

Genetic Evolution and Characteristics of Ichthyotoxic *Cochlodinium polykrikoides* (Gymnodiniales, Dinophyceae)

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This study presents a molecular phylogenetic analysis of the harmful dinoflagellate *Cochlodinium polykrikoides*, by use of partial sequence of small subunit (SSU) rRNA gene from most of the major taxa (24 species) in dinoflagellates. The class Dinophyceae clade formed a strong monophyletic relationship with *C. polykrikoides* and several taxa. On the basis of deeper nodes, the phylogenetic relationships placed *C. polykrikoides* closer to the order Prorocentrales rather than to the order Gymnodiniales, which was supported by a strong bootstrap value (100%) in the analyses of Neighbor-Joining and Parsimony methods. There is strong support for *C. polykrikoides* being placed in the same branch as Gymnodiniaceae and being connected in a clade with *Prorocentrum micans* among Prorocentrales. Morphological data show that *C. polykrikoides* is well associated with the genus *Gyrodinium*; however, this species is genetically closer to *Gymnodinium* than to *Gyrodinium*. The placement of *C. polykrikoides* always formed an independent branch separated from other dinoflagellates. In conclusion, planktonic *P. micans* plays an important role as an ancestor of *Gymnodinium*, whereas *C. polykrikoides* appears to be used an intermediate position between *P. micans* and *Gymnodinium* based on evolution.

Key words : *C. polykrikoides*, harmful, dinoflagellates, evolution, SSU, *Gymnodinium*, *P. micans*

Introduction

The class Dinophyceae belongs to a dinoflagellate, a member of Protista. Dinoflagellates are large and diverse, containing over 4,000 species [10]. Dinoflagellates are considered to be among the most primitive of the eukaryotic group, as they combine characteristics of prokaryotes with advanced eukaryotic features [37,38,48,49]. Furthermore, dinoflagellates are an ecologically important group of unicellular protists, with diverse nutrition (autotrophic, heterotrophic, and mixotrophic), and complex life cycles (sexual and asexual, motile and non-motile) [3,10,37]. It is also understood that some species should provide insights into massive blooms in the waters and are notorious for killing fish and shellfish [18,30].

HABs (Harmful Algal Blooms) are increasing in frequency or severity in many coastal environments and worldwide. Most HAB species belong to the class Dinophyceae, which can be resolved to five types (Gymnodinioids, Gonyaulacoids, Dinophysoids, Prorocentroids, and Peridinoids) on the morphological features paradigm as described by Taylor [50]. Next, Zardoya [56] reported that molecular analysis firmly

supported the morphological phylogenesis. Among the section of protists, *Cochlodinium polykrikoides* Margalef is unarmoured and chain-forming, and containing eyespots which are located under the anterior portion of the sulcus [6,35,38]. This species is found to be the most ichthyotoxic genera in Korea, which is associated with suffocation due to oxygen depletion by the production of mucus during massive blooms [4,29]. Recently, we compared and determined the nucleotide sequences of *C. polykrikoides* with Korean HABs targeted SSU (Small Subunit [31]), LSU (Large Subunit [38]), and ITS (Internal Transcribed Spacer [7]) in order to analyze their phylogenetic relationship. Most of our current understanding suggests that this species should be close to the genus *Gymnodinium* on the basis of morphological features, but appear to be not genetically adjacent.

It is known that most photosynthetic dinoflagellates have plastids that contain chlorophylls *a* and *c₂* as well as peridinin as the major photosynthetic pigments exist in the genome as unique single-gene mini-circles [11,25,58]. However, some species have found fucoxanthin derivatives (19'-hexanoyloxy-fucoxanthin and 19'-butanoyloxy-fucoxanthin) as the major pigments which are similar to haptophytes [8,9,19,25]. In this role, extensive research and discussion on the aspects of the degree of evolutionary lineage and history in dinoflagellates concerning the origin of

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ancestors for endosymbiotic relationships are still carried out [12,17,21,42,45-47,53,54,57]. However, dinoflagellate evolution has not been fully explained to date.

C. polykrikoides has been regarded as a photosynthetic dinoflagellate. More recently, *C. polykrikoides* could feed on small phytoplankton species and had a considerable grazing impact on the populations of cryptophytes described by Jeong [27]. Understanding the nuclear gene sequence-based studies on the evolutionary process of *C. polykrikoides* has been limited. For the host cell, the basal lineage is ambiguous, and the evolutionary relationship among different taxa is controversial for several lineages. SSU has proven to be useful for the study of intra and inter-specific genetic variation in Dinophyceae. Here, we sequenced SSU to investigate phylogenetic relationships, and the evolutionary process by undertaking a detailed examination of the mixotrophic *C. polykrikoides* to establish its association with related species. We also obtained other sequences through GenBank search to form a combined data set.

Materials and Methods

Cultures

In this study, a total of 24 species (Bacillariophyceae, 5 species; Chlorophyceae, 9 species; Cyanophyceae, 2 species; Dinophyceae, 7 species; Haptophyceae, 1 species) were obtained from Korea Marine Microalgae Culture, Pukyong University in Busan (Table 1). *C. polykrikoides* sample was taken from red tide waters in Yeosu, Korea, and was isolated using capillary pipette under microscope as axenically as possible. These organisms were grown in cell-culture flasks with 20 ml of f/2+Si medium [16] at 20°C under 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from white fluorescent tubes in 12:12 light:dark cycle, and maintained by serial transfers of an inoculum to fresh medium once in a month.

DNA extraction

Cultures were harvested during the exponential phase by centrifugation (12,000 rpm, 10 min). Pellets were imme-

Table 1. Isolates of microalgal species used in the phylogenetic analyses. Accession numbers of their SSU rRNA partial sequences were input into DNA databases (NCBI). *Cochlodinium polykrikoides* was collected from red tide waters in Korea, 2004. A total of 24 species were provided from Korea Marine Microalgae Culture, Pukyong University in Busan.

Species	Strain	Location	Habitat	Year	GenBank accession number
<i>Nitzschia apiculata</i>	B-100	Deukryang bay	Marine	1995	DQ887490
<i>Lithodesmium undulatum</i>	B-171	Nakdong river	Marine	1995	DQ887491
<i>Melosira nummuloides</i>	B-561	Uljin	Marine	2001	DQ887492
<i>Dunaliella tertiolecta</i>	C-009	Incheon	Marine	1985	DQ887493
<i>Nannochloris oculata</i> *	C-031	York river, USA	Marine	1974	DQ887494
<i>Cochlodinium polykrikoides</i>	YS2004	Yeosu	Marine	2004	DQ887495
<i>Gloeocystis gigas</i>	C-133	Yeosu	Marine	1998	DQ887496
<i>Chroomonas salina</i>	CR-002	Haeundae	Marine	1997	DQ887497
<i>Lyngbya taylorii</i>	CY-004	Mokpo	Marine	1986	DQ887498
<i>Chroococcus minutus</i>	CY-042	Busan	Marine	1995	DQ887499
<i>Prorocentrum micans</i>	D-008	Busan	Marine	1998	DQ887500
<i>Heterocapsa triquetra</i>	D-009	Yeosu	Marine	1998	DQ887501
<i>Gyrodinium impudicum</i> **	D-85	Narodo	Marine		DQ887502
<i>Gyrodinium aureolum</i>	D-97	Geojedo	Marine	1999	DQ887503
<i>Amphidinium caterae</i>	D-19	Yoido	Marine	1999	DQ887504
<i>Melosira</i> sp.	EB-004	Busan	Estuary	1999	DQ887505
<i>Chlorella vulgaris</i>	EC-003	Hwajinpo	Estuary	1995	DQ887506
<i>Oocystis lacustris</i>	EC-016	Busan	Estuary	1999	DQ887507
<i>Cyclotella choctawhatcheeana</i>	EB-001	Hwajinpo	Estuary	1995	DQ887508
<i>Prymnessium parvum</i>	H-20	Jejudo	Marine	1996	DQ887509
<i>Microcystis aeruginosa</i> **	FC-070	Nakdong river	Freshwater	1995	DQ887510
<i>Prorocentrum balticum</i>	D-71	Busan	Marine	1999	DQ887511
<i>Gymnodinium catenatum</i> ***	D-99		Marine		DQ887512
<i>Nannochloropsis salina</i>	EUS-001		Estuary		DQ887513
<i>Chlorella ellipsoidea</i> *	FC-006		Freshwater		DQ887514

Note: These species were obtained from UTEX (The University of Texas at Austin) Culture Collection of Algae*, Inje University**, and Pukyong University*** in Korea, respectively.

diately preserved at -20°C until required. Total DNA was extracted by the method of Asahida [2]. Amplification and sequencing of the partial region of the nuclear rRNA gene were conducted using primers NS1 and NS2 [23]. The primer sequences are as follows: NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS2 (5'-GGCTGCTGGCACCAGACTTGC-3'). PCR (Polymerase Chain Reaction) reactions were performed under the following conditions in 25 μl reaction volumes: 20 pmol of each primer; 0.5 mM dNTPs; 1.25 unit *Taq* DNA polymerase (FastStar *Taq* DNA polymerase, Roche Co.); $10 \times$ PCR reaction buffer (Roche Co.); 5-30 ng total genomic DNA. The thermocycling profile included an initial denaturation step of 95°C for 3 min, followed by 35 cycles of 1 min at 95°C , primer annealing for 1 min at 50°C , and extension for 5 min at 72°C . The final extension step was increased to 5 min. The PCR was carried out by iCycler Thermocycle (Bio-Rad). Products from specific PCR amplification reactions were analyzing using 2% agarose run at 50 V for 50 min, and visualized after staining in $0.5 \mu\text{g ml}^{-1}$ ethidium bromide. The PCR product was purified using PCR Purification kit (NucleoSpin[®] Extract) by following manufacturer's instruction. Purified DNA fragment was stored at -20°C until use.

Nucleotide sequence

The purified DNA using an Applied Biosystem model ABI 3730XL automated sequencer and a Big Dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, UK). For the sequencing reaction, 30 ng of purified PCR products, 2.5 pmol of primer, and 1 μl of Big Dye terminator were mixed and adjusted to a final volume of 7 μl with dH_2O . The reaction was run with 5% DMSO for 30 cycles of 15 s at 95°C , 5 s at 50°C , and 4 min at 60°C . Both strands were sequenced for crosscheck.

Phylogenetic analysis

Sequence data were aligned using the multiple alignment program Clustal W [51] and determined by parsimony and distances methods incorporated in PHYLIP (Phylogeny Inference Package) ver. 3.5c [13] as a subprogram NEIGHBOR. PHYLIP was used dinoflagellates such as *Protoperidinium thulesense* AB261522, *Noctiluca scintillans* DQ388461, *Gyrodinium aureolum* D-97, *Dinophysis acuminata* AJ506972, *D. fortii* AB073118, *D. norvegica* AY260470, *Prorocentrum lima* AB189780, *P. minimum* AJ415520, *P. triestinum* AB183673, *Gambierdiscus toxicus* DQ388463, *Alexandrium*

tamarense AJ415510, *A. ostenfeldii* AJ535384, *Coolia monotis* AJ415509, *Ostreopsis ovata* AF244939, *Ceratium furca* AJ276699, *C. fusus* AF022153, *Gonyalulax polygramma* AJ833631, *Gymnodinium mikimotoi* AF022195, *Karena brevis* AF352822, *Pfiesteria shumwaye* AF080093, *P. piscicida* AY033488, *Gymnodinium fuscum* AF022194, *G. galatheanum* AF272049, *Gyrodinium spirale* AB120001, *Scrippsiella trochoidea* AJ415515, *Takayama pulchellum* AY800130, *Akashiwo sanguinea* AJ415513. This search for parsimony analysis was repeated several times from different random starting points using the stepwise addition option to make certain the most parsimonious tree was found. For distance analysis, subprogram DNADIST in PHYLIP was used to obtain a matrix of Kimura's two-parameter distance [32]. Distance matrix was analyzed by subprogram NEIGHBOR in PHYLIP with algorithms based on Saitou and Nei's NJ method [41]. All nucleotide substitutions were equally weighted and unordered alignment gaps were treated as missing information. Reliability of the tree was constructed using subprogram CONSENSE in PHYLIP after pairwise sequence distances were estimated by Kimura's two-parameter method, which attempts to correct observed dissimilarities for multiple substitutions in sequences evolving with a transition bias.

GenBank accession number

The determined rRNA gene sequences were deposited at the NCBI (National Center for Biotechnology Information) data library. Their accession numbers are indicated in Table 1.

Results

The use of oligonucleotide primers NS1 (forward) and NS2 (reverse) following agarose gel electrophoresis enabled us to successfully amplify a partial portion of an SSU region in an RNA gene. This allowed us to obtain a 502 bp fragment in *Nannochloropsis salina* EUS-001 (Fig. 1). The alignment of nucleotide sequences of the rRNA gene for 25 species revealed considerable variation of sequences between each species (Fig. 2). The nucleotide lengths of SSU regions were significantly different. The shortest length of the partial portion of an SSU region was 498 nucleotides for *Prorocentrum balticum* D-71, and the longest length of the SSU regions was 579 nucleotides of *Prorocentrum micans* D-008. Among 25 species sequenced in this study, the difference between the shortest and the longest segment was

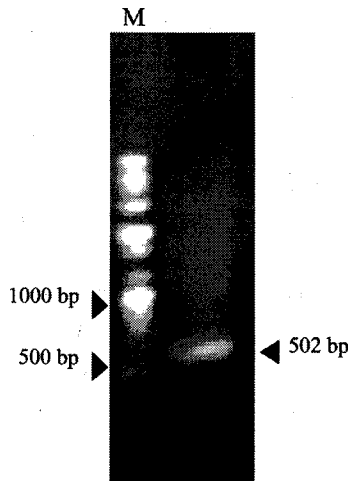


Fig. 1. Amplification product obtained with the primers NS1 (forward) and NS2 (reverse) for *Nannochloropsis salina* EUS-001. 100 bp DNA ladder was used as molecular size marker in this study.

81 nucleotides. In particular, the amplified PCR product of *C. polykrikoides* had a length of 510 nucleotides which was a moderate nucleotide size compared with other tested species. The SSU rRNA gene sequence was determined for 25 species which were retrieved from the GenBank database by way of PHYLIP. The Kimura two-parameter NJ analysis and substitution rate calibration analysis each produced phylogenetic trees, and the substitution rate calibration tree was shown in Fig. 3. The PHYLIP NJ and parsimony analyses produced a tree that was similar to the substitution rate calibration tree and all groups were recovered. The aspects of topology were retained. Tree members of each clade by NJ and parsimony analyses in PHYLIP included their own groups, forming monophyletic groups. The essential aspects of topology were that the class Dinophyceae (DI) clade formed a strong monophyletic relationship (bootstrap >70% based on NJ and parsimony analyses in PHYLIP) with *C. polykrikoides* and several taxa (*Amphidinium*, *Gymnodinium*, *Prorocentrum*, *Heterocapsa*, and *Gyrodinium*). *Prorocentrum*, *Heterocapsa*, and *Gyrodinium* showed the most basal section. That was nested to *G. catenatum* and *C. polykrikoides*, which were supported by a strong bootstrap value of >85% in NJ and parsimony analyses. Although *G. aureolum* was considered to be a member of Dinophyceae on the basis of morphological taxonomy, a phylogenetic analysis it was placed with *G. aureolum* in the class Chlorophyceae rather than Dinophyceae. On the basis of deeper nodes, the phylogenetic relationships placed *C. polykrikoides* closer to the order

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D-009 -----TAGTCATATGCTTGCTCAAAGATTAAGCCATGCATGTCT
H-20 AACCTGGTTGATCCTGCCAGTAGTCATATGCTTGCTCAAAGATTAAGCCATGCATGTCT
B-100 -----TAGTCATACGCTCGTCTCAAAGATTAAGCCATGCAAGCGT
D-008 -----T
D-71 -----GTCT
D-85 -----
D-99 -----
YS2004 -----
D-19 -----
C-009 -----GCCTAGTT
C-031 -----CGTCTAGTA
C-133 -----CGGTTA
CY-042 -----A
EC-003 -----
FC-006 -----
CY-004 -----CTGGACTA
EB-001 -----GCAGCCTA
QR-002 -----GCCGTCTGA
EUS-001 -----GA
EC-016 -----CTCGGCCCGAAAA
D-97 -----AGACATGCAGGCCIT
B-561 -----
EB-004 -----
B-171 -----
FC-070 -----
    
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D-009 CAGTATAAGCTTTTATACGGCGAAACTGCGA-ATGG CTCAATAAACAG-TTAT
H-20 AAGTATAAACITTTTATACGGTTAAACTGCGA-ATGG CTCAATAAACAG-TTAT
B-100 TAGTATAAATATCT-TACTTTGAAACTGCGA-ACGG CTCAATAATCAG-TTAT
D-008 CAGTATAAGCTTCTATACGGCGAAACTGCGA-ATGG CTCAATAAACAG-TTAT
D-71 -----AGCTTCTATCGGCGAAACTGCGA-ATGG CTCAATAAACAG-TTAT
D-85 CAGTATAAGCTTTTACACGGCGAAACTGCGA-ATGG CTCAATAAACAG-TTAT
D-99 -----AAACTGCGA-ATGG CTCAATAAACAG-TTAT
YS2004 -----TTTCTATACGGCGAAACTGCGA-ATGG CTCAATAAACAG-TTAT
D-19 -----AGCATAGCGATCTTACAGCAAGGTCGAA-ATGG CTCAATAAACAG-TTAT
C-009 ATAAACTGCTTATACTGT-----GAAACTGCGA-ATGG CTCAATAAACAG-TTAT
C-031 ATAA-CTGCTTTT-CTGT-----GAA-CTGCGA-ATGG CTCAATAAACAG-TTAT
C-133 GTTAACTGCTTAT-CTGT-----GAA-CTGCGA-ATGG CTCAATAAACAG-TTAT
CY-042 GTTAACTGCTTATACTGT-----GAAACTGCGA-ATGG CTCAATAAACAG-TTAT
EC-003 -----GCTTTATACTGG-----GAAACTGCGA-ATGG CTCAATAAACAG-TTAT
FC-006 -----ACTGCTTTATACTGGT-----GAAACTGCGCAATGG CTCAATAAACAG-TTAT
CY-004 GTTAGCACCTTATACTGG-----GAAACTGCTA-ATGG CTCAATAAACAG-TTAT
EB-001 GTTTACCA-TTATACAGGG-----AAACTGCGA-ACGG CTCAATAATCAG-TTAT
QR-002 AATAAGAGTTTTCTCTG-----AATCTGCGA-ATGG CTCAATAATCAG-TTAT
EUS-001 AAAAAAGATTTTTCTCTG-----AATCTGCGA-ATGG CTCAATAATCAG-TTAT
EC-016 AAAAAAGATTTTTCTCTG-----AATCTGCGA-ATGG CTCAATAATCAG-TTAT
D-97 AGTATAACGACTCTATACGGTAAAAACGCGA-ATGG CTCAATAATCAG-TTAT
B-561 -----GCTCATTATATCAG-TTAT
EB-004 -----GCTCATTATATCAG-TTAT
B-171 -----GCTCATTATATCAG-TTAT
FC-070 -----GCTCATTATATCAG-TTAT
    
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D-009 AGTT-TATTTGATGGTCATT-CTTA-----CATGGA-TAA-CGGT-GGTAATTC-TAGAG
H-20 AGTT-TACTTGATAACTCTTACTTA-----CATGGA-TAA-CGGT-GGTAATTC-TAGAG
B-100 AGTT-TATTTGATAGTCCCTTACTA-----CTTGGAAATC-CGGT-AGTAATTC-TAGAG
D-008 AGTT-TATTTGATGGTCACCTCTTTA-----CATGGA-TAA-CTGT-GCTAATTC-TAGAG
D-71 AGTT-TATTTGATGGTCATTCTTTA-----CATGGA-TAA-CTGT-GCTAATTC-TAGAG
D-85 CGTT-TATTTGGTGGTCATTCTTA-----CATGGA-TAA-CGAT-GGTAATTC-TAGAG
D-99 AGTT-TATTTGATGGTCATTCTTTA-----CATGGA-TAA-CGGT-AGTAATTC-TAGAG
YS2004 AGTT-TATTTGATGGTCGCTTCTTA-----CATGGA-TAA-CGGT-GGTAATTC-TAGAG
D-19 AAT-TTCGAGTGCCTTCAAC-ACA-----CATGGA-TAA-CTGT-GGAAATTC-TAGAG
C-009 AGTT-TATTTGATGGTACCTACT-A-----CTGCGA-TAA-CGGT-AGTAATTC-TAGAG
C-031 AGTT-TATTTGATGGTACCT-CT-A-----CTGCGA-TAA-CGGT-AGTAATTC-TAGAG
CY-042 AGTT-TATTTGATGGTACCT-CT-A-----CTGCGA-TAA-CGGT-AGTAATTC-TAGAG
EC-003 AGTT-TATTTGATGGTACTTACT-A-----CTGCGA-TAC-CGGA-AGTAATTC-TAGAG
FC-006 AGTT-TATTTGATGGTACTTACT-A-----CTGCGA-TAC-CGGTAGTAATTC-TAGAG
CY-004 GGTT-TATTTGATGGGACTTACT-A-----CTTGGG-TAA-CGGT-AGTAATTC-TAGAG
EB-001 TGTT-TCTTTGATAGTCCCTTACTA-----CTTGGG-TAA-CTGT-AGTAATTC-TAGAG
QR-002 AGTT-TATTTGATAGTCCCTTACTA-----CTTGGG-TAA-CGATG-TAATTC-TAGAG
EUS-001 AGTT-TATTTGATAGTCCCTTACTA-----CTTGGG-TAA-CGATG-TAATTC-TAGAG
EC-016 AGTTATATTGAGTAGGTCCTTAAACACTTGGG-AAAGCCGTAAGTATTAGTATAG
D-97 AGTT-TATTTGATAGTCACTTACTA-----CTTGGG-TAA-CGGTAG-TAATTC-TAGAG
    
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B-561 AGTT-TATTTGATAGTTCCTACT-A-----TATGGA-TAA-CGGT-AGTAATTC-TAGAG
EB-004 AGTT-TATTTGACATCTTACT-A-----TTTGGG-TAA-CGGT-AGTAATTC-TAGAG
B-171 -----ACTGCTG-TCTAGT-TAG-----CGAAGCAACTGGAATG
FC-070 -----ATCCTGCTCA-----GGATGAACCTTGGCGGCTGCC-TAACA
    
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D-009 C-----TAATACATGCGCCAAAAACCCGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
H-20 C-----TAATACATGCGAAAAAACTCGACTTTAT-----GGAAGGGTGTGTTTATTAGATAAA
B-100 C-----TAATACATGCGTCAATACCTTCT-----GGGGTAGTATTATTAGAT-TG
D-008 C-----TAATACATGCGCCAAAACCCGACTTCGA-----GGAAGGGTGTGTTTATTAGTACA
D-71 C-----TAATACATGCGCCAAAACCCGACTTATA-----GGAAGGGTGTGTTTATTAGTACA
D-85 C-----TAATACATGCGCCAGACCCGACTTCGT-----GGAAGGGTGTGTTTATTAGTACA
D-99 C-----TAATACATGCGCCAAAACCCGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
YS2004 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
D-19 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
C-009 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
C-133 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
CY-042 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
EC-003 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
FC-006 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
CY-004 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
EB-001 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
QR-002 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
EUS-001 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
EC-016 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
D-97 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
B-561 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
EB-004 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
B-171 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
FC-070 C-----TAATACATGCGTCA-ATACCTGACTTCT-----GGAAGGGTGTGTTTATTAGTACA
    
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Fig. 2. Continued

D-009 AAACCAACCCAGGCTCCGCCTGGCTTTCTGGTATTCAATAAACCAGGAATCGC
H-20 GAACCAATAATAA TTTGGTACTATAAATAAATTTACCGATAC
B-100 AAACCAACCCCTTCG GGGTGTATGGTATTCAATAAAGCTTGGGATCGC
D-008 GAACCAACCCAGGCTCCGCCTGGCTTTCTGGTATTCAATAAACCAGGAATCGC
D-71 GAACCAACCCAGGCTCCGCCTGGCTTTCTGGTATTCAATAAACCAGGAATCGC
D-85 GAACCAACCCAGGCTCCGCCTGGCTTTCTGGTATTCAATAAACCAGGAATCGC
D-99 GAACCAACCCAGGCTCCGCCTGGCTTTCTGGTATTCAATAAACCAGGAATCGC
YS2004 GAACCAACCCAGGCTCCGCCTGGCTTTCTGGTATTCAATAAACCAGGAATCGC
D-19 GAATCGGCGAGGCTCCGCCTGGCTTTCTGGTATTCAATAAACCAGGAATCGC
C-009 AGGCCG-ACCGAGCT-TTGGCTGC-TTGGCGTGAATCATGATAAATTCACGAATCGC
C-031 AGGCCG-ACCGAGCT-TTGGCTGC-TTGGCGTGAATCATGATAAATTCACGAATCGC
C-133 AGGCCA-GCCGAGCT-TTGGCTGCAC-CTGGCGTGAATCATGATAAATTCACGAATCGC
CY-042 AGGCCG-ACCGAGCT-TTGGCTGCAC-CTGGCGTGAATCATGATAAATTCACGAATCGC
EC-003 AGGCCG-ACCGAGCT-TTGGCTGCAC-CTGGCGTGAATCATGATAAATTCACGAATCGC
FC-006 AGGCCG-ACCGAGCT-TTGGCTGCAC-CTGGCGTGAATCATGATAAATTCACGAATCGC
CY-004 AAACCA-ACCTGGCT-TGC-GGGT-GAGTCTGAGTCACTAAGCTTTTCCAATCGC
EB-001 AGACCA-ACACGCTT-CGGGGTTG-CITTTGGTATTCAATAAATTCGGATCGC
CR-002 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
EUS-001 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
EC-016 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
D-97 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
B-561 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
EB-004 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
B-171 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
FC-070 AAACCAATCGGGCCAAACCCG-TA-TTGGTGAATCATGATAAATTCGGGATCG-
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D-009 ATGGCATC-CGCTGGCGATGAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
H-20 ATTAAT GTGACATATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
B-100 ATGCCTC-TGGCGGCGATGGATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
D-008 ATGGCATC-CGCTGGCGATGAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
D-71 ATAGC-TT-CGCTGGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
D-85 ATGGCCTC-GCCTAGCGATGAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
D-99 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
YS2004 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
D-19 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
C-009 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
C-031 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
C-133 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
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EC-016 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
D-97 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
B-561 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
EB-004 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
B-171 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
FC-070 ATGGCCTC-GCCTAGCGATGAATCAATCAAGTTTCTGACCTATCAGCTTCCGACGGTAG
*** ** *

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B-100 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
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D-71 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
D-85 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
D-99 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
YS2004 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
D-19 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
C-009 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
C-031 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
C-133 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
CY-042 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
EC-003 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
FC-006 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
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EB-004 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
B-171 GGTATTGGCTACCGTGGCAATGACGGGTAAACGGAGAAATAGGGTTCGATTCGGAGAGG
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*** ** *

Fig. 2. Continued

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FC-070 -TGACACAGGAGGTAGTGACAAGAAATAACAATACAGGGCATCTTT-TGTCTGT-AA
*** ** *

D-009 TTGGAATGAGTGAAT-TAAATCC
H-20 TTGGAATGAGTGAAT-TAAATCC
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D-71 TTGGAATGAGTGAAT-TAAATCC
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*** ** *

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FC-070 TCCAATAGCGTA
*** ** *

Fig. 2. Continued

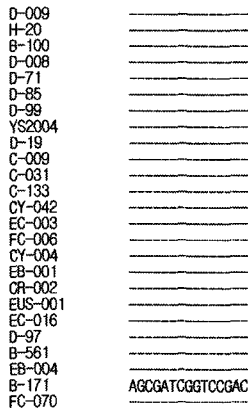


Fig. 2. Sequence alignment of 24 microalgae including harmful dinoflagellates obtained from a partial portion of SSU region. A hyphen represents a gap. An asterisk represents an identical sequence on vertical lines. Sequences have been deposited in GenBank (accession numbers DQ887490-DQ887514).

Prorocentrales (*P. balticum*, *P. minimum*, *P. triestinum*, and *P. micans*) than to the order Gymnodiniales, which was supported by an extremely strong bootstrap value of 100% in NJ and parsimony analyses (Fig. 4). Although the sequences of Gymnodiniales retrieved from GenBank database were added, the topology of the phylogenetic trees was similar to the clustering pattern that *C. polykrioides* had when joined to *P. micans* (Fig. 5a). This indicates that *C. polykrioides* is located far away in term of genetic distances from other species belonging to the order Gymnodiniales and formed a slightly different cluster. It was fragmented, with *C. polykrioides* and *P. micans* in close relationship to Gymnodiniales (bootstrap >90% in NJ and parsimony analyses, Fig 5a). To better illustrate the relationships within this group, we performed a branch and bound search on a broad data set consisting of *P. balticum*, *P. triestinum*, and *P. minimum* retrieved from the GenBank database. The resulting phylogenetic tree was shown in Fig. 5b. The order Prorocentrales displayed a strong sister-ship relationship (98/95 bootstrap support in NJ and parsimony analyses) with the family Gymnodiniaceae. There is strong support for *C. polykrioides* being placed in the same branch as Gymnodiniaceae and being connected in a clade with *P. micans* among Prorocentrales.

Discussion

C. polykrioides

Dinoflagellates used in this study are annually occurred

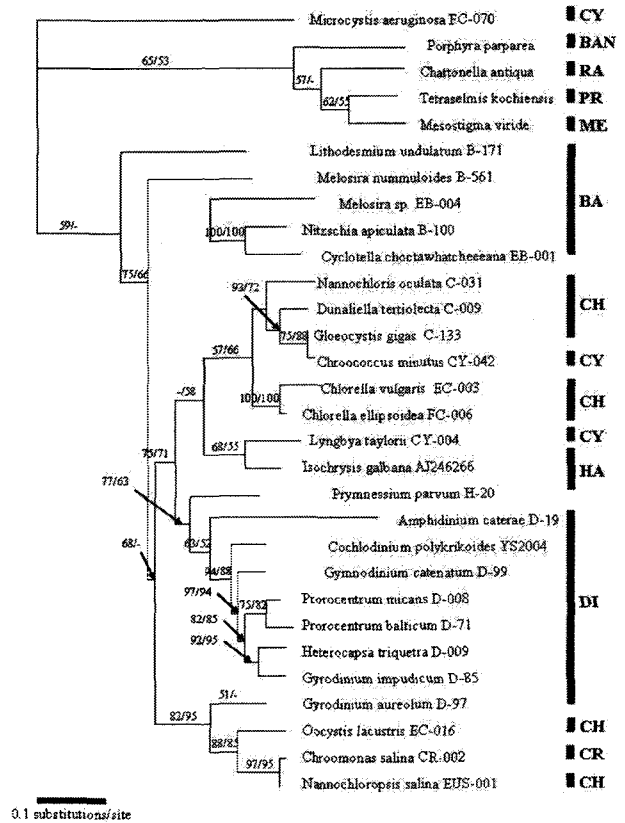


Fig. 3. PHYLIP analysis based on the class of 30 species obtained from GenBank database including microalgae used in this study. Phylogram was constructed by inferring from nucleotide sequences of partial SSU region. The tree was obtained using subprogram NEIGHBOR in PHYLIP with the option of Kimura's two-parameter method. The tree was rooted using *Microcystis aeruginosa* FC-070. The numbers shown on branches, which represent bootstrap values for 100 replications, were obtained from using the subprogram CONSENSE. Numbers at the nodes refer to the percentage (>50%) of bootstrap support in NJ and parsimony analyses. Bootstrap of <50% represents a hyphen on node. CY, cyanobacteria, BAN, bangiophyceae, RA, raphidophyta, PR, prasinophyceae, ME, mesostigmaviride, BA, bacillariophyceae, CH, chlorophyceae, HA, haptophyceae, DI, dinophyceae, CR, cryptophyceae.

and bloomed in Korean coastal waters up to present [30]. Among them, three species of *C. polykrioides*, *G. impudicum*, and *G. catenatum* appear simultaneously during the summer season, but it is easy to misidentify them under a light microscope because of their similar morphological features [5,6]. Cho [5,6,7] have analyzed gene sequences based on ITS region and determined that the three species were genetically different. The genetic distance between *C. polykrioides* and *G. impudicum*/*G. catenatum* based on SSU in this study was also similar to the result obtained

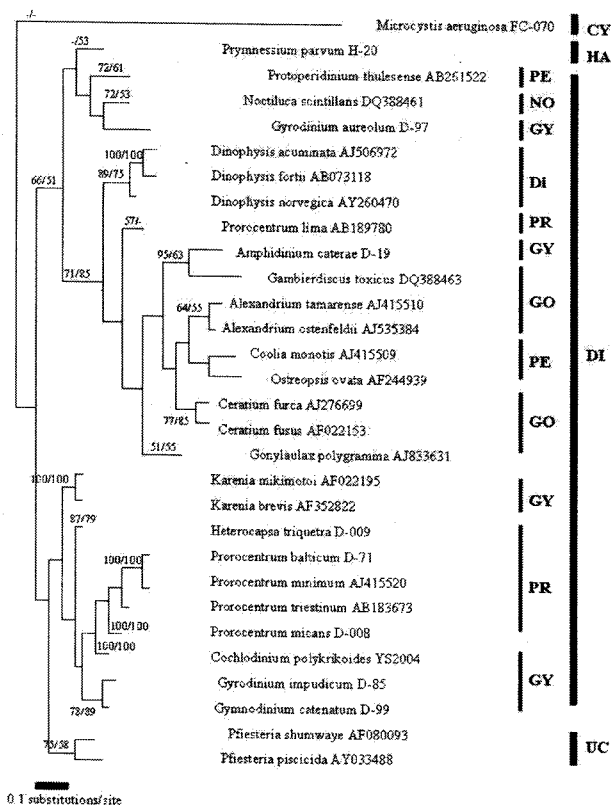


Fig. 4. PHYLIP analysis based on the order of 30 species obtained from GenBank database including harmful dinoflagellates. Phylogram was constructed by inferring from nucleotide sequences of partial SSU region. The tree was obtained using subprogram NEIGHBOR in PHYLIP with the option of Kimura's two-parameter. The topology represents the consensus tree from a heuristic search yielding two equally most parsimonious. The tree was rooted using *Microcystis aeruginosa* FC-070. The numbers shown on branches, which represent bootstrap values for 100 replications, were obtained from using the subprogram CONSENSE. Numbers at the nodes refer to the percentage (>50%) of bootstrap support in NJ and parsimony analyses. Bootstrap of <50% represents a hyphen on node. CY, cyanobacteria, HA, haptophyceae, DI, dinophyceae, UC, unclassified, PE, peridinales, NO, noctilulaceae, GY, gymnodiniales, Di, dinophysiales, PR, proro-centrales, GO, gonyaulacales.

from the ITS region and revealed that the genetic divergence between them was higher in *C. polykrikoides*. Subsequently, this present study reestablished that *C. polykrikoides* has a clear and distinct genetic position related to *G. impudicum* and *G. catenatum*. However, the three species were found to be members of Dinophyceae and Gymnodiniales and even Gymnodiniaceae (Fig. 3, 4, 5a), but did not belong to the same genus. Phylogenetic analysis of the present SSU also strongly supported their mem-

bership between *C. polykrikoides* and *G. impudicum*/*G. catenatum*.

The typical different character between *C. polykrikoides* and the genus *Gymnodinium* on the basis of morphological features reveals that a girdle in *C. polykrikoides* makes 1.8-1.9 turns around the cell [18], whereas cingular displacement is less than one-fifth of the cell's body length for *Gymnodinium* [8,22,52]. This study suggests that members of Gymnodiniaceae should not genetically exclude *C. polykrikoides*. An important factor to consider is that *C. polykrikoides* does not transfer to the genus *Gyrodinium*, but to the genus *Gymnodinium*, although the identification of *Gymnodinium* and *Gyrodinium* genera is very confusing based on their morphological features [20,40,43,44]. It is well known that a taxonomical tool to distinguish between the two genera is the degree of displacement of the cingulum separating the epicone and hypocone. Specifically, cingular displacement must be less than one-fifth of the cell's body length for *Gymnodinium* and greater than one-fifth of the body length for *Gyrodinium* [22]. Taylor [48] suggested that phylogenetic placement of *Cochlodinium* inferred from morphological features was branched from *Gyrodinium*. However, our present molecular data showed that genetic divergence between *C. polykrikoides* and *Gyrodinium* was higher than that of *C. polykrikoides* and *Gymnodinium*. We also discovered a stronger phylogenetic relationship between *C. polykrikoides* and *Gymnodinium*, which was genetically closely related to *Gymnodinium*.

Some researchers have suggested that the origin of the paralytic shellfish poisoning (PSP)-toxin producing dinoflagellate, *Alexandrium*, was the result of a process and factors intrinsic to the species [1,24]. Kodama [33] suggested that the origin of the toxin production was associated with undergoing the process of mobile genes caused by bacteria. At present, the inheritance of plastids in most eukaryotic phytoplankton has been obtained by incorporating them into a eukaryotic host cell via an endosymbioses [12,28,34,36,49]. In this role, endosymbiotic-origin hypothesis is perhaps the pacemaker in explaining the toxin production origin of *Alexandrium*. According to a recent study by Jeong [26,27], some harmful dinoflagellates could excessively feed on cyanobacteria in the field and laboratory. Among them, *G. catenatum*, *G. impudicum*, *P. minimum*, *H. triquetra*, and *P. micans* including *C. polykrikoides* were used in this study and are also able to ingest cyanobacteria as described by Jeong [26,27]. On the basis of these interactions, the genetic structure in mixotrophic dinoflagellates

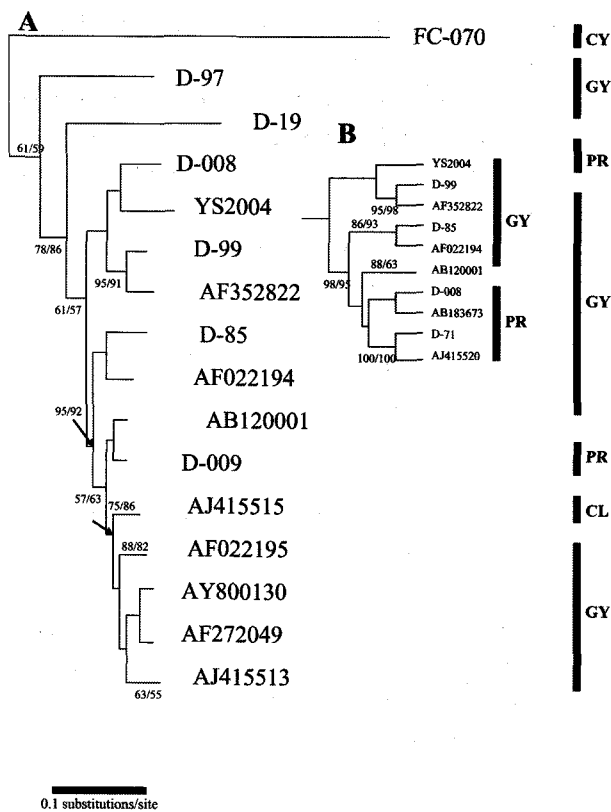


Fig. 5. PHYLIP analysis of harmful dinoflagellates obtained from GenBank database. A, phylogenetic analysis of 16 species within the family Gymnodiniaceae and Claciadiniellaceae and *Prorocentrum*, B, phylogenetic analysis of 10 species within the family Gymnodiniaceae and *Prorocentrum*. Phylogram was constructed by inferring from nucleotide sequences of partial SSU region. The tree was obtained using subprogram NEIGHBOR in PHYLIP with the option of Kimura's two-parameter. The topology represents the consensus tree from a heuristic search yielding two equally most parsimonious. The tree was rooted using *Microcystis aeruginosa* FC-070. The numbers shown on branches, which represent bootstrap values for 100 replications, were obtained from using the subprogram CONSENSE. Numbers at the nodes refer to the percentage (>50%) of bootstrap support in NJ and parsimony analyses. Bootstrap of <50% represents a hyphen on node. CY, cyanobacteria, GY, gymnodiniaceae, PR, prorocentrum, CL, claciadiniellaceae, FC-070, *Microcystis aeruginosa*, D-97, *Gyrodinium aureolum*, D-19, *Amphidinium caterae*, D-008, *Prorocentrum micans*, YS2004, *Cochlodinium polykrikoides*, D-99, *Gymnodinium catenatum*, AF352822, *Karenia brevis*, D-85, *Gyrodinium impudicum*, AF022194, *Gymnodinium fuscum*, AB120001, *Gyrodinium spirale*, D-009, *Heteroscaps triquetra*, AJ415515, *Scrippsiella trochoidea*, AF022195, *Gymnodinium mikimotoi*, AY800130, *Takayama pulchellum*, AF272049, *Gymnodinium galatheanum*, AJ415513, *Akashiwo sanguinea*, AB183673, *Prorocentrum triestinum*, D-71, *Prorocentrum balticum*, AJ415520, *Prorocentrum minimum*.

may lead to the possible existence of gene transfer over time and could lead to an independent evolutionary lineage. However, our current molecular data show that mixotrophic harmful dinoflagellate taxa are not randomly distributed in the class, order, and family, inferring phylogenetic relationships. Moreover, tested dinoflagellates which are isolated from seawater do not show phylogenetic relationships with the species obtained from estuary and freshwater. This study strongly suggests that the SSU genes in mixotrophic dinoflagellates remain to be conserved, even through successive generations.

Evolution

Several classical taxonomists suggested that armored Dinophyceae was differentiated from unarmored species based on evolutionary lineage [10,14,38,48]. Likewise, our study shows that unarmored species belonging to Gymnodiniales have a different cluster with thecate species (Dinophysiales, Noctilucales, Gonyaulacales, Peridinales, and Prorocentrales). These unarmored species are supported by a weak bootstrap, as revealed by the analysis of NJ and parsimony (Fig. 4). However, four species of *Prorocentrum* (*P. balticum*, *P. minimum*, *P. triestinum*, and *P. micans*) have a cluster with Gymnodiales instead of armored species. This indicates that current molecular data do not support a specific DNA signature to completely distinguish between the phylogenetic relationship between unarmored and armored species, as suggested in a similar study by Grzebyk [15]. Grzebyk [15] suggested that *Prorocentrum* based on DNA sequences is separated into two types, the first being symmetrical morphology and benthic behavior, the second being asymmetrical morphology and planktonic behavior. This study was also similar in that benthic *P. lima* joined armored species, not planktonic *Prorocentrum*. Since planktonic *Prorocentrum* is considerably different from benthic *Prorocentrum* based on ecological behavior and our current phylogenetic relationship, this study also suggests that it is desirable for two different-type *Prorocentrum* to have separate genus, as previously described by Zardoya [56] and Grzebyk [15].

Loeblich [37,38] and Taylor [48] suggested that armored species represented a more primitive state than unarmored species. However, our study strongly suggested that planktonic *Prorocentrum* among armored species contained a more advanced genetic characteristics than armored dinoflagellates and even benthic *Prorocentrum*. Comparison of

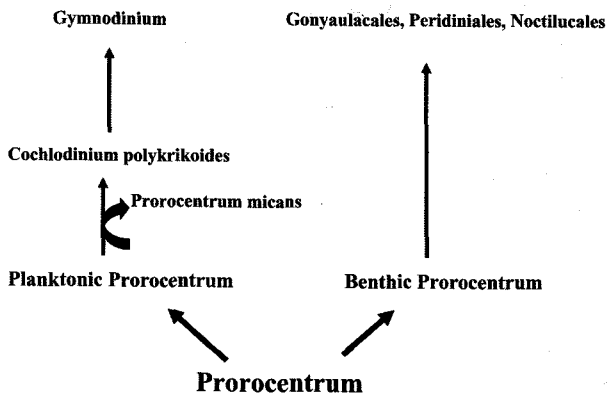


Fig. 6. A putative evolutionary lineage for *Cochlodinium polykrikoides* rRNA gene.

sequence divergence for planktonic *Prorocentrum* showed that it was far from *Gymnodinium*, indicating that planktonic *Prorocentrum* formed a genetically primitive state to construct the cluster of *Gymnodinium* throughout the evolutionary lineage. As can be seen in Fig. 3 and 4, the phylogenetic position of *C. polykrikoides* always formed an independent branch separated from other dinoflagellates. However, *C. polykrikoides* had a relatively long branch suggesting an early divergence of this species. It also suggested a closer evolutionary relationship with *P. micans* than that of *Gymnodinium*, which was strongly supported by bootstrap (Fig. 5a). Based on phylogenetic distance, *P. micans* was close to *P. balticum*, *P. minimum*, and *P. triestnium* (Fig. 5b) and somewhat far from *C. polykrikoides* and *Gymnodinium*. In this role, it is assumed that *P. micans* may be seen as the ancestor of *Gymnodinium*, whereas *C. polykrikoides* represents an intermediate position between *P. micans* and *Gymnodinium* (Fig. 6). *Cochlodinium* recorded a higher level in the analysis of dinoknot/desmokont organization [48], but recorded a lower level based on evolutionary relationship and lineage. Consequently, *C. polykrikoides* showed contradictory results obtained from morphology and molecular phylogeny. However, more data are required to test these relationships.

Acknowledgements

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초록 : 어류치사성 *Cochlodinium polykrikoides* 적조생물의 유전적 진화 및 특성

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본 연구는 유해성 적조생물인 *Cochlodinium polykrikoides*의 유전적 계통진화를 설명하기 위하여 24 종의 개체에 대한 SSU를 대상으로 분석했다. *C. polykrikoides*는 와편모조류와 밀접한 단일 계통군을 형성하고 있다. Neighbor-joining 혹은 parsimony 분석에 의하면 *C. polykrikoides*는 Gymnodiniales 보다 Prorocentrals 목 (order)에 훨씬 근접한 100% 유연관계를 보이고 있으며, 과 (family)로 분석해 보면 Gymnodiniaceae에 속해 있고, 특히 *Prorocentrum micans*와는 매우 밀접한 관계를 보이고 있다. 형태적으로는 *Gyrodinium* 속 (genus)에 가깝지만, 유전적으로는 *Gymnodinium* 속에 근접하고 있다. *C. polykrikoides*는 와편모조류 중에서 독립적인 계통군을 유지하고 있다. 따라서 *P. micans*는 *Gymnodinium*의 조상으로 추측되며, *C. polykrikoides*는 *P. micans*와 *Gymnodinium* 속의 중간단계인 것으로 보인다.