Phylogenetic Relationships Using ITS2 Sequence and RAPD-PCR Data from Four Species of Korean *Pseudo-nitzschia* (Bacillariophyceae)

Eun Seob Cho* and Young Sik Lee

South Sea Fisheries Research Institute, Coastal Environment Management, NFRDI, Yeosu 556-823

Received October 9, 2003 / Accepted November 26, 2003

A portion of ribosomal internal transcribed spacer (ITS) 2 was sequenced from the samples of *Pseudo-nitzschia* (*P. delicatissima*, *P. multiseries*, *P. pungens* and *P. subfraudulenta*) to investigate the genetic characteristics by measuring the magnitude of genetic diversity and the degree of similarity coefficient using random amplified polymorphic DNAs (RAPD)-PCR patterns. The phylogenetic trees inferred from the genetic distance analyses showed the placement of *P. delicatissima* formed a quite long distance from *P. multiseries*, *P. pungens*, and even *P. subfraudulenta*. The phylogenetic tree from RAPD patterns showed that *P. multiseries* and *P. pungens* had dissimilarity coefficient of 0.31, while *P. delicatissima* and three species of *Pseudo-nitzschia* had that of 0.81. It is likely thought that the genetic position of *P. delicatissima* formed far from *P. multiseries*, *P. pungens*, and *P. subfraudulenta*. These results imply that ITS2 region is expected to support a useful molecular characters for recognizing at the species level and for even discriminating *P. multiseries* from *P. pungens*. RAPD method also will be used to differentiate the species of *Pseudo-nitzschia* in a short time.

Key words – gene sequence, ITS2, phylogenetic analysis, *Pseudo-nitzschia*

Harmful algal blooms (HABs) occur in coastal regions worldwide, causing human illness and mortality as well as millions of dollars in annual economic lossess. Among red tide micoralgae in waters, the genus *Pseudo-nitzschia* Peragallo is difficult to identify and enumerate each strain of *Pseudo-nitzschia* because of closely morphological features. In particular, certain *Pseudo-nitzschia* species produce the neurotoxin domoic acid (DA), which causes amnesic shellfish poisoning (ASP) in humans and wildlife [17]. ASP was first recognized in 1987, after several people died and over one hundred became ill following the consumption of blue mussels from Prince Edward Island [3,17]. In Korea, the shellfish industry has a long history and a great deal of production, but recently the toxin DA produced by *Pseudo-nitzschia multiseries* (Hasle) Hasle was detected in cultured shellfish [6,7].

Recent advances in DNA amplification and sequencing, new approaches in harmful algal species are based on the information of genotypic properties and nucleic acid sequences of each HAB, which tend to combine morphological approach with molecular analysis. Ribosomal DNA (rDNA) genes are tandemly repeated multigene families containing both genic and nongenic, or spacer regions. Each repeat unit contains a copy of the small-subunit (SSU), 5.8S, and large-subunit (LSU) and two spacers, the internal transcribed spacer (ITS) and an intergenic spacer (IGS). Hills and Dixon [12] indicated that ITS regions were attractive candidates for genetic markers at higher and lower taxonomic levels. Interspecific and intraspecific ITS sequence variation has been examined in *Alexandrium* spp. [1], *Gymnodinium catenatum* Graham [2], *Gyrodinium impudicum* Fraga et Bravo [14] and *Cochlodinium polykrikoides* Margarlef [5]. It is thought that the ITS regions have been regarded as nonfunctional sequences, but these regions have some functions in processing of precursor molecules of rRNA [13].

The introduction of random amplified polymorphic DNAs (RAPD) analysis provides a powerful tool for rapid detecting of DNA polymorphism among individuals or populations [20,21]. Recently RAPD has been applied to know the intraspecific and interspecific variations and genetic relationships among various organisms. Although this technique has many weaknesses, it also offers some important advantages such as its relative ease and speed, the degree of polymorphisms and the virtually inexhaustible pool of possible genetic markers. However, a little is investigated to identify HAB using RAPD-PCR method [2,8].

In the present study, we investigated phylogenetic analysis for *Pseudo-nitzschia* species based on separate and combined partial gene analyses of the ITS2 regions sequence data, and assessed the support that the molecular data

***Corresponding author**
Tel : +82-61-685-8959, Fax : +82-61-686-1588
E-mail : eun-5657@hanmail.net

provided for a recent taxonomic revision of the genus of *Pseudo-nitzschia*. Also we have focused on how much do RAPD amplification contribute to support phylogenetic trees using gene sequences and how much to assess the verified genetic markers.

Materilas and Methods

Cultures

P. multiseries, *P. pungens* (Grunow) Hasle, *P. subfraudulenta* (Hasle) Hasle and *P. delicatissima* (Cleve) Heiden in this study were provided from Inje University, Kimhae, Korea, which were isolated from Jinhae Bay in 1999 and were exhibited in nonaxenic cultures. After a clonal culture was established, it was maintained and cultured in f/2+Si medium [10]. These strains were grown in 100 mL plastic containers at 20℃, with a light intensity of 100 mol m^{-2} s^{-1} continous light provided by cool-white fluorescent lamps.

DNA extraction

Cultures were harvested during the exponential phase by centrifugation. Pellets were immediately preserved at -20℃ until molecular analysis. Approximately 0.05g of algal pellets were suspended in 500 L of extraction buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA), 150 L of 10% (w/v) sodium dodecyl sulphate (SDS) and incubated at 55℃ for 30 min. The supernatant was extracted twice with phenol:chloroform: isoamylalchol (25:24:1, v/v/v) prior to ethanol precipitation and RNase (1 mg mL^{-1}) was added. The algal cells were pelleted and the supernatant mixed with 40 L of 3 M sodium acetate and incubated on ice for at least 1 h. The DNA was precipitated from the tube at room temperature for 10 min by adding 2.5 volumes of 100% ice-cold ethanol. The pellet was washed with 2 volumes of 70% ethanol and resuspended in distilled water. The DNA was kept at -20℃.

PCR amplification and sequencing

The ITS2 region was amplified by using primers 5.8S-R (5′-TCGATGAAGAACGCAGC-3′) and LR3 (5′-GGTCCG-TGTTTCAAGAC-3′) were derived from the conserved regions of SSU and LSU rDNA, respectively [4]. PCR amplifications were carried out with Perkin-Elmer 2400 Thermocycler. PCR reactions typically contained a 50 L mixture: 1.25 unit *Taq* DNA polymerase (Ex *Taq*, TaKaRa Co., JAPAN); 10 Ex *Taq* buffer (TaKaRa Co., JAPAN); 0.2 mM dNTP; 20-100 ng total genomic DNA; and 100 pmol of each primer. The thermocycling profile included an initial denaturation step of 95℃ for 30 sec, followed by 35 cycles of 30 sec at 95℃, primer annealing for 1 min at 50-53℃, and extenstion for 5 min at 72℃. Amplification products were separated electrophoretically on ethidium bromide-stained 1.5% agarose 0.5 TBE gels to check the yield, purity and length of the amplified products. After purification with QIAGEN gel elution kit (Qiagen, Wartworth, CA), all PCR products were sequenced directly on the Perkin-Elmer Applied Biosystems (ABI) 377A DNA sequencer using a ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer ABI, Foster City, CA) following the manufacture's protocol.

RAPD analysis

Prior to PCR, DNA concentrations were adjusted to 50 ng L^{-1}. PCR reactions were performed in a final volume of a 20 L mixture: 1.25 unit *Taq* DNA polymerase (Ex *Taq* ™, Bioneer Co.); 10 Ex *Taq*™ buffer (Bioneer Co.); 2.5 mM dNTPS; 50 ng template DNA; and 100 pmol of RAPD primer. Amplifications were performed with the Perkin-Elmer thermocycler 2400. The thermocycling profile included an initial denaturation step of 94℃ for 4 min, followed by 35 cycles of 1 min at 94℃, primer annealing for 1 min at 55℃, and extenstion for 2 min at 72℃. Amplification products (20 L) were separated for 40 min at 100V on 1.8% agarose TBE gels. These were stained with 10 L ethidium bromide added to the gel and 0.25 L ethidium bromide mL^{-1} TBE buffer to the TBE running buffer. Gels were illuminated with UV light and photographed with Polaroid 667 positive/negative instant film. Twelve random primers (Seoulin UniPrimer™ Kit I) were screened (Seoulin Scientific Co., Ltd., Seoul), two of which, URP-3 and URP-9 were used for analysis. RAPD bands were scored as present/absent (p/a = 1/0) and only well-resolved profiles were considered. Individual data set from each primer were combined to make data matrix, and phylogenetic relationship was constructed using NTSYS-PC program ver. 2.02 [18].

Phylogenetic analysis

Sequence data were aligned using CLUSTAL W and phylogenetic analysis was performed using distance method. Homologous sequences of *P. multiseries* (AF001456), *P. pungens* (AF001458) and *P. australis* (AF001460) obtained from GenBank search [4] were used. This study used Neighbor-joining (NJ) method in PHYLIP (Phylogeny Inference Package) ver. 3.5c [9]. To obtain phylogenetic tree, the data

set was first iterated 1,000 times using the subprogram SEQBOOT. Each iterated data set was run using the subprogram DNADIST to obtain distance matrix between pairs of sequences with the option of Kimura two-parameter correction [15]. Finally, a consensus tree representing reliability at each branch in the tree was obtained using the subprogram CONSENSE.

Results and Discussion

Amplified gene sequences targeted to ITS2 region from *P. pungens, P. multiseries, P. subfraudulenta,* and *P. delicatissima* including relative taxa were shown in Fig. 1. Four species of *Pseudo-nitzschia* appeared to be considerable nucleotide variations in the ITS2 regions, but little variation of *P. pungens* and *P. multiseries* isolated from Jinhae Bay, Korea and Monterey Bay, USA. The phylogenetic tree inferred from the distance analysis of the aligned data set (NJ method) was shown in Fig. 2. Although two species of *P. multiseries* and *P. pungens* were closely contributed with morphological features, the phylogenetic position was different, which was supported by moderate bootstrap value of 83%. *Pseudo-nitzschia australis* formed the closed group with *P. multiseries* and *P. pungens*. However, *P. subfraudulenta* and *P. delicatissima* were quite long distance taxa compared with *P. multiseries, P. pungens* and *P. australis*. In particular,

```
P.pungens               --CCCACTCAGCGCTGGCTTCGTGCTGGCTGCTGGTC-TTTTG----GCCGTGACTGAAC 50
P.multiseries           --CCCACTCAGCGCTGGCCTCGTGCTGGCTGCTGGTTGTTTTG----GCTTTGACTGGCA
P.delicatissima         GTTGCGATCTGTGCGAATTGAG-ACTTCTTGTATGTAATCGCC----ATAGCAGCAGGGA
P.subfraudulenta        CCCGCGATGCGCGCGATTTGTA-GATCTCTATATGTTATCACCCCATGTTGCAGCAGGAC
AF001458                --CCCACTCAGCGCTGGCTTCGTGCTGGCTGCTGGTC-TTTTG----GCCGTGACTGAAC
AF001456                --CCCACTCAGCGCTGGCCTCGTGCTGGCTGCTGGTTGTTTTG----GCTTTGACTGGCA
AF001460                --CCCACTCAGCGCTGGTTTAG-GCCAGTCGCTGGTTGTTTTG----GCCTTGACAGCTA
                            *  *  * **                    **  *             * *

P.pungens               TTGTT--TAGTCTC-GGCTTAAGTT--TTAC-GTCAAGTACGTGCATAGATCTAGAAAGC 100
P.multiseries           CTATTGCCTGTCTC-TGCTTAAGTT--CTACTGTATAGTACGTGCATAGACGTGGAAGGC
P.delicatissima         TCTTGCGCTAGCAC-TGTGAGATTAGATGACTGGCTTCCACATGCTTTCATATTCTACGG
P.subfraudulenta        TGTTTCTACACAACATGCACGGCCAGACCATAGAAATCCACTTA-ATGGAAGTTTTATGG
AF001458                TTGTT--TAGTCTC-GGCTTAAGTT--TTAC-GTCAAGTACGTGCATAGATCTAGAAAGC
AF001456                CTATTGCCTGTCTC-TGCTTAAGTT--CTACTGTATAGTACGTGCATAGACGTGGAAGGC
AF001460                CTAGTAGCTGTCTC-TGCTTAAGTT--CTACGGTATCGTACGTGCATAGATTTAGAGACC
                                  *  *           *  *      ** *   *  *  *

P.pungens               TCTGCCCGTCTAGTTTAAAACACGGCGCTGACACTCTTGTC-TATCTCTG-GTAGGTT-- 150
P.multiseries           TTGACCTGTCTAGTT--AAAGACGGCGTTGACACTCTTGTC-TATCTCTG-GTAGATTGT
P.delicatissima         ATCACACACCAGGTTGGCTATTCGGTGAGAGGTAACTCAAC-TGGATCTATGCAAGTACC
P.subfraudulenta        CCTGCTGGAGTTGGTGCCTACACTCTCAAGTATCCTCTAGCGTAGAACCGTACTGGAGTT
AF001458                TCTGCCCGTCTAGTTTAAAACACGGCGCTGACACTCTTGTC-TATCTCTG-GTAGGTT--
AF001456                TTGACCTGTCTAGTT--AAAGACGGCGTTGACACTCTTGTC-TATCTCTG-GTAGATTGT
AF001460                TCAACCTGTCTTGTC--AA-GACGGCGTTGACATTCTTGTC-TATCTCTG-GTAGCTTGT
                            *       *         *                  * *    *

P.pungens               -----TATT------CAACTGGAGTTTACTAGGAGT--CGTCTGTAGCTGTTTTGAAACT 200
P.multiseries           ACTTTTAGTATGAAATATCTGGAGTTTACTATGAGT-TTGTTTTCAGCTGTTTTGAAACT
P.delicatissima         G----TAGAAG-CAGTAATTATACCCGTTACGGACAGGTGTATTCTA-TGTTTGTGGCTT
P.subfraudulenta        T----AAGTGCTTTAACACAAAAGACTTTGTAGATC-ACATACGACGTGACAATGCATCT
AF001458                -----TATT------CAACTGGAGTTTACTAGGAGT--CGTCTGTAGCTGTTTTGAAACT
AF001456                ACTTTTAGTATGAAATATCTGGAGTTTACTATGAGT-TTGTTTTCAGCTGTTTTGAAACT
AF001460                A--TTCATTAC-AAATATCTGGAGTTTGTTAAGATTGTCATGTTCAGCTGTTT-GAAACT
                              *               *         **      *                 *

P.pungens               ACTGGAACGAGCGGTTTTAGATCTAACCAGCTGGAAACAG 240
P.multiseries           GATGGAACGAGCACTTCTATGCCT----------------
P.delicatissima         GCTTGTACGTTAAGAATTACCAT-----------------
P.subfraudulenta        AATTCTATCTATAGGAC-----------------------
AF001458                ACTGGAACGAGCGGTTTTAGATCTAACCAGCTGGAAACAG
AF001456                GATGGAACGAGCACTTCTATGCCT----------------
AF001460                TTTCGATTGAGCGTCACTAGATCTAGCTA-----------
                          *
```

Fig. 1. Sequence alignments of the ITS2 rDNA region for *Pseduo-nitzschia pungens, P. multiseries, P. subfraudulenta,* and *P. delicatissima.* CLUSTAL W generated the alignment. A hypen represents a gap and a n asterisk represents an identical sequence on vertical lines.

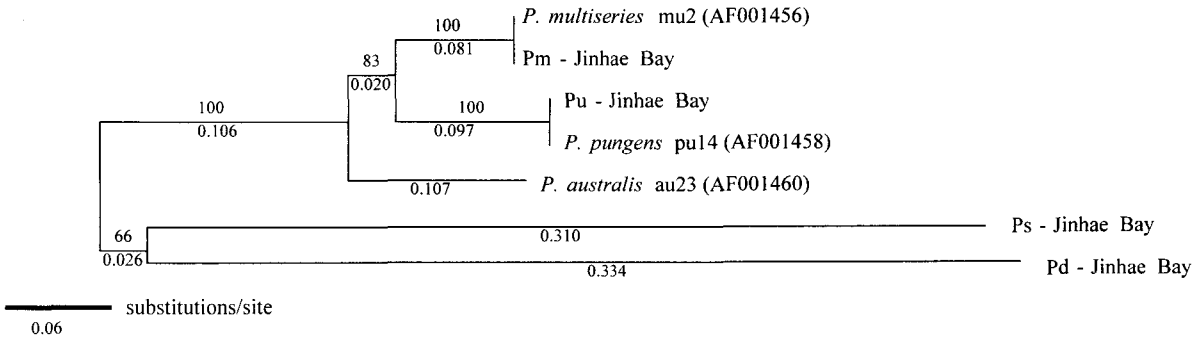

Fig. 2. Phylogenetic analysis of relationships among four strains of *Pseudo-nitzschia* performed on ITS2 region using a distance method. The tree was obtained using the subprogram NEIGHBOR incorporated in the PHYLIP package with option of Kimura's 2-parameter method (1980). Bootstrap values (% of 1,000 replications) are given above the internal nodes. The upper numbers are bootstrap values and the lower numbers show genetic distance values. Values lower than 50 have not been included. The letters of Pm, Pu, Pd, Ps represent *Pseudo-nitzschia multiseries*, *P. pungens*, *P. delicatissima* and *P. subfraudulenta*, respectively.

P. delicatissima was longest genetic distance of 0.334 than any other of *Pseudo-nitzaschia* species used in this study. Amplified PCR products using URP-3 and URP-9 showed various polymorphic fragments on agarose gels (Fig. 3). A total of 29 DNA fragments was obtained from *P. pungens*, *P. multiseries*, *P. subfraudulenta*, and *P. delicatissima* using the two primers. The number of amplified products by two primers ranged from 12-17, with average 14.5 bands per primer. RAPD data was shown in Fig. 4. *P. pungens* and *P. multiseries* had higher degree of dissimilarity coefficient of 0.31, while the relationship between *P. delicatissima* and the rest species of *Pseudo-nitzschia* showed significantly lower degree of 0.81.

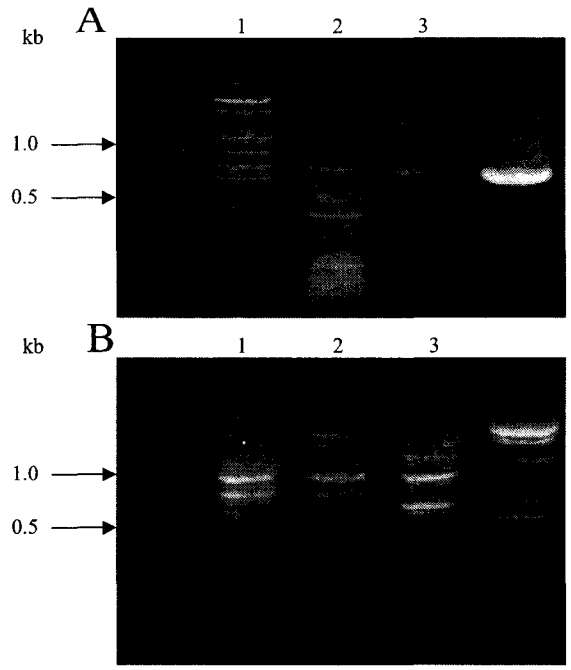

Fig. 3. Agarose gel electrophoresis pattern of randomly amplified four strains of *Pseudo-nitzschia* templeate DNA. The template DNA was amplified using the primer URP-3 (A) and URP-9 (B) and separated on a 1.8% agarose gel. Left lane represents a 1 kb DNA ladder in both (A) and (B). Light lane 1, 2, 3, 4 mean *P. pungens*, *P. multiseries*, *P. subfraudulenta*, *P. delicatissima*, respctively.

Our results show that there are a much higher level of variability in ITS2 region of Korean *P. pungens*, *P. multiseries*, *P. subfraudulenta*, and *P. delicatissima*, including *P. australis* (American strain). Previously, Manhart *et al.* reported that the divergence of ITS1 sequences between *P. pungens* and *P. multiseries* was relatively high (27%) [16]. Scholin *et al.* and Manhart *et al.* observed that a comparison of SSU and LSU rDNA sequences in *P. pungens* and *P. multiseries* had a low genetic variation. Both researcher suggested that the rDNA sequences allowed us to confirm that *P. pungens* and *P. multiseries* were closely related but separated [16,19]. However, ITS2 region used in this study is expected to support a useful molecular character for differentiating *P. multiseries* from *P. pungens* and ITS1 will be also used to

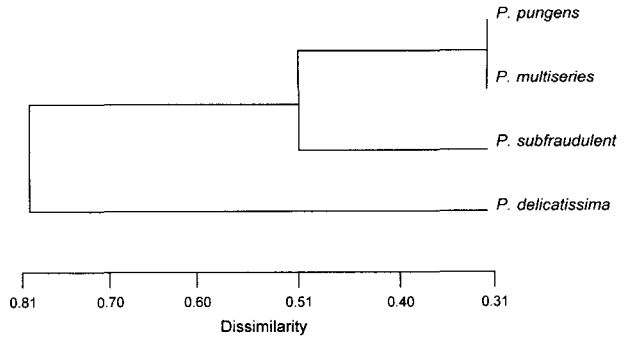

Fig. 4. RAPD band pattern of four strains of *Pseudo-nitzschia* based on the NTSYS program version 2.02.

recognize them at the species level. Recently, Hasle reported that *P. multiseries* was closer to *P. seriata* (Cleve) Peragallo on the basis of the striae of the valve face, valve mantle and bands than to *P. pungens* [11]. In morphological ambiguities, gene sequences targeted to ITS regions will thus support to useful genetic markers.

The restriction fragment length polymorphism (RFLP) analyses have been performed for differentiation and characterization of *P. pungens* and *P. multiseries* [16,19]. In addition, the recognition of *P. pungens* and *P. multiseries* at the species level is also supported on RAPD-PCR patterns in this study (Fig. 3). Thus, RAPD method is expected to prove to be effective in detecting the genus of *Pseudo-nitzschia*. At interspecies levels, two species of Korean *P. pungens* and *P. multiseries* from Chinhae Bay showed the same nucleotide sequences as American *P. pungens* and *P. multiseries* collected from Monterey Bay (Fig. 1). This result suggests that there is minimized genetic heterogeneity and is no genetic distance over a relatively wide geographic area. According to Dr. James Manhart (personal communication), samples of *P. multiseries* from all over the world were invariant while there was variation in samples of *P. pungens*. This indicates to me that *P. multiseries* is probably recently introduced by human activities, but is unclear where it come from originally. Thus, we require to investigate distribution of haplotypes, degree of gene flow, and the resultant population genetic structure of *P. pungens* and *P. multiseries* in order to broad my understanding of the genetic aspect of the species.

Bootstrapping of *P. pungens*, *P. multiseries* and *P. australis* alignment in the ITS2 regions reveals the branching pattern of them to be in agreement with Scholin *et al.* result [19]. This is indicated that a statistically significant grouping of *P. pungens*, *P. multiseries*, and *P. austalis* can be enough and may cluster together in gene sequences targeted to any of regions. In morphology, *P. multiseries* and *P. pungens* have no central nodule, while *P. subfraudulenta* and *P. delicatissima* have it. Thus, the presence of central nodule may play an important role in a species identification tool. *P. delicatissima* was significantly different morphometric data of *P. pungens*, *P. multiseries*, and *P. subfraudulenta* on the basis of apical axis, central nodule, the number of fibulae in 10 m and the number of poroids in 1 m [7]. Likewise, phylogenetic relationship based on RAPD-PCR patterns showed that the placement of *P. delicatissima* was separated with *P. subfraudulenta* and also was quite low degree of dissimilarity coefficient with *P. pungens* and *P. multiseries* (Fig. 4). Thus, on the basis of phylogenetic analysis and genetic comparison (sequence analysis of ITS2 region and RAPD patterns), *P. delicatissima* appears to be high degree of separation from the genus of *Pseudo-nitzschia*.

In conclusion, the analysis of ribosomal ITS2 gene sequences of *P. pungens*, *P. multiseries*, *P. subfraudulenta*, and *P. delicatissima* to understand genetic divergence of the species showed that the phylogenetic placement of *P. delicatissima* generated by PAUP and NJ methods located in far genetic distance from the rest of species of *Pseudo-nitzschia*. The phylogenetic relationship of *P. delicatissima* inferred from RAPD-PCR patterns also observed the same genetic position using ITS2 sequences. This conclusion is that ITS2 region analysis is a useful method for resolving the systematic relationships of Korean *Pseudo-nitzschia*. We want to suggest that the analyses of gene sequences and RAPD-PCR are desirable to determine high resolution of even deep branches with any degree of confidence for diverse assemblages and the degree of phylogenetic signal loss of organisms.

Acknowledgements

This work was supported by a grant from the Maritime Affairs and Fisheries Ministry of Korea. We sincerely thank Dr. J.G. Park, Inje University, Kimhae for providing the strains of *Pseudo-nitzschia* and Dr. I.S. Kim, National Institute of Agricultural Science and Technology, Suwon for the helpful comments and suggestions.

References

1. Adachi, M., Y. Sako and Y. Ishida. 1996. Analysis of *Alexnadrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *J. Phycol.* **32**, 424-432.
2. Adachi, M, Y. Sako and Y. Ishida. 1997. Analysis of *Gymnodinium catenatum* (Dinphyceae) using sequences of the 5.8S rDNA-ITS regions and random amplified polymorphic DNA. *Fish. Sci.* **63**, 701-707.
3. Bates, S. S., C. J. Bird, A. S. W. Freitas, R. Foxall, M. Gilgan, *eds.* 1989. Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *Can. J. Fish. Aquat. Soi.* **46**, 1203-1215.
4. Cangelosi, G. A., A. M. Hamlin, R. Marin and C. A. Scholin. 1997. Detection of stable pre-rRNA in toxigenic *Pseudo-nitzschia* species. *Appl. Enviro. Micro.* **63**, 4859-4865.
5. Cho, E. S., H. G. Kim and Y. C. Cho. 2000. Sequence analysis

of *Cochlodinium polykrikoides* isolated from Korean coastal waters using sequences of Internal Transcribed Spacers and 5.8S rDNA. *J. Kor. Soc. Oceano.* **35**, 158-160.
6. Cho, E. S. 2003. Molecular discrimination of dinoflagellates *Cochlodinium polykrikoides* Margarlef, *Gyrodinium impudicum* Fraga et Bravo and *Gymnodinium catenatum* Graham using RAPD-PCR method. *Kor. J. Lif. Sci.* **13**, 651-657.
7. Cho, E. S., Y. Kodaki and J. G. Park. 2001. The comparison of two species toxic *Pseudo-nitzschia multiseries* (Hasle) Hasle and non-toxic *P. pungens* (Grunow) Hasle isolated from Chinhae Bay. *Algae* **16**, 275-285.
8. Cho, E.S., J. G. Park, B. C. Oh and Y. C. Cho. 2001. The application of species specific DNA-targeted probes and fluorescently tagged lectin to differentiate several species of *Pseudo-nitzschia* (Bacillariophyceae) in Chinhae Bay, Korea. *Sci. Mar.* **65**, 207-214.
9. Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) ver. 3.5c. Department of Genetics, University of Washington, Seattle.
10. Guilalrd, R. R. L. and J. H. Ryther. 1962. Studies of marine planktonic diatoms 1. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* **8**, 229-239.
11. Hasle, G. R. 1995. *Pseudo-nitzschia pungens* and *P. multi*series (Bacillariphyceae): nomenclatural history, morphology, and distribution. *J. Phycol.* **31**, 428-435.
12. Hills, D. M. and M. T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Rev. Biol.* **66**, 411-453.
13. Jung, J. W., G. Y. Kim, M. G. Ha, T. H. Lee and J. D. Lee. 1999. Phylogenetic analysis of the genus *Phellinus* by comparing the sequences of internal transcribed spacers and 5.8S rDNA. *Kor. J. Mycol.* **27**, 124-131.
14. Kim, G. Y., M. G. Ha, E. S. Cho, T. H. Lee, S. J. Lee and J. D. Lee. 1999. Molecular identification of *Gyrodinium impudicum* and *Gymnodinium sanguineum* by comparing the sequences of the internal transcribed spacers 1, 2 and 5.8S ribosomal DNA. *J. Fish. Sci. Tech.* **2**, 66-77.
15. Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* **116**, 111-120.
16. Manhart, J. R., G. A. Fryxell, M. C. Villac and L. Y. Segura. 1995. *Pseudo-nitz*schia *pungens* and *P. multiseries* (Bacillariophyceae): nuclear ribosomal DNAs and species differences. *J. Phycol.* **31**, 421-427.
17. Perl, T. M., L. Bedard, T. Kosatsky, J. C. Kockin, E. C. D. Todd and R. S. Remis. 1990. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N. Engl. J. Med.* **322**, 1775-1778.
18. Rohlf, S. B. 1992. NTSYS-PC numerical taxonomy and multivariate analysis syste, version 1.7. Applied Biostatistics Inc., New York, USA.
19. Scholin, C. A., M. C. Villac, K. R. Buck, J. M. Krupp, D. A. Powers, G. A. Fryxell and F. P. Chavez. 1994. Ribosomal DNA sequences discriminate among toxic and non-toxic *Pseudo-nitzschia* species. *Nat. Tox.* **2**, 152-165.
20. Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**, 7213-7218.
21. Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski and S. V. Tingey. 1990. DNA polymrophisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531-6535.

초록 : ITS2 부위의 염기서열 및 RAPC-PCR에 의한 *Pseudo-nitzschia* 4종의 유연관계

조은섭* · 이영식
(국립수산과학원 남해수산연구소)

ITS2 부위를 시퀀싱하여 *Pseudo-nitzschia delcatissima, P. multiseries, P. pungens, P. subfraudulenta* 상호간의 유전자 다양도를 조사함과 아울러 RAPD-PCR pattern을 이용하여 유사도를 구하였다. 유전자 거리를 근거로 했을 때 *P. delicatissima* 종은 *P. multiseries*와 *P. pungens*와는 유전적 거리가 상당히 요원하였고, 심지어 *P. subfraudenlta*와도 거리를 보였다. 유사도의 경우 *P. multiseries*와 *P. pungens*는 0.31로 보인 반면에, *P. delicatissima*는 다른 세종과 0.81를 나타내었다. 따라서 *P. delicatissima* 종은 *P. multiseries, P. pungens, P. subfraudulenta*와는 유전적으로 밀접하지 않는 관계로 보였다. ITS2 부위는 *Pseudo-nitzschia* 동정에 사용될 수 있는 유용한 도구로 보이며 형태적으로 구분할 수 없는 *P. multiseries*와 *P. pungens*을 구분할 수 있다. 또한 RAPD-PCR 방법도 단시간에 *Pseudo-nitzchia*을 분리시키는데 사용될 것으로 보인다.