ026070	
G459	Middle Banks: Penang: Malaysia	12/10/07	E. Phiak	JQ026076	
G052	Hongsha river: Sanya: Hainan: China	01/31/10	M.S. Kim	JQ026077	JN605793
***Gracilaria textorii* (Suringar) De Toni**					
PS100117-10	Pyosun: Jeju: Korea	01/17/10	M.S. Kim	HQ322061	
PS100317-2	Pyosun: Jeju: Korea	03/17/10	M.S. Kim	HQ322051	
JD100404-9	Jongdal: Jeju: Korea	04/04/10	E.G. Han	HQ322062	
GT0616	Chagwido: Jeju: Korea	06/14/09	M.S. Kim	HQ322064	JQ026036
GT0615	Udo: Jeju: Korea	06/17/09	M.Y. Yang	HQ322065	
G095	Hyodon: Jeju: Korea	01/17/10	E.G. Han	HQ322069	
HG100404-14	Hagwi: Jeju: Korea	04/14/10	J.C. Kang	HQ322063	
MI53	Misaki: Miura: Kanagawa: Japan	04/30/10	J.C. Kang and H.W. Lee		JQ026037
***Gracilaria vermiculophylla* (Ohmi) Papenfuss**					
G068	Molunde: Busan: Korea	11/15/09	M.S. Kim	HQ322059	
GM01	Godo: Geomundo: Yeosu: Korea	06/12/10	M.Y. Yang and E.G. Han	HQ322053	
G099	Geumneung: Jeju: Korea	01/15/10	M.Y. Yang	HQ322048	
G100	Sinyang: Jeju: Korea	01/18/10	J.C. Kang	HQ322039	
HW100415-47	Haengwon: Jeju: Korea	04/15/10	M.Y. Yang		
SS100404-8	Sungsan: Jeju: Korea	04/04/10	E.G. Han	HQ322049	
GV1001	Woljung: Jeju: Korea	10/15/05	M.S. Kim		JQ026039
YG012	Nokonosima: Fukuoca: Japan	03/04/10	M.S. Kim and M.Y. Yang	HQ322041	
GV0405	Asamushi: Japan	04/26/05	M.S. Kim	HQ322046	

CN-G212	Shimoda: Shizuoka: Japan	03/18/03	M.S. Kim	HQ322086	
G183	China	01/28/00	M.S. Kim	JQ026079	
***Gracilaria* sp.1**					
GT0310	Japan	03/29/07	M.S. Kim	JQ026052	JQ026051
GI0301	Japan	03/29/07	M.S. Kim	JQ026053	JQ026050
***Gracilaria* sp.2**					
G091	Bulusan: Sorsogon: Philippines	02/02/10	S.Y. Kim and P.J.L. Geraldino	JQ026059	JQ026033
G092	Bulusan: Sorsogon: Philippines	02/02/10	S.Y. Kim and P.J.L. Geraldino	JQ026060	JQ026034
G405	Brgy: Piblaum: Philippines	09/30/10	P.J.L. Geraldino	JQ026058	
***Gracilaria* sp.3**					
G3021	Batu Feringii: Penang: Malaysia	12/12/07	M.S. Kim	JQ026068	JQ026035
***Gracilaria* sp.4**					
PH020	Tuburan: Cebu: Philippines	02/20/10	P.J.L. Geraldino	JQ026056	JQ026048
***Gracilaria* sp.5**					
G083	Bulusan: Sorsogon: Philippines	02/02/10	S.Y. Kim and P.J.L. Geraldino	JQ026078	JQ026027
***Gracilariopsis heteroclada* J.Zhang & B.M.Xia**					
G174	Big Korean Island: Penang: Malaysia	12/10/07	M.S. Kim	JQ026096	
G3018	Batu Pucat: Penang: Malaysia	12/10/07	M.S. Kim	JQ026097	JQ026029
G057	Yulin harbour: Sanya: Hainan: China	01/30/10	M.S. Kim	JQ026098	JQ026028
G335	Bantayan: Bantayan Island: Philippines	04/02/10	P.J.L. Geraldino	JQ026099	
***Gracilariopsis chorda* (Holmes) Ohmi**					
BY18	Biyangdo: Jeju: Korea	05/20/10	J.C. Kang	HQ322090	
G121	Jongdal: Jeju: Korea	05/29/10	M.Y. Yang and E.G. Han	HQ322079	

G3030	Jongdal: Jeju: Korea	01/24/08	M.S. Kim	HQ322066	
G3029	Ojori: Jeju: Korea	01/23/08	M.S. Kim	HQ322050	JQ026038
CN-Gr6	Dangmokri: Wando: Korea	10/15/08	M.S. Kim	HQ322081	
CN-Gr8	Jangheung: Jeollanamdo: Korea	11/10/07	M.S. Kim	HQ322080	
CN-G214	Shimoda: Shizuoka: Japan	02/18/03	M.S. Kim	HQ322084	
CN-G213	Chiba: Chiba: Japan	02/20/03	M.S. Kim	HQ322085	
MI62	Misaki: Miura: Kanagawa: Japan	04/30/10	J.C. Kang and H.W. Lee	HQ322073	
MI42	Misaki: Miura: Kanagawa: Japan	04/30/10	J.C. Kang and H.W. Lee	HQ322074	
CN-G210	Daeryeon: China	10/27/02	M.S. Kim	HQ322087	
***Hydropuntia changii* (B.Xia & I.A.Abbott) M.J.Wynne**					
G081	Bulusan: Sorsogon: Philippines	11/15/09	S.Y. Kim and P.J.L. Geraldino	JQ026061	JQ026026
***Hydropuntia edulis* (S.G.Gmelin) Gurgel & Fredericq**					
G054	Qingshui Bay: Sanya: Hainan: China	01/29/10	M.S. Kim	JQ026081	JN605795
G320	Currimao: Ilocos Norte: Philippines	05/26/10	P.J.L. Geraldino	JQ026082	
G325	Bmbm: Oslob: Cebu: Philippines	04/10/10	P.J.L. Geraldino	JQ026080	
G411	Saugali Bay: Zamboanga Ciry: Philippines	04/10/10	P.J.L. Geraldino	JQ026085	
G323	Bmbm: Oslob: Cebu: Philippines	04/01/10	P.J.L. Geraldino	JQ026086	JQ026047
G171	Phuket: Thailand	05/05/08	M.S. Kim	JQ026087	
G163	Phuket: Thailand	05/05/08	M.S. Kim	JQ026088	
GE0501	Phuket: Thailand	05/05/08	M.S. Kim	JQ026089	JQ026046
G167	Big Korean Island: Penang: Malaysia	12/10/07	M.S. Kim	JQ026084	
G180	Big Korean Island: Penang: Malaysia	12/10/07	M.S. Kim	JQ026083	

***Hydropuntia fisheri* (B.M.Xia & I.A.Abbott) M.J.Wynne**					
GT0511	Ao Tabg Kare: Phuket: Thailand	05/04/08	M.S. Kim	JQ026062	
G168	Phuket: Thailand	05/05/08	M.S. Kim	JQ026065	JQ026049
G164	Phuket: Thailand	05/05/08	M.S. Kim	JQ026067	
G181	Morib: Malaysia	11/23/07	M.S. Kim	JQ026063	
G173	Big Korean Island: Penang: Malaysia	12/10/07	M.S. Kim	JQ026064	
G165	Big Korean Island: Penang: Malaysia	12/10/07	M.S. Kim	JQ026066	
***Hydropuntia multifurcata* (Børgesen) M.J.Wynne**					
G3023	Big Korean Island: Penang: Malaysia	12/11/07	M.S. Kim	JQ026092	JQ026041
G3024	Batu Feringii: Penang: Malaysia	12/12/07	M.S. Kim	JQ026093	JQ026042
G3025	Batu Pucat: Penang: Malaysia	12/11/07	M.S. Kim	JQ026091	JQ026044
G166	Batu Feringii: Penang: Malaysia	12/12/07	M.S. Kim	JQ026094	JQ026043
***Gracilaria perplexa* Byrne, K., Zuccarello, G.C., West, J., Liao, M.-L.& Kraft, G.T.**					
YG009	Howaito beach: Uruma: Okinawa: Japan	03/03/10	M.S. Kim and M.Y. Yang	JQ026090	JQ026030
***Hydropuntia preissiana* (Sonder) Gurgel & Fredericq**					
WAR-66	Ricey beach: Rottnest Island: Australia	11/17/10	M.S. Kim and M.Y. Yang	JQ026095	JQ026025

Table 2. Oligonucleotide primers used for amplification and sequencing

gene	Primer	Direction	Sequence (5' - 3')	Reference
*rbc*L	F7	Forward	AAC TCT GTA GAA CGN ACA AG	Gavio and Fredericq, 2002
	F645	Forward	ATG CGT TGG AAA GAA AGA TTC T	Gavio and Fredericq, 2002
	R753	Reverse	GCT CTT TCA TAC ATA TCT TCC	Gavio and Fredericq, 2002
	R-rbcS start	Reverse	ATC TTT CCA TAG ATC TAAA AGC	Gavio and Fredericq, 2002
COI	GazF1	Forward	TCA ACA AAT CAT AAA GAT ATT GG	Saunders, 2005
	GHalF	Forward	TCA ACA AAT CAT AAA GAT ATY GG	Saunders, 2005
	GazR1	Reverse	ACT TCT GGA TGT CCA AAA AAY CA	Saunders, 2008
	COX1R1	Reverse	GTA TAC ATA TGA TGH GCT CAA	Saunders ,2008

Table 3. Thermal cycling conditions of polymerase chain reaction for *rbc*L and COI gene

	step	Temperature	Time	Cycles
*rbc*L	Initial denaturation	96℃	4 min	1
	Denaturation	94℃	1 min	35
	Annealing	50℃	1 min	
	Extension	72℃	2 min	
	Final extension	72℃	7 min	1
COI	Initial denaturation	96℃	4 min	1
	Denaturation	94℃	30 sec	40
	Annealing	45℃	30 sec	
	Extension	72℃	1 min	
	Final extension	72℃	7 min	1

Ⅲ. RESULTS

1. Characteristics of plastid *rbc*L gene

The 1,171 nucleotides of the *rbc*L gene were determined for 73 taxa including 51 GenBank sequences. No insertion or deletion mutations were found in the *rbc*L sequences, permitting unambiguous alignment of all sequences. Of these, 482 positions (41.2%) were variable and 411 (35.1%) were parsimoniously informative. The data file had an unequal frequency of bases (A=30.7%, C=14.8%, G=21.8%, T=32.7%). The sequence divergence ranged from 1.1% to 14.6% among Gracilariaceae species.

Comparisons of informative signal between *rbc*L and COI sequences revealed a slightly greater in *rbc*L (35.1%, n=51 taxa) than in COI (34.6% n=115 taxa). The overall genetic variation of *rbc*L gene in Gracilariaceae displayed that our *rbc*L sequences were more variation (Table 4). The range of genetic variation within genera, slightly expanded in all group.

2. Characteristics of mitochondrial COI gene

The 616 nucleotides of the COI gene were determined for 115 taxa including 13 GenBank sequences. No insertion or deletion mutations were found in the COI sequences, permitting unambiguous alignment of all sequences. Of these, 232 positions (37.7%) were variable and 213 (34.6%) were parsimoniously informative. The data file had an unequal frequency of bases (A=26.5%, C=15.1%, G=18.5%, T=39.9%). The sequence divergence ranged from 2.8% to 14.7% among Gracilariaceae species. The ranges of COI sequence variation within group have wider range than *rbc*L, although COI data are comparatively included small number of species.

3. Phylogenetic relationships within the Gracilariaceae

The family Gracilariaceae formed a well defined monophyletic group with 100% bootstrap support for both ML and MP analysis using *rbc*L gene. The maximum likelihood (ML) tree (Fig. 1) was compared with the maximum parsimony (MP) tree (Fig. 2). Both phylogenetic analyses were identified as two major assemblages: *Gracilaria sensu lato* clade and *Gracilariopsis* clade. *Gracilariopsis* was early diverged with strong bootstrap supports in ML (98%) and MP (99%) trees. *Gracilaria sensu lato* formed a sinagle assemblage without bootstrap support composed three large groups, treated here as *Gracilaria sensu stricto, Hydropuntia* and *G. vermiculophylla* clade. *Hydropuntia* represent an independent clade in ML tree but no bootstrap support. This monophyly does not hold in MP tree, due to *H. changii, H. fisheri* and *G. punctata* grouping with other clade. *G. vermiculophylla* clade are sustainedly monophyly with low and lacking bootstrap support in ML and MP tree.

Most specimens are grouped together as conspecific with verified *rbc*L sequences supported strong bootstrap, while several specimens are formed their independent clade. Clade composed four *Gracilaria* species, *G. articulata, G. blodgettii, Gracilaria* sp.3 and sp.4, are newly derived and clustered basally within *Gracilaria sensu stricto* in ML Tree (Fig. 1). *Gracilaria* sp.1 and sp.3 are proposed as new species with distinct morphology to comparing any other species. *Gracilaria* sp.2 is closely related *G. arcuata* with strong bootstrap support (100%). *H. multifurcata* clustered with the clade containing *H. edulis,* and we provide published sequences for this species for the first time. *H. fisheri* closely related to *H. changii* with similar morphology, but clearly distinct in the *rbc*L tree. *Gracilaria* sp.5 is included in *G. vermiculophylla* clade and closely related to *G. tenustipitata* with genetic variable.

4. DNA barcoding

NJ tree using K2P model (Fig. 3) showed the genetic distance for all specimens based on the COI DNA barcoding. DNA barcoding approach separated 22 species including five unidentified species from Asia-Pacific region with 11 sequences from GenBank. COI sequences of the conspecific members were either totally identical or very similar to each other, and formed distinct separate clusters. Among sequences acquired from in this study, 13 species represented by more than two individuals but 9 species are one individual only. We deal with intraspecific divergence for these 13 species except 9 species. The range of interspecific divergence across all taxa in Gracilaiaceae was 2.8-16.9%. The maximum genetic divergence (16.9%) produced between *G. pacifica* and *Gp. longissima*. Species comparisons within genera are summarized in Table 5. Low interspecific divergence levels were observed in two pairs, between *G. textorii* and *Gracilaria* sp.1 (2.8%), and between *G. tenuistipitata* and *Gracilaria* sp.5 (2.9%). The minimal sequence divergences of two pairs are also showed in *rbc*L tree. The range of intraspecific divergence within Gracilariaceae was 0-1.5%. Each conspecific intra-divergence was shown in Figure 4. In four species as *G. textorii, Gracilaria* sp.1, *H. fisheri,* and *H. multifurcata,* conspecific members are all identical. *Gracilaria salicornia* from six countries has the maximum intraspecific divergence among all species.

5. New geographical records and cryptic species

Our wide geographic sampling allowed us to extend and clarify the geographic range of a number of the species. *Gracilaria perplexa,* previously known from Australia as type locality, is here reported for the first time from Okinawa, Japan. In addition, *Hydropuntia fisheri,* previously known from Thailand and Vietnam, is reported for the first time from Malaysia.

Finally, *Gracilariopsis heteroclada,* previously known from Hainan in China and the Philippines, is reported for the first time from the Malaysia.

Comparisons of sequences within the study area as well as comparisons with published sequences revealed five cryptic species in this group. Two species of them, *Gracilaria* sp.1 and sp.3, have potential to be new species with morphological characters. More information of which, we shall mention below in discussion.

Table 4. Comparison of genetic information from *rbc*L and COI in the Gracilariaeae.

		Plastid *rbc*L	Mitochondria COI
Number of taxa		52	115
Number of species		54	31
Alignment length (bp)		1171	616
Number of Constant sites (%)		689 (58.8%)	384 (62.3)
Number of variable sites (%)		482 (41.2)	232 (37.7)
Number of informative sites (%)		411 (35.1)	213 (34.6)
Base frequency	π_A	0.307	0.265
	π_C	0.148	0.151
	π_G	0.218	0.185
	π_T	0.327	0.399

Table 5. Comparison of genetic diversity between *rbc*L and COI sequences.

	COI (This study)	*rbc*L (This study)	*rbc*L (Gurgel and Fredericq 2004)
Gracilaria sensu stricto	2.8 - 14.2 (n=16)	1.1 - 9.6 (n=22)	2.10 - 9.05 (n=33)
Hydropuntia	5.9 - 14.5 (n=6)	2.5 - 12.0 (n=10)	0.40 - 10.08 (n=11)
G. vermiculophylla Clade	2.9 - 13.7 (n=3)	2.3 - 13.8 (n=3)	12.12 - 12.67 (n=3)
Gracilaria sensu lato	2.8 - 14.7 (n=25)	1.1 - 14.6 (n=35)	2.00 - 13.61 (n=47)
Gracilariopsis	6.8 - 12.1 (n=5)	1.3 - 7.8 (n=17)	2.37 - 7.47 (n=13)

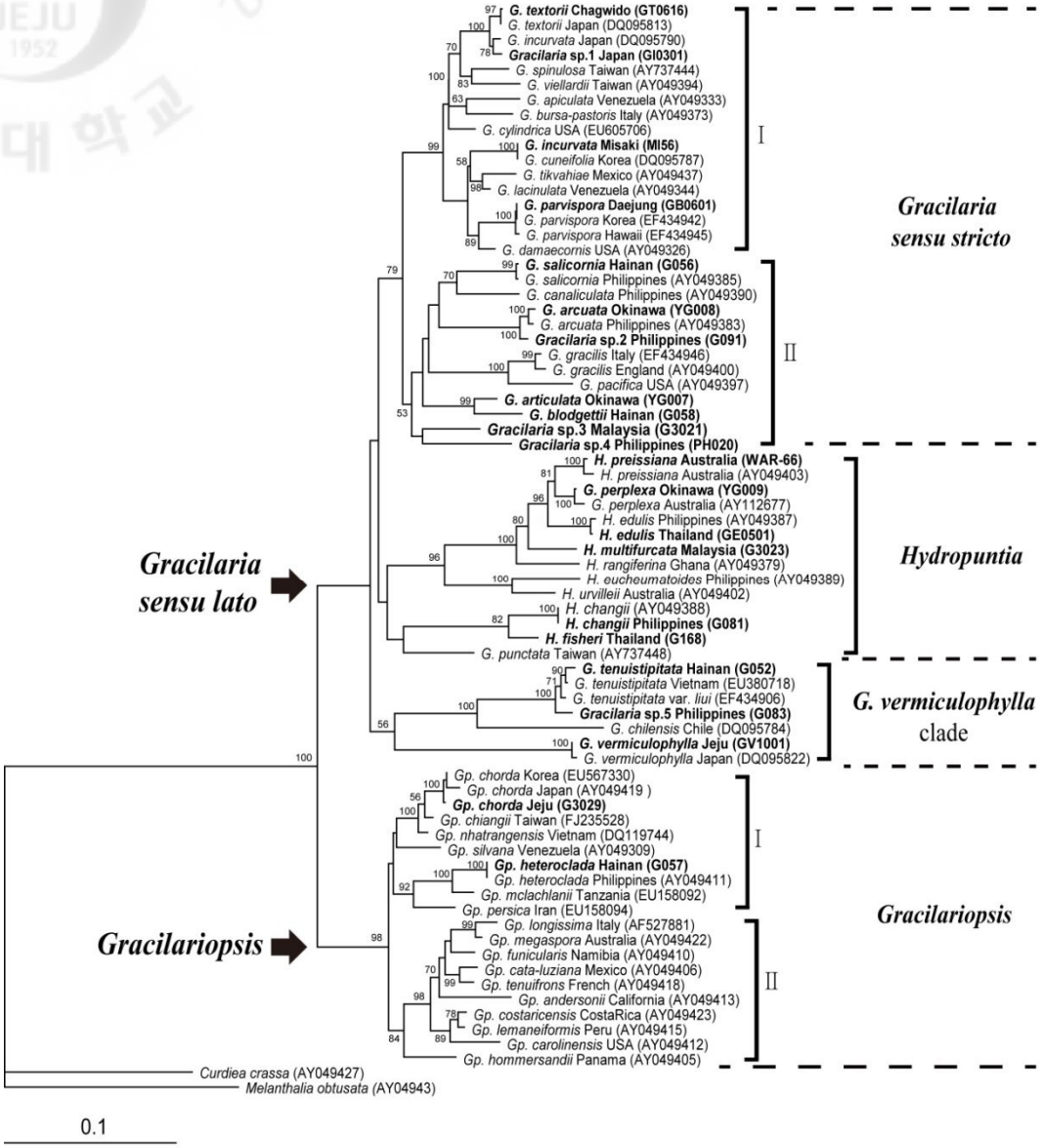

Fig. 1. Maximum likelihood tree for Gracilariaceae inferred from the *rbc*L sequences

The supporting values shown above the branches are from 100 bootstrap values. GenBank accession numbers of the *rbc*L gene sequences are reported in brackets; sequences generated in the present study are indicated in bold.

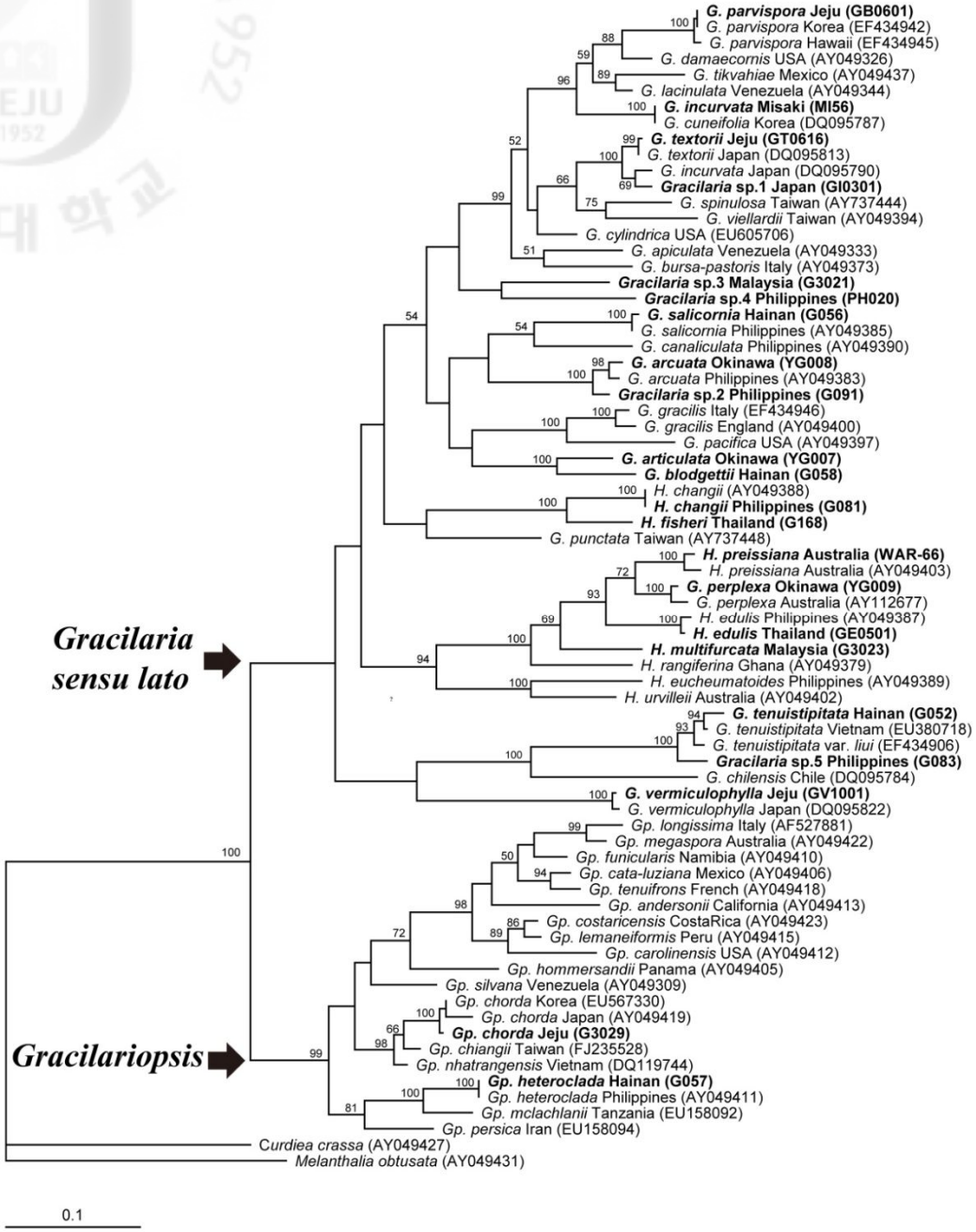

Fig. 2. Maximum parsimony tree for Gracilariaceae inferred from the *rbc*L sequences. The supporting values shown above the branches are 1000 bootstrap values. GenBank accession numbers of the *rbc*L gene sequences are reported in brackets; sequences generated in the present study are indicated in bold.

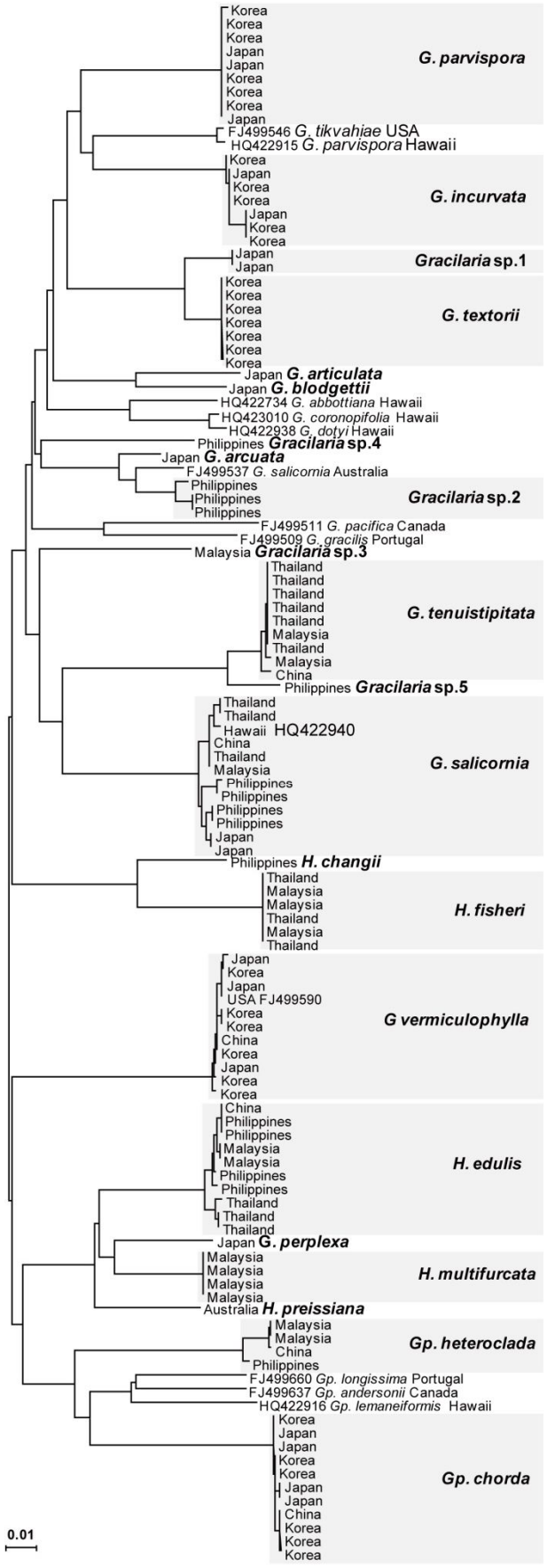

Fig. 3. Neighbor joining tree of COI barcode of 115 specimens in Gracilariaceae. Species collected in the present study highlighted.

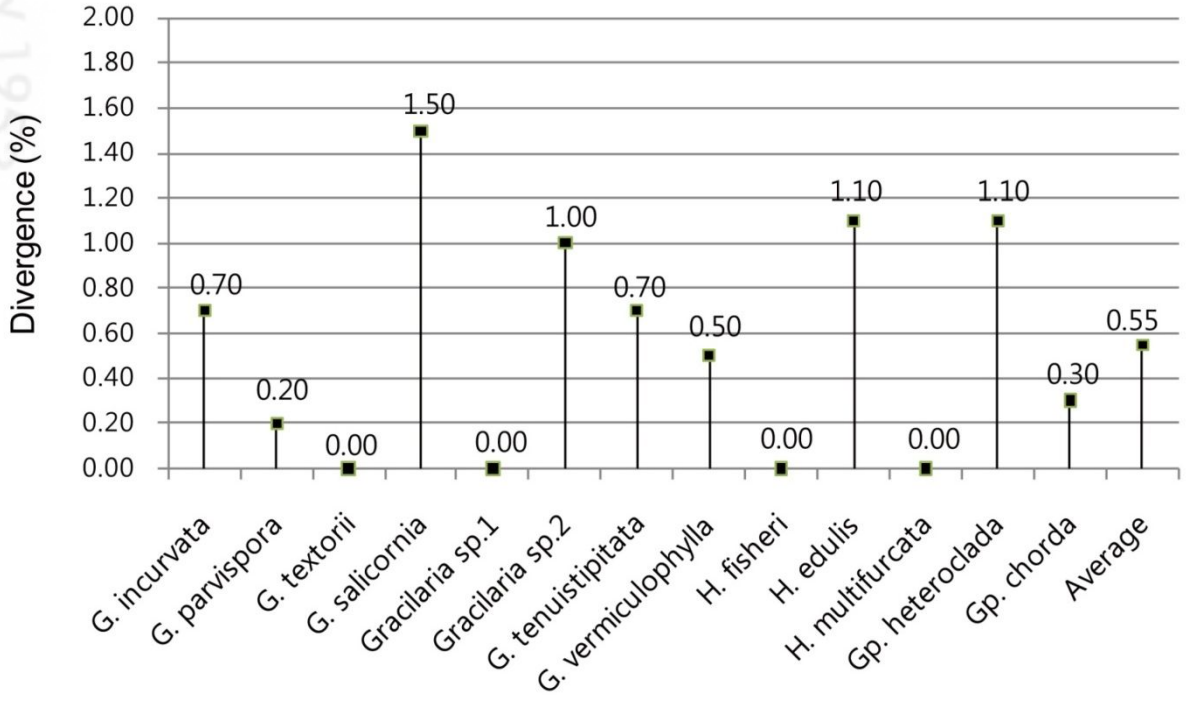

Fig. 4. Comparioson of intraspecific divergence in COI among Gracilariaceae species. Range in values is shown.

Members of *Gracilaria textorii, Gracilaria* sp.1, *Hydropuntia fisheri, H. multifurcata* are all identical.

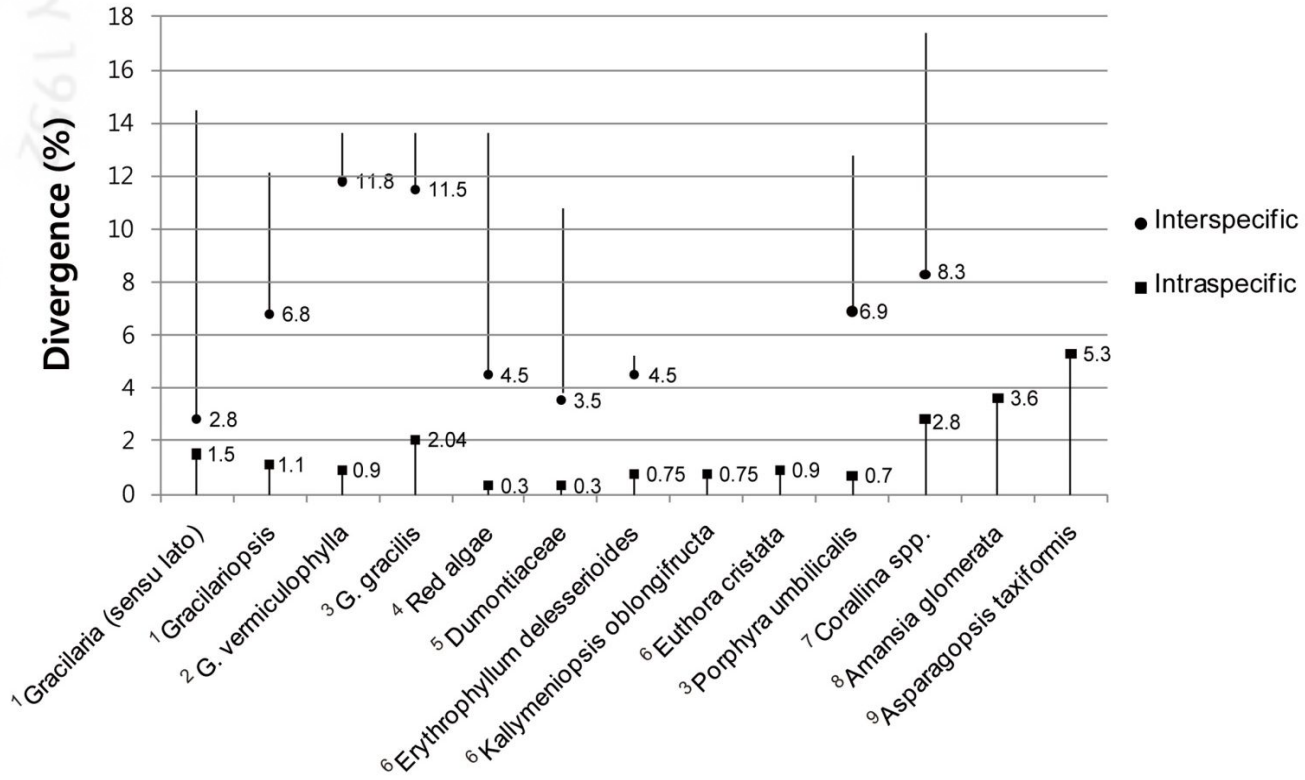

Fig. 5. Inter- and intraspecific divergences in several red algae. Range in values is shown.

References and molecular markers: [1]this study (COI), [2]Yang et al. 2008 (*cox*1), [3]Robba et al. 2006 (*cox*1), [4]Saunders 2005 (COI), [5]Saunders 2008 (COI), [6]Clarkston and Saunders 2010 (COI), [7]Walker et al. 2009 (*cox*1), [8]Sherwood et al. 2011, [9]Sherwood 2008 (COI).

Ⅳ. DISCUSSION

1. Phylogeny of Gracilariaceae inferred from *rbc*L gene

1.1 *Gracilaria* clade

Gracilaria is a species-rich genus and one of the most taxonomically difficult genera in the Rhodophyta. In this study, 11 species from the Asia-Pacific region were resolved based on *rbc*L gene sequence data (Fig. 1).

Subgroup I: The phylogenetic relationships among deeper nodes within the most derived *Gracilaria* lineage were composed of four Asian *Gracilaria* species with high bootstrap support in ML and MP (99/99) analyses. In this lineage, three flat *Gracilaria* species, namely, *G. incurvata*, *G. textorii*, and *Gracilaria* sp.1 from Japan, and one compressed species, *G. parvispora*, were included. In Pacific Asia, three of the flat species (*G. cuneifolia*, *G. incurvata*, and *G. textorii*) were very similar in having proliferous thalli and shallow spermatangial conceptacles, and were difficult to identify because of variation in frond morphology and inconsistent branching patterns (Yamamoto, 1978). Kim *et al*. (2006) provided molecular evidence of *G. textorii* and *G. cuneifolia* from Korea and Japan using the plastid-coding *rbc*L and *psb*A genes. Their molecular phylogenetic analyses revealed the entities in each species, each of which has a considerable amount of gene sequence diversity. In addition, both the *rbc*L and *psb*A sequences clearly separated Japanese *G. incurvata* samples from *G. textorii*. We recently collected *G. incurvata* from the type locality in Misaki, Kanagawa Prefecture, Japan, and analyzed this species' *rbc*L sequence. The data suggested that *G. incurvata* from Misaki and a previously published *G. cuneifolia* from Korea were identical. Our numerous specimens collected from intertidal and subtidal regions of Korea were also identical.

Although *G. incurvata* is very similar to *G. textorii* and *G. cuneifolia,* it is distinguished by narrower blades, incurved apices, and twisted branches. *G. textorii* has a relatively high divergence (5.9–6.0%) from *G. incurvata* regardless of their similar morphologies, and is sister to *Gracilaria* sp.1 from Japan. This raises questions about *Gracilaria* sp.1. Sequence divergence between *G. textorii* and *Gracilaria* sp.1 was 1.1–1.3%, ambiguous values in terms of separating Gracilariaceae species. Nevertheless, the 100% bootstrap value and obvious differences in morphology convinced us that this represented a new species. *Gracilaria* sp. 1 from Japan is characterized by cylindrical stipes, and produces a round to elliptical lobe with roundish apices and smooth margins. At present, it does not fit any reported Japanese *Gracilaria* species; therefore, study of its anatomical and reproductive features is needed before giving it a formal taxonomic status. On the other hand, *G. parvispora* was substituted for *G. bursa-pastoris* from Korea and Japan by Kim *et al*. (2008a). We showed that *G. parvispora* is monophyletic with a Hawaiian sample in this study. This species has compressed thalli and is restricted to the Pacific, and is sister to *G. damaecornis* from the USA, with 2.9% divergence.

Subgroup II: This clade is composed of six terete species from the Asia-Pacific region: *G. arcuata*, *G. articulata*, *G. blodgettii*, *G. salicornia*, *Gracilaria* sp. 2, and *Gracilaria* sp. 4, and one flat unknown species from Malaysia, *Gracilaria* sp. 3. Many species included in this clade, and *G. salicornia* has been confused with several related species (Millar and Xia, 1997; Xia, 1986). *G. salicornia*, *G. articulata*, and *G. blodgettii* have cylindrical and constricted thalli as common morphological features. A degree of thalli constriction and phenotypic plasticity renders species delimitation based solely on morphological grounds problematic (Xia, 1986). In a tree derived from *rbc*L sequence data, however, these species are related, but taxonomically distinct (Fig. 1 and 2). The presence of *G. blodgettii* in Asian countries has been questioned by Fredericq and Norris

(1992), but Terada and Yamamoto (2000) confirmed that the species is found in Japan. Our molecular evidence also supported the existence of *G. blodgettii* in Hainan, China. *G. blodgettii* is distinguished from *G. articulata* and *G. salicornia* by its acute apices (Terada and Shimada, 2005). Although *G. salicornia* and *G. articulata* have blunt apices with club-shaped branches in common, *G. salicornia* is distinguished by short branches and enlarged branch apices; intercalary articulations are present only in *G. articulata*. Our *G. salicornia* specimens are group together with published *rbc*L sequences with 100% bootstrap values. And this species is sister to *G. canaliculata* from the Philippines, with 6.1–6.3% divergence. *G. arcuata* does not share a pattern of thallus constriction with other species. This species is distinguished by its tapering branches that curve toward the apex (Ohno *et al*., 1999). *G. arcuata* is sister to *Gracilaria* sp. 2 from the Philippines with 100% bootstrap support. These two species have different morphology but little genetic divergence (1.1–1.2%). Genetic divergence between *G. arcuata* and *Gracilaria* sp. 2 is more remarkable according to COI data (Fig. 3). The distinct characteristics of this unknown species are the arcuate, irregularly secund to subdichotomous branches that are curved to the main axes. *Gracilaria* sp. 4, also collected from the Philippines, is morphologically very similar to *G. canaliculata*. However, this species is far from *G. canaliculata* in the *rbc*L tree, and so requires additional anatomical observation. In this clade, on the other hand, one flat species from Malaysia (*Gracilaria* sp. 3) was included. We identified this specimen as *G. textorii* at first; however, it is more related to cylindrical species from Asia, and is sister to *Gracilaria* sp. 4 with 5.7% divergence. Four species located in the base of subgroup II are newly derived and their classification as species was confirmed by the molecular evidence in this study. Unknown species should now be the focus of research in order to elucidate the species and phylogeny of this group.

1.2 *Gracilariopsis* clade

The genus *Gracilariopsis* was separated from members of *Gracilaria* based on the characteristics of the reproductive organs; i.e., tubular nutritive cells are absent in the cystocarp and spermatangial cells are produced from superficial cortical cells (Fredericq and Hommersand, 1990a). This genus has repeatedly been confirmed as valid by molecular data (Gurgel and Fredericq, 2004; Iyer *et al*., 2005; Kim *et al*., 2008). While 20 species are currently recognized globally (Guiry and Guiry, 2011), three (*Gp. nganii* Pham-Hoàng Hô, *Gp. phanthietensis* Pham-Hoàng Hô, and *Gp. rhodotricha* E.Y. Dawson) of these are ambiguous. The existence of *Gp. nganii* and *Gp. phanthietensis* has not been reported since their first collection, and *Gp. rhodotricha* was unsuitable for inclusion in the genus *Gracilariopsis* because the spermatangia originate in deep pockets, unlike the superficial spermatangia in *Gracilariopsis* (Hau and Lin, 2006). *Gracilariopsis* species have a highly conserved morphology - cylindrical thalli - that render the definition of species boundaries problematic. A phylogenetic tree based on *rbc*L data (Fig. 1 and 2) allows the members of *Gracilariopsis* to be easily distinguished, with 1.3–7.8% differences among 17 species globally. This also confirmed monophyly with strong bootstrap support (ML 98/MP 99).

The *Gracilariopsis* clade is divided into two subgroups.

Subgroup I: Subgroup I is composed of Asian *Gracilariopsis* specimens, with the exception of two species from Venezuela and Tanzania. In this study, the two *Gracilariopsis* species were resolved as *Gp. chorda* and *Gp. heteroclada*. Many Asian species are mostly endemic, or have a very narrow distribution. *Gp. chorda* is the first member of the genus reported in Asia, and is distributed in Korea, Japan, China, and Taiwan. *Gp. chorda* groups together with 100% bootstrap values and is sister to *Gp. chiangii*, with 1.3–2.2% divergence. *Gp. heteroclada* was originally described from

Yinngehai, Hainan Island, China, based on its mix of long and short spine-like branches (Chang and Xia, 1988), and is distributed in Vietnam and the Philippines. This species resembles *Gp. chorda*, but differs in terms of its small medullar cells, thicker cell walls, and a non-constricted cystocarp base (Chang and Xia, 1988). The specimens we collected from the type locality are identical to those from the Philippines with 100% bootstrap values. Molecular data confirmed the occurrence of *Gp. heteroclada* in Hainan as the type locality, and in the Philippines and Malaysia. Its geographical distribution now includes Malaysia. *Gp. heteroclada* is sister to *Gp. mclachlanii* from Tanzania, with 2.9% divergence.

Subgroup II: This group has moderate bootstrap support (84%) in ML tree, and includes the type species of genus *Gracilariopsis*, *Gp. andersonii.* Dawson (1949) designated *Gp. sjoestedtii* as the type species, now synonymized to *Gp. andersonii. Gp. lemaneiformis* was shown not to have a worldwide distribution but to be restricted to the vicinity of Peru in South America. This species is most likely identical to *Gp. costaricensis* from Costa Rica (Gurgel and Fredericq, 2004). There was only 0.8% divergence between *Gp. lemaneiformis* and *Gp. costaricensis*, and the range of variation in *rbc*L is 1.3–7.8%, with the exception of two species.

1.3 *Hydropuntia* clade

This clade includes six *Hydropuintia* species from the Asia-Pacific region. *Hydropuntia* Montagne was originally grouped with *H. urvillei*, based on their elaborate compound spermatangial conceptacles (Wynne, 1989). *Hydropuntia* has been validated by Fredericq and Norris (1992) on the basis of its “indirect” production of gonimoblast, basally directed, nutritive filaments; more rigidly programmed pericarp and cystocarp cavity development; and ramified, spreading, fusion cells. Gurgel and Fredericq (2004)

confirmed the independence of the genus *Hydropuntia* in Gracilariaceae using *rbc*L sequence evidence. The generic status of *Hydropuntia*, however, is still in doubt when both morphological and molecular data are considered (Bellorin *et al*., 2002; Gargiulo *et al*., 2006). In our ML tree derived from *rbc*L data (Fig. 1), *Hydropuntia* are monophyletic and represent an independent clade with a type species but no bootstrap support. This monophyly does not hold in an MP tree (Fig. 2), due to three species grouping with the *Gracilaria sensu stiricto* clade.

The type species of *Hydropuntia* (*H. urvillei*) is found in the clade, and sister to *H. eucheumatoides* from the Philippines with 5.3% divergence. *H. edulis*, a widely distributed species, was collected from the Philippines, Malaysia, Thailand, Taiwan, and Hainan, China, in this study. This species is closely related to two Australian species, *H. preissiana* and *G. perplexa*. *H. preissiana* was originally reported from Southwestern Australia in 2004. We collected this species from Rottnest Island, Western Australia, and it exhibits only 0.8% divergence from the published sequence. Specimens have a compressed thallus with many small branchlets arising from the blade margins. Another species is *G. perplexa*, reported only in Australia. Byrne *et al*. (2002) mentioned that this species has an ambiguous morphology making it difficult to distinguish from several other Australian species; however, they confirmed a new species of *G. perplexa* using RuBisCo and *cox*2-3 spacers. Our *rbc*L data also demonstrated its distinctiveness, using a specimen we collected in Okinawa, Japan. Japan is the only region reported outside the type locality. Also, we suggest that *G. perplexa* should be transferred to *Hydropuntia* due to its *Hydropuntia*-type spermatangial conceptacles (Byrne *et al*., 2002) and molecular data. Sequence data using *Rbc*L (this study) and RuBisCo and *cox*2-3 spacers (Byrne *et al*., 2002) indicated that *G. perplexa* is sister to *H. preissiana*. *H. preissiana* and *G. perplexa* have 2.5–3.0% interspecific divergence in *rbc*L data and moderate bootstrap

values (ML 81/MP 72). In terms of the morphological characteristics of these species, *G. perplexa* is easily distinguished from *H. preissiana* by its terete thallus with acute tips.

H. multifurcata was originally reported from Mauritius, Africa. We collected four samples of this species in Penang, Malaysia, each of which exhibited a compressed and multiaxial thallus. This species has *Hydropuntia*-type spermatangial conceptacles (Terada *et al*., 2004). We confirmed this species' inclusion in *Hydropuntia* based on molecular data.

Two cylindrical species were derived in this study, *H. changii* and *H. fisheri*. *H. changii* from the Philippines was identical to published *rbc*L data, and characterized by a slightly constricted branch base and tapering apices. This species is sister to *H. fisheri* with 3.9% divergence and an 82% ML bootstrap value. *H. fisheri* was collected in Thailand and Malaysia in this study. To the best of our knowledge, this is the first report of *H. fisheri* in Malaysia. *H. fisheri* is characterized by an abruptly constricted branch base and inflated branches.

1.4 *Gracilaria vermiculophylla* clade

This clade is composed of *G. tenuistipitata*, *G. vermicuolophylla*, and *G. chilensis*, which were proposed to make up a new genus by Gurgel and Fredericq (2004). In the present study, this clade was monophyletic and independent in both ML and MP (Fig. 1 and 2), however, had a low bootstrap value (56%) in the ML analysis.

G. tenuistipitata was collected in Thailand, Malaysia, and Hainan, China, in this study. The *G. tenuistipitata* clade was resolved with 71% and 93% bootstrap support in the ML and MP analysis, respectively. This species is closely related to *Gracilaria* sp. 5 from the Philippines with 2.3% *rbc*L divergence and 2.9–3.1% divergence in COI data. These two

species are similar in morphology, although the genetic divergence is sufficient to separate them into distinct species.

G. vermiculophylla was also resolved with strong support (100%) in both ML and MP. We collected this species from Korea, Japan, and China.

2. DNA barcoding inferred from COI gene

The utility of a DNA barcoding system based on the COI mitochondrial gene for identification and discovery of species has been tested in several red algal groups (Clarkston and Saunders, 2010; Gall and Saunders, 2010; Sherwood *et al*., 2011). In fact, this technique may be useful for identifying cryptic species in which diagnostic morphological characteristics are lacking or difficult to analyze (Sherwood *et al*., 2008).

We aligned specimens of 17 species of *Gracilaria*, 5 of *Gracilariopsis*, and 6 of *Hydropuntia* for the COI barcoding data set. COI barcoding successfully differentiated the Gracilariaceae to the species level. The within-species divergence values ranged between 0% and 1.5% in all, whereas between-species divergences were greater than 2.8%. Intraspecific variation was slightly higher for *G. salicornia* (1.5%), *Gracilaria* sp. 4 (1.0%), *H. edulis* (1.1%), and *Gp. heteroclada* (1.1%) than is typically reported in red algae. In terms of *G. salicornia* and *H. edulis*, intraspecific divergence is likely due to the specimens being collected from 6–7 different nations, because the greatest divergence values occurred between Thailand and the Philippines in both species. However, *Gracilaria* sp. 2 and *Gp. heteroclada* exhibited high intraspecific divergence despite being collected from only one and three countries, respectively. Also, these four species, having high intraspecific variation, have specimens from the Philippines in common. More research is required before this divergence can be attributed to phenomena such as

incipient speciation and introgression. Intraspecific divergence is absent from *G. textorii*, *Gracilaria* sp. 1, *H. fisheri*, and *H. multifurcata*. On the other hand, the interspecific divergence between *G. textorii* and *Gracilaria* sp. 1 in COI was 2.8%; these species are closely related and showed the lowest *rbc*L value (1.1%). Moreover, *G. tenuistipitata* is closely related to *Gracilaria* sp. 5, with 2.9–3.1% interspecific divergence in COI. These interspecific divergence values are a little lower than those of other red algae (Saunders, 2005; Robba *et al*., 2006; Saunders, 2008; Walker *et al*., 2009).

A critical feature of any region used as a 'barcode' in species identification is that the intraspecific variation must be significantly lower than the interspecific variation; a so-called 'barcoding gap' must exist (Hebert *et al*., 2003; Moritz and Cicero, 2004). The genetic diversity and differentiation of several mitochondrial genes are compared in Figure 5, and demonstrate the barcoding gap. Analysis of the intraspecific divergence in the Gracilariaceae with respect to other *Gracilaria* taxa revealed intermediate values (0–1.5%), but *G. gracilis* had a relatively high intraspecific divergence (2.04%). Usually DNA barcoding of Gracilariaceae species uses an empirical 0–2% intraspecific divergence; however, this threshold was far exceeded in other red algal species including *Asparagopsis taxiformis* (5.3%) and *Amansia glomerata* (3.6%) (Sherwood, 2008; Sherwood et al., 2011). Sherwood *et al*. (2011) suggested that these two species may represent species complexes, a possibility that would require detailed anatomical study. Intraspecific divergence of *Corallina* spp. (2.8%), which occurred in specimens between Ireland and England, also exceeded the threshold of the Gracilariaceae. With the exception of the three taxa mentioned above, the intraspecific divergence of Gracilariaceae is somewhat higher than that of other red algae, while minimum interspecific divergence is lower. Therefore, the barcoding gap of Gracilariaceae is narrower than that of other taxa.

The COI-5P exhibits high variability, especially in the third codon, making it effective for discriminating between even closely related species (Hebert *et al*., 2003). Due to this high interspecific divergence, the COI marker has successfully been used to establish species identity in several red algal species, which are difficult to identify (Saunders, 2005; Teasdale and Klein, 2010). Hebert *et al*. (2004) proposed a standard sequence threshold of ten times the mean intraspecific variation for the group. If applied to the *Gracilaria sensu lato* species examined in this study, the average intraspecific variation would be 0.52% with a 5.2% threshold. Because the minimum interspecific divergence in this study was 2.8% based on the COI gene, it does not correspond to a standard sequence threshold in *Gracilaria sensu lato.* If applied to the *Gracilariopsis* genus, it has 0.7% average intraspecific variation with a 6.8% threshold. However, *Gracilariopsis* represents far fewer species than *Gracilaria sensu lato;* therefore, a change in the interspecific variation may occur if more species are added to this group. In terms of results, we have five times the species sequence threshold of the mean intraspecific variation for Gracilariaceae. Previous barcoding studies have indicated that interspecific divergences of 3.5–18% should be sufficient for distinguishing among red algal species (Saunders, 2008; Robba *et al*., 2006; Clarkston and Saunders, 2010; Walker *et al*., 2009). In the present work, we identified a barcoding gap based on COI, and DNA barcode values greater than 2.8% corresponded to unequivocally distinct species. At lower values (1–2%), divergent mitotypes may or may not correspond to distinct species, and additional investigation is then required to assess the status of the populations under study (Saunders, 2005; Lane *et al*., 2007). Meyer and Paulay (2005), Meirer *et al*. (2006), and Wiemers and Fiedler (2007) all question the notion of barcoding gaps, suggesting that such “gaps” are in fact an artifact of insufficient sampling across taxa. In this study, closely related Gracilariaceae species lessened the purported barcoding gap; therefore, an extension to

this study with additional red algae species is warranted to resolve this issue. However, Lukhtanov *et al*. (2009) discussed how the inclusion of geographically separated populations influenced DNA barcoding. They indicated that although expanded geographical coverage did substantially increase intraspecific variation, reducing the barcoding gap between species, this did not decrease species identification using NJ clustering. In our COI data (Fig. 3), all species were independently monophyletic and possessed distinct clusters, indicating that this DNA barcoding method can effectively identify these Gracilariaceae species.

Ⅴ. CONCLUSIONS

Our phylogenetic reconstruction of Gracilariaceae confirmed the previously recognized subgeneric classification, but highlighted several species-level cases. We derived a total of 22 Gracilariaceae species from the Asia-Pacific region and elucidated their phylogenetic relationships and clarified the classifications of previously unclear entities. *Rbc*L and COI sequences were sufficiently differentiated at the species level in Gracilariaceae. With these two molecular datasets we have contributed to the assessment of species boundaries in Gracilariaceae. Using *rbc*L data, morphologically distinct and well-defined species usually have more than 2% interspecific *rbc*L sequence divergence. Lower interspecific divergence occasionally exists between closely related species; therefore, before final conclusions can be made regarding the taxonomy of this group, it is necessary to perform a comprehensive investigation that includes phylogenetic analysis of several taxa. The COI barcoding used here allowed the clear distinction of the different species in this group, and COI sequences exhibited a barcoding gap. Intraspecific diversity plays a fundamental role in delimiting species boundaries. Generally, COI barcoding of Gracilariaceae species showed 0–2% intraspecific divergence, which is the standard threshold for separation of species. Thus, studies of intraspecific divergence that include additional sampling should be conducted to define the species boundaries. For large-scale phylogeny and species identification, a consensus regarding the most suitable gene is essential. To better understand the evolutionary relationships within this group, an extension to this study with additional species is necessary.

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국 문 초 록

홍조류 꼬시래기과는 아가로즈를 생산하는 주요 해조류로서 현재 150 여종의 생육이 전 세계적으로 널리 보고되었다. 꼬시래기과는 유용해조류라는 중요성 때문에 생활사, 배양, 분류 등 폭넓은 연구가 진행되어 왔으나 종을 식별하는데 사용하는 뚜렷한 기준형질이 모호하고, 개체간, 지역간 환경에 따른 형태변이가 심하며, 과 내의 많은 종 수로 인하여 분류학적 및 종의 경계에 대한 문제가 많았다. 본 연구는 아시아-태평양지역에 서식하고 있는 유용 홍조류 꼬시래기과의 계통학적 유연관계와 종 다양성을 밝히고, 종의 경계를 명확히 하는 데에 그 목적이 있다. 이를 위해 한국, 일본, 말레이시아 등 7 개국 연안의 조간대 및 조하대에서 채집된 꼬시래기과의 115 개체를 연구재료로 사용하였으며, 색소체 *rbc*L 과 미토콘드리아 COI 유전자 분석이 이루어졌다. *Rbc*L 1,171 염기쌍과 COI 616 염기쌍이 분석된 결과, 5 종의 잠재종을 포함한 총 22 종의 생육 및 지리적 분포가 확인되었다. *Rbc*L 유전자 분석을 통해 추론된 분자계통 결과에서 *Gracilaria*, *Gracilariopsis*, *Hydropuntia* 속은 단계통을 이루었고, 22 종의 분류학적 위치 및 계통학적 유연관계를 확인하였다. 최대절약(Maximum parsimony), 최대유사 (Maximum likelihood) 방법을 이용한 분석에서는 *Hydropuntia* 와 *G. vermiculophylla* 그룹을 포함하는 *Gracilaria sensu lato* 와 *Gracilariopsis* 으로 크게 나뉘었으며, 꼬시래기과내의 유전적 변이율은 1.1-14.6%로 나타났다. COI 유전자 분석을

통한 DNA 바코딩 연구결과를 통해 7 종의 *Gracilaria*, 2 종의 *Hydropuntia*, 2 종의 *Gracilariopsis* 를 확인하였다. 0-1.5%의 종내 변이율과 2.8-16.9% 종간 변이율이 관찰되었고, 모든 종이 근린결합분석 (Neighbor joining)을 이용한 계통수에서 각각의 독립적인 계통군을 형성하고 있어 DNA 바코딩 기법이 꼬시래기 종의 식별에 효율적임을 보여주고 있다. 한편, 아시아-태평양지역 7 개국에서의 채집을 통하여 3 종의 미기록종을 확인하였다. 기준생육지인 호주에서만 생육이 보고되어 왔던 *G. perplexa* 는 일본의 오키나와섬에서 생육을 확인하였고, 태국과 베트남에 보고된 *H. fisheri* 와 중국의 하이난섬, 필리핀에서 보고된 *Gp. heteroclada* 는 말레이시아에서의 생육을 확인하였다. 또한 현재까지 보고된 종과는 다소 차이를 나타내는 외부형태와 DNA 염기서열을 가지는 5 개의 잠재종을 확인하였으며, 이에 대한 분류학적 위치 및 계통학적 유연관계를 규명하기 위하여 추가적인 연구가 필요할 것으로 사료된다.

감사의 글

어린 시절, 마냥 과학자가 꿈이었던 저는 생물을 좋아하는 고등학생이 되었고, 지금의 저는 해조류와 분류학을 사랑하는 생물학도가 되었습니다. 많은 것을 배우고 느끼느라 바빴던 2 년간의 석사과정을 마무리하면서, 그리고 좀 더 성장한 모습으로 맞이할 박사과정을 앞두면서 저를 믿어주시고 응원해주시는 모든 분들께 감사하다는 말과 함께 더욱 발전하는 제가 되리라는 다짐의 말을 드리고 싶습니다.

우선, 새로운 학문의 길을 알게 해 주신 김명숙 교수님께 진심 어린 감사와 존경의 마음을 전합니다. 넓은 세상에서 다양한 경험을 할 수 있도록 많은 기회를 주셨던 교수님의 마음을 잊지 않고, 교수님의 열정을 닮고자 하는 마음과 앞으로 더욱 성장하는 모습으로 보답하겠습니다. 그리고 학위논문을 완성하기까지 세심한 심사와 큰 격려를 주신 김문홍 교수님과 이준백 교수님께 깊은 감사의 마음을 전하며, 생물학의 다양한 분야에서 노력하시며 학부과정부터 많은 가르침을 주신 오덕철 교수님, 이화자 교수님, 고석찬 교수님, 김세재 교수님, 이선령 교수님께도 깊은 감사를 드립니다.

바쁘신 와중에도 제가 혼자서도 실험할 수 있도록 정말 도움 많이 주신 조가연 박사님과 이진성 박사님, 그리고 프로그램분석방법을 가르쳐주신 이정형교수님, 덕분에 크게 발전할 수 있었습니다. 감사합니다.

언제나 함께했던 해산식물분류학실험실에 정찬선생님, 병석선생님, 형우오빠, 영호오빠. 함께 채집 다니고 실험하며 쌓아온 소중한 추억과 신뢰로 형성된 우리이고, 바쁘고 힘들수록 더 멋진 팀워크를 보여줬던 우리이기에, 많이 의지할 수 있었고 힘을 낼 수 있었습니다. 그리고 스쿠버다이빙을 가르쳐주신 고용덕 선배님에게도 감사의 마음을 전합니다. 생태학실험실에 현화자, 송국만 박사님과 은영언니, 강대현오빠, 분자생물학실험실에 성일선생님, 형식선생님, 혜선언니, 민진언니, 승우오빠, 정환오빠, 생명공학실험실에 구슬언니와 성훈오빠, 수경언니, 미생물실험실에 김지영 선배님, 한수언니, 후돈오빠를 비롯한 생물학과 원우회 식구들과,

갑작스런 질문에도 자세하게 대답해주셨던 박지권 박사님, 항상 밝은 모습의 효민오빠와 세영언니에게도 고마움의 마음을 전합니다. 소중한 동기들인 선아, 영준이, 종현이, 민곡이, 구동환, 항상 잊지 않고 날 챙겨주는 우리 홍가, 너무나도 고맙습니다.

태어날 때부터 인연이 된 신례리 패밀리 승일, 성환, 남진, 동현, 기문, 정원, 동규, 민범, 윤정, 현규, 라나, 진아, 은주, 은애, 은혜, 효진. 자주 만나지 못하더라도 각자의 자리에서 서로를 응원해주는 큰 힘이 되고 있으리라 믿습니다. 또한 앞으로도 지금처럼 내 곁에서 좋은 친구로 남아줄 소희, 소영, 지현, 공희, 유나, 영규와 나의 학교생활의 활력소가 되어준 제주대 테니스동아리 선후배님들께 감사의 마음을 전합니다.

사랑하는 부모님, 두 분이 계셨기에 제가 지금 이 자리에서 하고 싶은 공부 마음껏 하며 행복할 수 있었습니다. 딸이 하는 일을 전적으로 믿어주시고 기다려주셔서 너무 감사합니다. 겉으로 표현할 줄 모르는 무뚝뚝한 맏딸이지만, 저를 위해 부모님이 흘리시는 땀방울을 가슴 깊이 새기며 앞으로 제가 제일 존경하는 두 분을 위해 더 큰 사람이 되도록 노력하겠습니다. 항상 건강하시고 행복하시길 바랍니다. 이제 어엿한 요리사가 된 준호, 스스로 잘 하고 있는 모습에 누나가 너무 고맙고, 많은 걸 못 챙겨준 것에 미안하고, 그리고 하나뿐인 동생에게 사랑의 마음을 전합니다.

마지막으로 5 년전이나 지금이나 늘 내 곁에서 믿음과 격려로 날 지켜준 한은규에게, 그 변치 않는 마음에 진정한 고마움의 마음을 전합니다.

모든 분들에게 다시 한번 진심으로 감사드립니다. 더욱 발전하는 생물학도가 되겠습니다.