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Taxonomy and Phylogeny of *Neosiphonia japonica* (Rhodomelaceae, Rhodophyta) Based on *rbc*L and *cpe*A/B Gene Sequences

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Neosiphonia japonica is a rhodomelacean red alga that occurs in Korea, Japan, China, far-east Russia, northwest America, and New Zealand. Although it is distinguished by a bush-like habit having four pericental cells with cortication and numerous branches on axes, the taxonomy of *N. japonica* is still problematic. To investigate the taxonomy and phylogeny of the species, we analyzed *rbc*L and phycoerythrin (*cpe*A/B) genes from 19 samples of *N. japonica* and putative relatives. Phylogenetic trees from both genes show that *N. japonica* from Korea, Japan, New Zealand, and USA is clearly separated from *N. decumbens*, *N. harlandii*, and *N. flavimarina* from the Pacific Ocean. Instead, *N. harveyi* from the Atlantic Ocean was more related to *N. flavimarina* than to *N. japonica*. This result supports morphological and distributional differences between *N. japonica* and *N. harveyi*. However, the close relationship between these species suggests that they might have a recent most common ancestor. This is the second report to use the *cpe*A/B gene for evaluating species diversity in the Rhodophytes.

Key Words: *Neosiphonia japonica*, *rbc*L, *cpe*A/B, Rhodomelaceae, Rhodophyta, Taxonomy

INTRODUCTION

Neosiphonia M.S. Kim *et* I.K. Lee is a rhodomelacean red algal genus that is commonly encountered in the intertidal zone of temperate waters in the world. The genus *Neosiphonia*, which was established based on species previously named under the genus *Polysiphonia*, is well distinguished by procarps bearing a three-celled carpogonial branches, spermatangial branches arising from a branch of the trichoblasts, and tetrasporangia arranged in spiral series (Kim and Lee 1999). The phylogenetic difference of *Neosiphonia* from *Polysiphonia* is clearly shown by both cladistic analyses of morphological features and phylogenetic analyses of nuclear ribosomal small subunit (SSU) region sequences (Choi *et al*. 2001). Recently, *Neosiphonia* is found in Malaysia (Masuda *et al*. 2001; Tani *et al*. 2003), Vietnam (Abbott *et al*. 2002), Brazil (Guimarães *et al*. 2004), Hawaii (Kim and Abbott 2006), and Japan as well as Korea (Kim 2005). To date, 22 species previously placed in *Polysiphonia* have been transferred into *Neosiphonia* and 1 new species reported.

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Neosiphonia japonica (Harvey) M.S. Kim *et* I.K. Lee was originally described from Hakodate, Hokkaido, Japan, as *Polysiphonia japonica* by Harvey (1856). The species has a bush-like habit, four pericental cells, cortication on axes, numerous branches, and three-celled carpogonial branches on females. The thalli occur commonly as epiphytes on other seaweeds from Korea (Kim 1995), Japan (Yoshida 1998), China (Tseng 1984), and Pacific Russia (Perestenko 1994). Despite studies on the morphology (Yoon 1986; Kudo and Masuda 1986; Kim 1995), life history (Kudo and Masuda 1986), lectotypification (Masuda *et al*. 1995), and distribution (Kim 1995), the taxonomy of *N. japonica* is still problematic. Kudo and Masuda (1986) reported that *N. japonica* (as *Polysiphonia japonica*) is very similar to *N. harlandii* (Harvey) M.S. Kim *et* I.K. Lee, *N. decumbens* (Segi) M.S. Kim *et* I.K. Lee, and *P. akkeshiensis* Segi. Yoon (1986) also concluded *N. savatieri* (Hariot) M.S. Kim *et* I.K. Lee, *P. forfex* Harvey and *N. teradomariensis* (Noda) M.S. Kim *et* I.K. Lee as varieties of *N. japonica*, based on the number of pericentral cells and the presence or absence of cortication at the base. Recently, McIvor *et al*. (2001) proposed that *N. japonica*, together with *P. akkeshiensis* Segi, *P. acuminata* N.L. Gardner and *P. strictissima* J.D. Hooker & Harvey were conspecific with *N. harveyi* (J. Bailey) M.S. Kim, H.G. Choi, Guiry &

G.W. Saunders.

DNA sequence data are very useful tools for elucidating the taxonomic status of morphologically confused species in red algae, determining which morphological characters are important for recognizing species, and understanding the phylogenetic relationships of species (e.g. Gavio and Fredericq 2002; Seo *et al.* 2003; Zuccarello and West 2003). Previously used molecular markers for taxonomy of *Polysiphonia* and *Neosiphonia* are protein-coding plastid *rbc*L (McIvor *et al*. 2001; Kim *et al*. 2004; Kim and Yang 2005) and nr DNA SSU region (Choi *et al*. 2001).

In the present paper, we analyzed both *rbc*L and phycoerythrin-coding plastid gene (*cpe*A/B) from *Neosiphonia japonica* and we specially pointed out *N. decumbens*, *N. harlandii* and *N. flavimarina* from the Pacific Ocean and *N. harveyi* from the Atlantic Ocean as putative relatives. The *rbc*L is one of the most commonly used molecular markers in red algae (Yang and Boo 2004) and the *cpe*A/B has proved useful for differentiating morphologically similar species and genera of the callithamniod red algae (Yang and Boo 2006).

MATERIALS AND METHODS

Samples

Specimens, their collection sites, and the GenBank accession numbers of *rbc*L and *cpe*A/B sequence data are listed in Table 1. Samples collected in the field were transported live back to the laboratory in sterilized seawater, and removed organisms attached on the thalli under a dissecting microscope. The cleaned thalli were dry in air and preserved in silica gel desiccant for DNA extraction. All voucher specimens were deposited in the herbarium of the Department of Biology (CNUK), Chungnam National University, Daejon, Korea.

Analyses of the *rbc*L and *cpe*A/B sequences

Genomic DNA was extracted from dry thalli ground in liquid nitrogen using the Invisorb Spin Plant Mini Kit (Invitek), according to the manufacturers' instructions. The *rbc*L region was amplified using primers F7 – R753 and F645 - RrbcS start and sequenced using primers F7, F645, R753, and RrbcS start (Lin *et al*. 2001; Gavio and Fredericq 2002). Amplifying and sequencing primers for each of the phycoerythrin gene (*cpe*A and *cpe*B) were PE-B5F, PE-B3F, PE-A5R and PE-A3R (Yang and Boo 2006). The PCR products were purified using a High Pure PCR Product Purification Kit (Roche), in accordance with the users' guide. The electrograms of the forward and reverse strands were constructed for all taxa using an ABI PRISM^{TM} 377 DNA Sequencer (Applied Biosystems) at Research Center, Chungnam National University, Daejon, Korea. The electropherogram output for each specimen was edited using the program Sequence Navigator v. 1.0.1 (Applied Biosystems). The alignment of each gene sequence was based on the alignment of the inferred amino acid sequence and was refined by eye. There were no gaps in alignments of both *rbc*L and *cpe*A/B sequences.

Phylogenetic analyses

Three data sets were used for the phylogenetic analyses: 29 taxa for *rbc*L, 20 taxa for *cpe*A/B, and 18 taxa for combined *rbc*L + *cpe*A/B data sets. We conducted the partition homogeneity test (PHT), implemented in PAUP* 4.0b10 (Swofford 2002). The PHT was done using 1,000 replicates, each with 100 random sequence-addition replicates using tree bisection-reconnection (TBR) branch swapping.

Maximum parsimony (MP) tree was constructed using PAUP* 4.0b10. Full heuristic search was carried out with 1,000 replicates, random addition sequences of taxa, keeping best trees only, holding one tree at each step, TBR branch swapping, collapsed of zero length branches and MULTREES on. Bootstrap values were calculated performing 1,000 replicates with following options selected: heuristic search, TBR branch swapping, collapse of zero length branches, and random sequence addition with one replicate.

Maximum likelihood (ML) analysis preformed the Akaike information criterion (AIC) method to determine the best-fitting model for each of three data. The model was general time reversible (GTR) model with a gamma correction for among-site variation (Γ) and invariant sites (I). Tree likelihoods were estimated using a heuristic search with 100 random addition sequence replicates, and TBR branch swapping. Bootstrap analyses were undertaken with 500 replicates.

Bayesian analysis was conducted using the GTR + Γ + I model, as was used in the ML analysis. The GTR rates and the proportion of invariable sites value were not fixed. For the data matrix, 1.3 million generations were performed with four chains and trees sampled every 100 generations. The burn-in period can be identified graphically by tracking the likelihoods at each generation. After of preliminary analyses, a burn-in period of 300,000 generations was determined to be appropriate for the data.

Table 1. Taxa, locality or data sources, and GenBank Accession number

Taxa	Locality (dates & collectors) or data sources	GenBank Accession No.			
		*cpe*A	spacer	*cpe*B	*rbc*L
Neosiphonia japonica (Harvey) M.S. Kim *et* I.K. Lee					
	Moonseom, Jejudo, Korea (18.iv.2003; MS Kim)	DQ787511	DQ787551	DQ787531	DQ787491
	Sinchun, Jejudo, Korea (11.v.2005; MS Kim)	DQ787510	DQ787550	DQ787530	DQ787490
	Sinnam, Samcheok, Korea (12.i.2002; SM Boo)	DQ787509	DQ787549	DQ787529	DQ787489
	Chiba, Japan (20.ii.2003; MS Kim)	DQ787512	DQ787552	DQ787532	DQ787492
	Shimoda, Japan (18.ii.2003; MS Kim)	DQ787513	DQ787553	DQ787533	DQ787493
	Sumiyoshi, Hakodate, Japan (08.iv.2004. MS Kim)	DQ787514	DQ787554	DQ787534	DQ787494
N. harlandii (Harvey) M.S. Kim *et* I.K. Lee					
	Cheongsapo 1, Busan, Korea (14.xii.2002; MS Kim)	DQ787501	DQ787541	DQ787521	DQ787482
	Sooryeomri 1, Geongju, Korea (23.xi.2003; MS Kim)	DQ787503	DQ787543	DQ787523	DQ787484
	Tonggumi, Ulreungdo, Korea (27.viii.2003; MS Kim)	DQ787504	DQ787544	DQ787524	DQ787485
	Cheongsapo 2, Busan, Korea (20.iii.2003; MS Kim)	DQ787502	DQ787542	DQ787522	DQ787483
N. decumbens (Segi) M.S. Kim *et* I.K. Lee					
	Haegeumgang, Geojedo, Korea (21.iii.2003; MS Kim)	DQ787497	DQ787537	DQ787517	DQ787477
	Namhaedaegyo, Namhaedo, Korea (03.xi.2002; MS Kim)	DQ787498	DQ787538	DQ787518	DQ787479
	Minamri, Tongyoung, Korea (20.i.2003; MS Kim)	DQ787499	DQ787539	DQ787519	DQ787480
	Sooryeomri 2, Geongju, Korea (23.xi.2003; MS Kim)	DQ787469	DQ787636	DQ787516	–
	Namhae, Namhaedo, Korea (02.xi.2002; MS Kim)	–	–	–	DQ787478
N. harveyi (Harvey) M.S. Kim, H.G. Choi, Guiry *et* G.W. Saunders					
	Wembury, Devon, England (28.vii.2003; SM Boo & EC Yang)	DQ787507	DQ787547	DQ787527	DQ787488
	Plymouth 1, Devon, England (28.vii.2003; SM Boo & EC Yang)	DQ787505	DQ787545	DQ787525	DQ787486
	Plymouth 2, Devon, England (28.vii.2003; SM Boo & EC Yang)	DQ787506	DQ787546	DQ787526	DQ787487
	Gape Tachimachi, Hakodate, Japan (9.iv.2004; MS Kim)	DQ787508	DQ787548	DQ787528	-
N. flavimarina M.S. Kim *et* I.K. Lee					
	Bangpo, Taean, Korea (16.vii.2003; MS Kim & EC Yang)	DQ787500	DQ787540	DQ787520	DQ787481
N. yendoi (Segi) M.S. Kim *et* I.K. Lee					
	Gijang, Busan, Korea (09.iii.2005; MS Kim)	DQ787515	DQ787555	DQ787535	DQ787495
Polysiphonia akkeshiensis Segi*					
	Akkeshi (230), Hokkaido, Japan (24.vi.1993; K Kogame)	–	–	–	AF342901
P. harveyi Bailey*					
	Monterey, California (321), USA (21.vii.1994; CAM)	–	–	–	AF342905
	Wilmington, North Carolina (448), USA (01.vi.1998; DW Freshwater)	–	–	–	AF342906
	Hayling I., Hampshire (175), England (04.x.1992; CAM)	–	–	–	AF342900
	Dale, Pembrokeshire (358), Wales (10.x.1996; CAM)	–	–	–	AF342899
	Skerries, Dublin (138), Ireland (30.viii.1992; CAM)	–	–	–	AF342898
	Maghery, W. Donegal (111), Ireland (02.viii.1992; CAM)	–	–	–	AF342897
	Wellington, New Zealand (460) (1998; W Nelson)	–	–	–	AF342907
P. japonica Harvey*					
	Oshoro (231), Hokkaido, Japan (01.vii.1993; T Abe)	–	–	–	AF342902
	Shimoda (284), Honshu, Japan (05.viiii.1993; CAM)	–	–	–	AF342903

*McIvor *et al*. 2001

The 10,000 trees sampled at stationarity were used to infer the Bayesian posterior probability (BP). Majority-rule consensus trees were calculated using PAUP*.

RESULTS

Characteristics of *rbc*L and *cpe*A/B

We determined a total of 29 *rbc*L sequences from *Neosiphonia japonica* and putative relatives; 19 sequences were newly determined in the present study, and 10 were derived from the GenBank database. In all, 1245 bp of the *rbc*L were aligned; 704 sites (56.06%) were variable and 17 sites (1.4%) were phylogenetically informative. There were excesses of adenine (32.33%) and thymine (30.2%) at all codon positions. Transitions were more common than transversions for all codon positions

Table 2. Information of *rbc*L and *cpe*A/B and statistics from MP analyses of both data

	*rbc*L	*cpe*A/B	combined
Number of taxa	29	20	18
Nucleotides base pairs	1245	922	2167
Base frequency of A/C/G/T	0.3233/0.1660/0.2088/0.3020	0.3492/0.1658/0.2055/0.2794	0.3343/0.1657/0.2075/0.2925
Number of transitions/transversions	3092/522	992/281	2098/443
(Ti/Tv ratio)	(5.9234)	(3.5302)	(4.7359)
Variable sites	704 (56.6%)	610 (66.2%)	710 (32.8%)
Informative sites	17 (1.4%)	8 (0.9%)	18 (0.8%)
Number of trees	5	1	2
Tree length	77	47	111
Consistency index	0.996	0.957	0.955
Retention index	0.995	0.941	0.922

(Ti/Tv = 5.9) (Table 2). The uncorrected sequence divergence (interspecific *p*-distance) values for the *rbc*L region within *Neosiphonia* ranged from 0.24% between *N. japonica* and *N. harlandii* to 4.33% between *N. flavimarina* and *N. yendoi*. *Neosiphonia japonica* from Korea and Japan differed by 0.08% sequence divergence, and there was a difference of 3.9% sequence divergence between *N. japonica* and *N. yendoi*. Most specimens of *N. japonica* were identical, but specimen from Chiba, Japan differed by one to two nucleotides from those other places. Within each of *N. harlandii*, *N. decumbens*, *N. harveyi*, the *rbc*L sequence was identical.

The 922 nucleotides (90.9% of full length, 1014 nt) of the *cpe*A/B gene were newly determined for 20 taxa. Of these, 610 positions (66.2%) were variable, and 8 positions (0.9%) were parsimoniously informative. There were excesses of adenine and thymine at all codon positions (34.92% and 27.94%, respectively). The transition/transversion (Ti/Tv) ratio was 3.53. The sequence divergence for the *cpe*A/B gene within *Neosiphonia* ranged from 0.1% (between *N. decumbens* and *N. harlandii*) to 5% (between *N. japonica* and *N. yendoi*). *Neosiphonia japonica* from Korea and Japan differed by 0.1% sequence divergence. As in the *rbc*L data set, base composition of the *cpe*A/B data was slightly AT-biased (Table 2). In the *cpe*A/B data, four specimens of *N. japonica* were identical, and those from the Shimoda and Chiba, Japan differed by one to two bp. Two *N. harlandii* specimens, six specimens of *N. decumbens*, and four specimens of *N. harveyi* were identical, respectively.

The combined *rbc*L + *cpe*A/B data set had 2167 characters, of which 1457 were constant and 18 sites were parsimoniously informative. Other characteristics of the combined data set are in Table 2.

Molecular phylogeny

The independent analyses of *rbc*L, *cpe*A/B, and *rbc*L+*cpe*A/B data sets resulted in congruent, though not identical, phylogenetic trees. The statistics for the MP analyses are compared among individual and combined data sets (Table 2), and the ML trees for all three data sets are shown in Figs 1, 2 and 3.

The ML analysis of *rbc*L data showed that *Neosiphonia japonica* was clearly separated from *N. harlandii*, *N. decumbens*, *N. harveyi* and *N. flavimarina* (Fig. 1). The monophyly of each species was supported by moderate BS values (81% for MP, 68% for ML, and 63% for BP). All

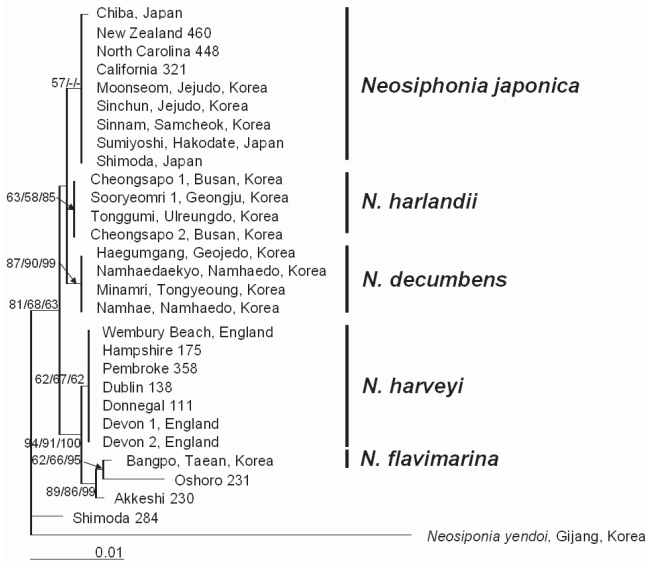

Fig. 1. Maximum likelihood tree for *Neosiphonia japonica* and relatives estimated from the *rbc*L sequence data (GTR + Γ + I model, -ln likelihood = 2125.49; Γ = 0.8742; I = 0.4345; A – C = 4796.14, A – G = 83706.39, A - T = 18278.33, C – G = 29800.00, C – T = 253001.46, G – T = 1). The bootstrap values shown above the branches are from MP/ML/BP methods and dashes indicate < 50% support of bootstrap.

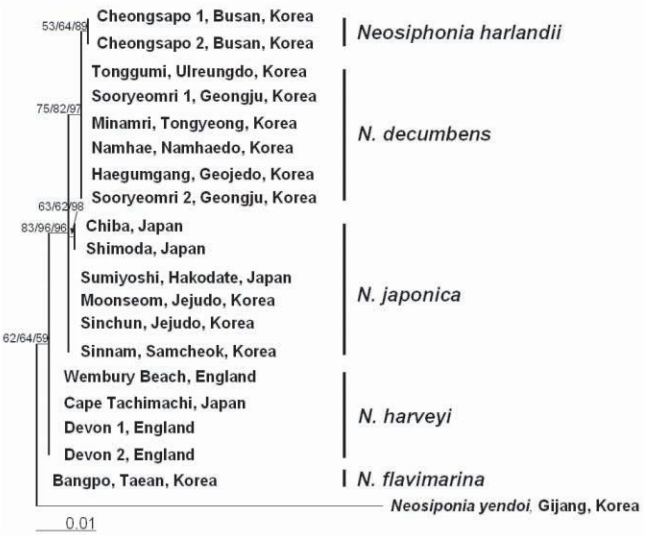

Fig. 2. Maximum Likelihood tree for *Neosiphonia japonica* and relatives estimated from the *cpe*A/B sequence data (GTR + Γ + I model, -ln likelihood = 1485.33; Γ = 0.1254; I = 0.3385; A – C = 2.2386, A – G = 3.3839, A – T = 1.8428, C – G = 2.1067, C – T = 29.3917, G – T = 1). The bootstrap values shown above the branches are from MP/ML/BP methods and dashes indicate < 50% support of bootstrap.

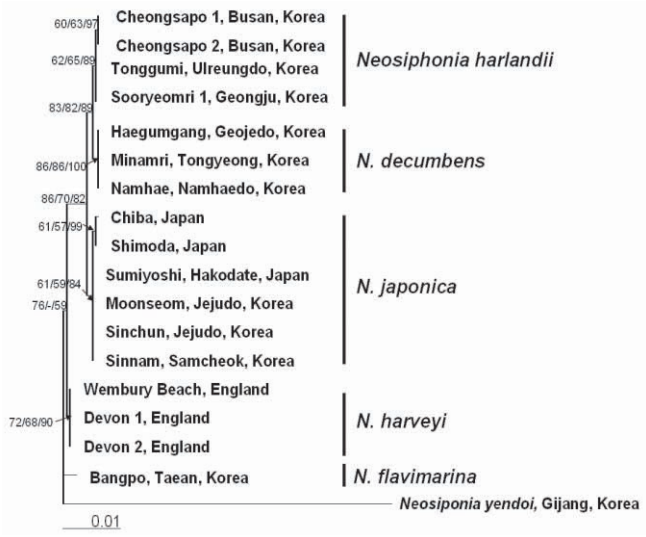

Fig. 3. Maximum likelihood tree for *Neosiphonia japonica* and relatives estimated from combined *rbc*L + *cpe*A/B sequence data (GTR + Γ + I model, -ln likelihood = 3516.78; Γ = 0.7754; I = 0.6724; A – C = 7.6707, A – G = 34.4701, A – T = 11.3466, C – G = 11.8066, C – T = 156.7648, G – T = 1). The bootstrap values shown above the branches are from MP/ML/BP methods and dashes indicate <50% support of bootstrap.

specimens of *N. japonica* investigated here formed a clade, and the species was related to *N. harlandii* and *N. decumbens*. The sequences of the specimen from the Sumiyoshi, Hakodate, Japan, the type locality of *N. japonica*, was identical with Korean samples of the species.

Specimens of *Neosiphonia* harlandii from the southeastern coast of Korea formed a clade (63%/58%/85% for MP/ML/BP, respectively). Specimens of *N. decumbens* from south coast of Korea were strongly monophyletic (87%/90%/99% for MP/ML/BP, respectively). *N. harveyi* was closely related to *N. flavimarina* and two specimens from Japan (94%/91%/100% for MP/ML/BP, respectively). The basal-most taxon of the *rbc*L tree was the specimen of Shimoda 284 (as *N. japonica* in McIvor *et al*. 2001) except *N. yendoi* as an outgroup.

The analysis of the *cpe*A/B data is consistent with that of *rbc*L in recognizing *N. japonica*, *N. decumbens*, *N. harlandii*, and *N. harveyi* (Fig. 2). The relationship between *N. japonica* and *N. decumbens* was strongly supported (83%/96%/96% for MP/ML/BP, respectively). Specimen from the type locality of *N. japonica* was identical with Korean specimens, but differed from those from Chiba and Shimoda. *N. harveyi* was placed in a terminal position and supported by low BS values (62%/64%/59% for MP/ML/BP, respectively)

The topology of the combined data for *rbc*L + *cpe*A/B sequences was similar to the *rbc*L tree than the *cpe*A/B, but all these trees were congruent in having *N. japonica* and three distinct species (Fig. 3). Each species was clearly separated with strong bootstrap values. *Neosiphonia japonica* was more closely related to *N. harlandii* and *N. decumbens* than *N. harveyi*. However, *N. flavimarina*, the type of the genus, was closely related to *N. harveyi* from Atlantic Ocean.

DISCUSSION

All analyses of *rbc*L, *cpe*A/B, and combined *rbc*L + *cpe*A/B data did not produce any strongly supported incongruent patterns of phylogenetic relationships. These results indicate that the *cpe*A/B gene is useful for resolving phylogeny of rhodomelacean red algal species. However, the combined *rbc*L+ *cpe*A/B data set gives higher bootstrap support values than each of *rbc*L and *cpe*A/B data, although the combined trees are more similar to those of the *rbc*L data. This may be due to the small size of the *cpe*A/B gene (922 bp), compared to *rbc*L with 1245 bp. This is the second report to use the *cpe*A/B gene for investigating species diversity in the rhodophytes (Yang and Boo 2006).

Our molecular phylogenetic analyses show that *Neosiphonia japonica* is clearly separated from *N. harlandii*,

N. decumbens and *N. flavimarina* from the Pacific Ocean, and *N. harveyi* from the Atlantic Ocean. *N. japonica* looks less variable genetically, all samples of the species investigated here being almost identical in both *rbc*L and *cpe*A/B. Although *N. japonica* and putative relatives studied here are morphologically similar, sharing four pericentral cells, cortication at the base of thalli, and branches not associated with trichoblasts in their origin, each species is easily identifiable. *N. japonica* is distinguished by two to four endogenous branches in the same node at the base of the main axis, a Y-shaped ramification at the lower part of thalli, and usually dichotomous branches (Yoon 1986; Kudo and Masuda 1986; Kim 1995). On the other hand, the species studied here are easily identified based on a combination of characters relating to habit and vegetative morphology. *N. harlandii* is recognized by distinct main axes, numerous cicatrigenous branchlets, and irregularly alternate branches (Segi 1951; Yoon 1986; Kim 2003). *N. decumbens* is characterized by dwarf and decumbent thalli and secund to alternate branches with wide angles (Segi 1951; Yoon 1986; Kim 2003). *N. flavimarina* is distinguished by ultimate branchlets abundant, short, obtuse, and spur-like in several orders, short cicatrigenous branching, short segments of L/B < 0.5, which differentiate it from *N. japonica* and *N. flavimarina* (Kim and Lee 1999). *N. harveyi* is distinguished by a transparent, glassy appearance due to the absence of plastids from outer cell walls (Maggs and Hommersand 1993).

In the present study, it is confirmed that *Neosiphonia japonica* occurs in the California, USA and New Zealand as well as Korea and Japan. The detailed collections may give more information on its distribution within the Pacific Ocean area. In view point of ecological characteristics, *N. japonica* is different from *N. harlandii*, *N. decumbens*, and *N. flavimarina*. For example, *N. japonica* occurs on the east to south coast of Korea and is usually epiphytic on other seaweeds. *N. harlandii* occurs mostly on the east coast of Korea and the thalli form a mat on rock in exposed areas and *N. decumbens* is usually found on the south coast of Korea and grows on various seaweeds in sheltered areas (Kim 2003). *N. flavimarina* is common in intertidal rocky pools attached to rock or at other plant on the west coast of Korea.

Kudo and Masuda (1986) reported that incompletely isolated northern and southern breeding groups between *Neosiphonia japonica* and *Polysiphonia akkeshiensis* are present among the local populations studied, which are entirely allopatric. They speculated that natural hybridization between these local populations does not occur and these groups may have reached a certain stage of gradual speciation before morphological differentiation. McIvor *et al*. (2001) also referred crossability data failed to sample from the Hokkaido breeding group. They suggested that the Hokkaido breeding group has recently extended its distribution in Hokkaido into the range of the Honshu group. In the present data of *rbc*L ML tree, *N. japonica* samples from Japan formed a group with little genetic variation. However, in the ML tree of *cpe*A/B (Fig. 2), two isolates from Hokkaido, Japan, the type locality of *N. japonica*, are grouped the clade of *N. japonica* in the North Pacific Ocean and the clade of *N. harveyi* in the North Atlantic Ocean, respectively. The *cpe*A/B's genetic diversity in sympatric samples of Honshu as well as allopatric ones of Hokkaido is consistent with the results of McIvor *et al*. (2001). It is possible that we presume the possibility of adaptive radiation in the parapatric region near by Japan where is the center of diversity.

Neosiphonia harveyi was originally described based on specimens from Connecticut, USA (Bailey 1848). The species is distributed from the east coast of the North America to the European coast (Maggs and Hommersand 1993). In both *rbc*L and *cpe*A/B trees, no samples from the Pacific Ocean area were included in the *N. harveyi* clade. The results indicate that the distribution of *N. harveyi* may be limited to the North Atlantic Ocean area, while *N. japonica* occurs in the Pacific Ocean area. However, it is interesting that a specimen from North Carolina belongs to *N. japonica* and a specimen from Japan belongs to *N. harveyi*.

It is therefore considered that *Neosiphonia japonica* is a good natural species, which is separated from its similar species in morphology, *rbc*L and *cpe*A/B sequences, biogeography, and ecology. On the same token, *N. harveyi* is limited to the Atlantic Ocean area. The present results do not support the broad concept of *N. harveyi* by McIvor *et al*. (2001), who treated *N. japonica*, *P. akkeshiensis*, *P. acuminata* and *P. strictissima* as conspecific species with *N. harveyi*. As McIvor *et al*. (2001) suggested, Japan is the center of diversity of *Neosiphonia*. All analyses of the *rbc*L and *cpe*A/B data reveal that the branch lengths of *N. japonica*, *N. decumbens*, *N. harlandii*, *N. flavimarina*, and *N. harveyi* were short and their genetic divergences were not high. These results indicate that it is possible to define *N. japonica* and putative relatives as recently diverged species and they might have a recent most common ancestor. They are not cryptic species because cryptic species means morphologically indistinguishable to the

researcher examining the population.

In conclusion, even though *Neosiphonia japonica* has a few characters to delimit species, molecular markers reveal the sequence variation, congruence between two genes, and robust bootstrap support for the lineages. Our results imply that five lineages recently diverged species and they share many of the criteria used to define species. It is unexpected that *N. japonica* and *N. harveyi* were closely related to each other because of marine algal species from the Pacific Ocean and the Atlantic Ocean regions. Further studies on the molecular clock for evaluating the evolutionary divergence time between both species and/or within the genus *Neosiphonia* will be interesting.

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