

## Sequence polymorphism in a novel noncoding region of Pacific oyster mitochondrial DNA

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**Abstract.** Nucleotide sequence polymorphism in a 641-bp novel major noncoding region of mitochondrial DNA (mtDNA-NC) of the Pacific oyster *Crassostrea gigas* was analysed for 29 cultured individuals within the Goseong population. A total of 30 variable sites were detected, and the relative frequency of nucleotide alteration was determined to be 4.68. Alterations were mostly single nucleotide substitutions. Transition, transversion, both transition and transversion, and both transversion and nucleotide deletion were observed at 18, 9, 2 and 1 sites, respectively. Among 29 specimens, 22 haplotypes were identified, and pairwise genetic diversity of haplotypes was calculated to be 0.988 from multiple sequence substitutions using the two-parameter model. A phylogenetic tree, obtained for haplotypes by the neighbor-joining method, showed a single cluster of linkages. The cluster comprised 11 haplotypes associating with 14 specimens, while the other 11 haplotypes associating with 15 specimens were scattered. This mtDNA-NC presenting a high nucleotide sequence polymorphism is a potential mtDNA control region. It therefore can serve as a genetic marker for intraspecies phylogenetic analysis of the Pacific oyster and is more useful than the less polymorphic mtDNA coding genes.

**Key words:** control region, *Crassostrea gigas*, mitochondrial DNA, noncoding region, Pacific oyster.

### Introduction

In both terrestrial and aquatic vertebrates, the major noncoding region of mitochondrial DNA (mtDNA) is known as a control region or displacement loop region and can become a useful genetic marker in research on population genetic structure and intraspecies phylogeny (Hoelzel 1993; Pollock et al. 2000; Bruford et al. 2003; Ross et al. 2003). Among over 25 000 species of bivalves (Brands 2004), the major mtDNA noncoding region was identified as the control region from only some *Mytilus* mussel species (Burzynski et al. 2003; Zbawicka et al. 2003; Cao et al. 2004). The nucleotide divergences of the mussel control region were reported to be comparable to those of the vertebrate counterpart region. In other bivalve species, we found 2 lengthy noncoding regions distributed on both sides of the NADH dehydrogenase (ND) subunit 4L gene from

the AB065375 mtDNA sequence of the short neck clam *Tapes philippinarum* (unpublished). We also found a short but major mtDNA noncoding region between glutamine tRNA and ND2 genes by gene orientation analysis of the NC005335 mtDNA sequence of the freshwater mussel *Lampsilis ornata* (unpublished). A short 218-bp portion between methionine tRNA and 12S ribosomal RNA (rRNA) genes of the scallop *Patinopecten yessoensis* mtDNA is reported to be the major mtDNA noncoding region termed as NcR2 (Sato and Nagashima 2001). None of these noncoding regions, however, have been identified to be the control region yet.

Cupped oysters *Crassostrea* are found everywhere in the world except in the north and south poles (Matthiessen 2000). They provide the basis for a large aquaculture industry worldwide, and this industry has grown over the past 40 years since the hanging culture method using ropes sus-

Received: October 4, 2004. Revised: December 19, 2004. Accepted: January 3, 2005.

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pended from long lines or rafts was introduced (FAO 1997; Rana and Immink 2001). The industrial aquaculture is dominated by 3 species: the Pacific oyster *Crassostrea gigas*, Eastern oyster *C. virginica* and Portuguese oyster *C. angulata*. These species naturally occur in East Asia (Japan, Korea, Taiwan, etc.), along the Atlantic from south Canada to the USA, and in South Europe from Portugal to France, respectively (Park et al. 1989). Other species of interest for oyster aquaculture are *C. rivularis*, *C. belcheri*, *C. plicatula* and a few tropical species (Lovatelli 1988). Among various cupped oyster species, the Pacific oyster is most consumed worldwide, and its distribution has been extended through the transplantation from Japan to Australia, Europe and America since the early 20th century (Chew 1990). In the late 20th century, oyster aquaculture developed not only in the regions where oysters occur naturally but also in those countries where they had been introduced (Matthiessen 2000). This resulted in the global production of cultured Pacific oysters ranking first among cultured aquatic animals in 1996 (Rana and Immink 2001).

Since the hanging culture method was first evolved in Japan, geographical variations and genetic relationships among regional wild populations and cultured broodstocks have become the major concern, because farmers demanded appropriate selective breeding programs to local environmental conditions. Hence, basing on regional differences in morphological and physiological characteristics, the Pacific oyster distributed around Japan was found to comprise a few local races (Imai and Sakai 1961; Okoshi et al. 1987). Although alternative genetic studies using allozyme analysis were conducted, the results obtained were complicated. The initial study demonstrated that the genetic population structure around Japan well corresponded to the morphologically divided population structure (Fujio 1979), but the same research group subsequently reported that there was little genetic differentiation among these populations (Ozaki and Fujio 1985). In order to clarify the interpretation of the population structure of the Pacific oyster, advanced studies using a novel genetic marker other than allozyme analysis are required, and the mtDNA control region could be the best candidate for the alternative marker. Among a few noncoding regions identified by us from the NC001276 mtDNA sequence of the Pacific oyster, this study focused on a novel major 641-bp

noncoding region, termed as the mtDNA-NC segment, and characterized its intraspecies sequence polymorphism within a cultured population of the Pacific oyster.

## Material and methods

### Oyster DNA

Adult oysters cultured on ropes suspended from long lines along coastal regions of Goseong Bay, Kyongnam, Korea (Kang et al. 2000), were harvested and then transported alive by air in a cooled package to Japan. Adductor muscle was collected from fresh specimens and immediately stored at  $-40^{\circ}\text{C}$  until DNA extraction. Total genomic DNA was prepared from frozen adductor muscle by the modified urea-SDS-Proteinase K method (Aranishi and Okimoto 2004). In brief, samples were incubated in TESU4 buffer (10 mM Tris-HCl, pH 7.5, 20 mM EDTA, pH 8.0, 1% SDS, 4 M urea) containing 25  $\mu\text{g}$  Proteinase K (Sigma) at  $55^{\circ}\text{C}$ , and then 5 M NaCl was added and mixed. DNA was extracted with phenol-chloroform-isoamyl alcohol, followed by chloroform-isoamyl alcohol, and finally precipitated with ethanol. DNA pellet was washed with ethanol, dried up, and resuspended in 10T0.1E (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, pH 8.0).

### PCR amplification

A 739-bp fragment comprising the mtDNA-NC segment and adjacent glycine tRNA and valine tRNA genes were amplified in a PCR mixture containing 2 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  each dNTP, 0.25 unit *Taq* DNA polymerase (Sigma), 0.5  $\mu\text{M}$  each primer (5'-TCACAAGTACATTTGTCTTCCA-3'; 5'-AACGTTGTAAGCGTCATGTAAT-3'), and template DNA in a Techgene thermal cycler (Techne). The PCR amplification protocol consisted of an initial denaturation at  $94^{\circ}\text{C}$  for 2 min, followed by 30 cycles of 10 s at  $94^{\circ}\text{C}$ , 10 s at  $58^{\circ}\text{C}$  and 40 s at  $72^{\circ}\text{C}$ , and a final extension at  $72^{\circ}\text{C}$  for 5 min. Amplicon was run on a 2.0% agarose gel at 15 V/cm for 40 min and visualized under UV illumination in an EDAS290 Gel Documentation System (Invitrogen).

### Sequence analysis

Nucleotide sequencing of both DNA strands of PCR products was accomplished by using a CEQ DTCS kit in a CEQ2000XL DNA Analysis System (Beckman Coulter). Multiple sequence align-

ments were performed with CLUSTAL W 1.7 software (Thompson et al. 1994). Phylogenetic trees were constructed for haplotypes by the neighbor-joining method (Saitou and Nei 1987) using TREEVIEW software (Page 1996) and the unweighted pair group method with arithmetic mean (UPGMA) using MEGA 2.1 software (Kumar et al. 2001). The reliability of the topology of the trees was assessed by 1000 bootstrap replications. Genetic distances among haplotypes were calculated from multiple sequence substitutions by the two-parameter model (Kimura 1980).

transversion and nucleotide deletion at 1 site (Table 1). Among 4 kinds of nucleotide, adenine altered most frequently, and this alteration was almost always derived from transition to guanine. Although the transitions from adenine, thymine and cytosine occurred more frequently than the transversions, 6 sites out of 7 guanine variable sites altered by the transversion to either cytosine or thymine. Multiple alterations involving both transition and transversion occurred at 1 site of thymine and 1 site of cytosine, while transversion and deletion occurred at only 1 site of thymine.

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TACGCTGTA GTTGAGACA ATTTTAAAGG TAGCGCGAGT AGAAAGTTTT AAAATTTGCT TACGCTAGG ATTGCTTCAA TTITGATATT TTTAATAACC
TATTTGTAAG CCTCTATTAC ATGACGAAA TTTTLAGAAT TGTCTTAAAG TTTAATTACA TGTAGCTTAT GTGTTAACCG TTATCTTTGT GAAGTGAAAT
ACAGCTTAAG TAAAAAATAA AAAATATCTT TTCCCTATGT AATAAAAGG GTTTTAGTGT CTTAACCTAT AGTGATTCGT GCTTAGTCTA AGCTGATAGT
GGCAGCCAAA TAAAAATTG GTGTTGATG AATTGCTTGC AAGCAGGTTA AAAAATCAAC TTGGTCTATT AGGGTGGTAA GGTAAAAGTA TTGGGTGAAG
CCTTATAAATA TATTGAAAAG TACTATAAGT TAATCTAAT AAGCATAACG GGAGTGCAAT AGGTTGGGTA TAATAATAAA TGITTAAGTA ATAAAAAATG
ATGGGTAGCA ATAAATGTT TGGGTATAAA AGTGATTAGC TTGGGTAGA AGAAATCATA AAGGCTTGGG TGTAAGTTTT AAAATAAAT ATGATAAGAT
CGAAGGTTAA GTTGAGTGC GCTTATAAGG CACATTAGTG T

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Figure 1. Nucleotide sequence for the mtDNA-NC segment of cultured Pacific oysters of the Goseong population. Underlined nucleotides with shadow indicate 30 variable sites from 29 individuals.

## Results

The nucleotide sequences of the mtDNA-NC segment were compared among 29 Pacific oyster specimens, which had been cultured in the restricted sea farming area of Goseong Bay. A total of 30 variable sites were detected from a 641-bp portion of the mtDNA-NC segment (Figure 1),

Among 29 cultured oyster specimens within the Goseong population, 22 haplotypes were identified from nucleotide sequence analysis of the mtDNA-NC segment. Most haplotypes were unique to particular specimens. The exceptions were haplotypes GB-1 and GB-2 (found in 3 specimens each) and haplotypes GB-3, GB-7 and GB-15 (found in 2 specimens each). Pairwise ge-

**Table 1.** Nucleotide diversity at 30 variable sites in the mtDNA-NC segment of cultured Pacific oyster of the Goseong population

Nucleotide	Variable sites	Frequency (%)	Nucleotide diversity					Total
			Ts	Tv	Ts+Tv	Tv+Del	Del	
G	7	1.09	1	6	0	0	0	7
A	10	1.56	9	1	0	0	0	10
T	8	1.25	5	1	1	1	0	8
C	5	0.78	3	1	1	0	0	5
Total	30	4.68	18	9	2	1	0	30

Ts = transition; Tv = transversion; Ts+Tv = transition and transversion; Tv+Del = transversion and deletion; Del = deletion.

and relative frequency of nucleotide alteration was determined to be 4.68%. Alterations were mostly single nucleotide substitutions. Transition was observed at 18 sites, transversion at 9 sites, both transition and transversion at 2 sites, and both

netic diversity of 22 haplotypes was calculated to be 0.988. Genetic distances among haplotypes were estimated, and the resulting phylogenetic trees constructed by the neighbor-joining method and UPGMA method showed almost the same to-

pology. The neighbor-joining tree illustrated that a single cluster of linkages comprised 11 haplotypes associating with 14 specimens, and the other 11 haplotypes associating with 15 specimens were scattered (Figure 2). It is noteworthy that similar results were obtained from a different phylogenetic analysis by means of the RAPD technique (Aranishi and Okimoto 2004). In that study, a total of 20 cultured oyster specimens of the Goseong population constituted 2 large clusters of linkages, one comprising 12 specimens and the other comprising 8 specimens.

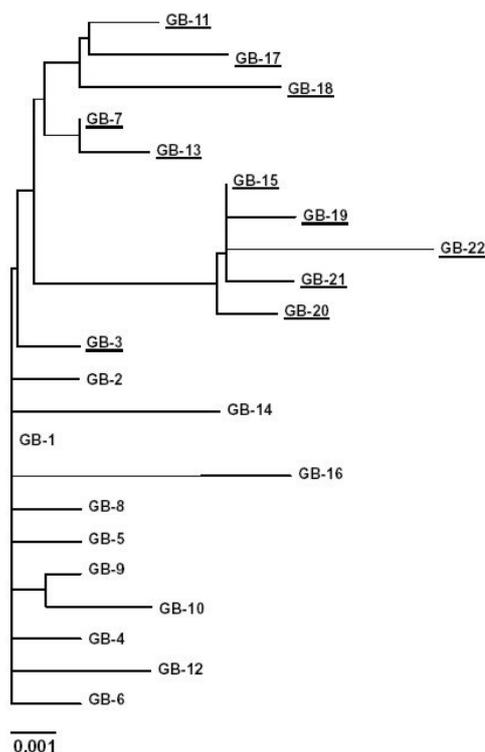


Figure 2. Neighbor-joining tree for 22 haplotypes in the mtDNA-NC segment of cultured Pacific oyster of the Goseong population, based on 1000 bootstrap replicates. Underlined haplotypes constitute a single cluster of linkages.

## Discussion

Among a few noncoding regions identified by us from the NC001276 mtDNA sequence of the Pacific oyster, the present study focused on a novel major noncoding region termed the mtDNA-NC segment (Figure 1). The NC001276 sequence was revised from the AF177226 data deposited in the GenBank in December 1999. The Pacific oyster is one of the most commercially important bivalves worldwide (Rana and Immink 2001). Surprisingly, no research was conducted earlier to verify genetic structures of cultured broodstocks

and wild populations of the Pacific oyster by using the nucleotide sequence analysis of variable mtDNA noncoding regions, such as the mtDNA-NC segment, although the reference sequences have been available for more than 4 years.

The mtDNA-NC segment of the Pacific oyster is a hypervariable region, where 30 variable sites mostly derive from single nucleotide substitutions (Table 1), and relative frequency of nucleotide alteration was determined to be 4.68%. Of the mtDNA coding regions of the Pacific oyster, intraspecies phylogenetic studies were performed for wild and cultured populations by PCR-RFLP analysis of the cytochrome oxidase C subunit I (COI) gene (Boudry et al. 1998) and by nucleotide sequence analysis of both the COI and 16S rRNA genes (Boudry et al. 2003). Results obtained in these studies were well consistent, while different techniques were applied. The latter study demonstrated that only 3 and 1 variable sites were detected from 547 bp and 415 bp portions of the COI and 16S rRNA genes, respectively, so relative frequencies of nucleotide alteration were only 0.54% and 0.24%, respectively. In view of these findings, the mtDNA-NC segment presenting a much higher nucleotide sequence diversity is obviously a good marker, more suitable for intraspecies phylogenetic analysis of the Pacific oyster than are the less polymorphic mtDNA coding regions, such as the COI and 16S rRNA genes.

The phylogenetic tree constructed by genetic distances among 22 haplotypes showed a single cluster of linkages of 11 haplotypes associating with 14 specimens, while the other 11 haplotypes associating with 15 specimens were not clustered (Figure 2). Oyster farming using ropes suspended from long lines or rafts has been executed by natural reproduction of cultured oyster, in which naturally bred spats attach to the suspended cultches and grow on them. This system allows maintaining the genetic diversity of oyster broodstocks, unlike other aquaculture systems using hatchery-reared seeds (Li and Hedgecock 1998). However, various unforeseen accidents necessitate transplantation of commercial spats produced by a limited number of mature oyster parents from hatcheries. In the last several years, mortality of cultured oyster sometimes occurred in Korea, because of protozoan parasites and deterioration of the environmental quality (Choi et al. 1997; Park et al. 1999a; Kang et al. 2000), and thus attributed to failure in spat collection by natural reproduction (Park et al. 1999a, 1999b).

Although we are unaware of whether abundant commercial spat were transplanted to Goseong Bay in such accidents or not, half the number of cultured oyster of the Goseong population forming a single cluster of linkages (Figure 2) might originate in genetically related hatchery-reared spat.

In conclusion, we analysed sequence polymorphism of the novel major noncoding region of the Pacific oyster mtDNA for 29 cultured specimens of the Goseong population. The mtDNA-NC segment was determined as a hypervariable region presenting a much higher nucleotide sequence diversity than the mtDNA coding regions, such as the COI and 16Sr RNA genes. In addition, its nucleotide sequence diversity and pairwise genetic diversity of haplotypes were comparable to the NcR2 major noncoding region of the scallop mtDNA, presenting nucleotide sequence diversity at 5.48% (12 variable sites in a 219-bp fragment) among 40 specimens within the Saroma population and pairwise genetic diversity of resulting 14 haplotypes at 0.869 (Sato and Nagashima 2001). Not only the mtDNA-NC segment of the Pacific oyster but also the NcR2 region of the scallop are the potential control regions of bivalve mtDNA due to the high sequence polymorphism. Our further investigation is therefore undertaken to perform their structural characterization, e.g. identifying functionally important domains, such as conserved sequence blocks and termination-associated sequences, which are highly conserved in the control regions of vertebrate mtDNA (Hoelzel 1993).

**Acknowledgements.** This work was supported in part by grants from the Agriculture, Forestry and Fisheries Research Council of Japan and Fisheries Research Agency of Japan.

#### REFERENCES

- Aranishi F, Okimoto T, 2004. Genetic relationship between cultured populations of Pacific oyster revealed by RAPD analysis. *J Appl Genet* 45: 435–444.
- Boudry P, Heurtebise S, Collet B, Cornette F, Gerard G, 1998. Differentiation between populations of the Portuguese oyster, *Crassostrea angulata* (Lamarck) and the Pacific oyster, *Crassostrea gigas* (Thunberg), revealed by mtDNA RFLP analysis. *J Exp Mar Bio Ecol* 226: 279–291.
- Boudry P, Heurtebise S, Lapegue S, 2003. Mitochondrial and nuclear DNA sequence variation of presumed *Crassostrea gigas* and *Crassostrea angulata* specimens: a new oyster species in Hong Kong? *Aquaculture* 228: 15–25.
- Brands SJ, 2004. *Systema Naturae 2000*. Amsterdam.
- Bruford MW, Bradley DG, Luikart G, 2003. DNA markers reveal the complexity of livestock domestication. *Nat Rev Genet* 4: 900–910.
- Burzynski A, Zbawicka M, Skibinski DOF, Wenne R, 2003. Evidence for recombination of mtDNA in the marine mussel *Mytilus trossulus* from the Baltic. *Mol Biol Evol* 20: 388–392.
- Cao L, Kenchington E, Zouros E, Rodakis GC, 2004. Evidence that the large noncoding sequence is the main control region of maternally and paternally transmitted mitochondrial genomes of the marine mussel (*Mytilus* spp.). *Genetics* 167: 835–850.
- Chew KK, 1990. Global bivalve shellfish introductions. *World Aquaculture* 21: 9–22.
- Choi WJ, Chun YY, Park JH, Park YC, 1997. The influence of environmental characteristics on the fitness of Pacific oyster, *Crassostrea gigas*, in Hansan-Koje Bay. *J Korean Fish Soc* 30: 794–803 (in Korean with English abstract).
- FAO 1997. Review of the State of World Aquaculture. FAO Fisheries Circular No. 886 Rev. 1. FAO, Rome. 163 pp.
- Fujio Y, 1979. Enzyme polymorphism and population structure of the Pacific oyster, *Crassostrea gigas*. *Tohoku J Agric Res* 30: 32–42.
- Hoelzel AR, 1993. Evolution by DNA turnover in the control region of vertebrate mitochondrial DNA. *Curr Opin Genet Develop* 3: 891–895.
- Imai T, Sakai S, 1961. Study of breeding of Japanese oyster, *Crassostrea gigas*. *Tohoku J Agric Res* 12: 125–171.
- Kang CK, Park MS, Lee PY, Choi WJ, Lee WC, 2000. Seasonal variations in condition, reproduction activity, and biochemical composition of the Pacific oyster, *Crassostrea gigas* (Thunberg), in suspended culture in two coastal bays of Korea. *J Shellfish Res* 19: 771–778.
- Kimura M, 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111–120.
- Kumar S, Tamura K, Jakobsen BI, Nei M, 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* 17: 1244–1245.
- Li G, Hedgecock D, 1998. Genetic heterogeneity, detected by PCR-SSCP, among samples of larval Pacific oysters (*Crassostrea gigas*) supports the hypothesis of large variance in reproductive success. *Can J Fish Aquat Sci* 55: 1025–1133.
- Lovatelli A, 1988. Status of oyster culture in selected Asian countries. FAO, Rome.
- Matthiessen GC, 2000. *Oyster Culture*. Fishing News Books Series, Blackwell Publishing, Ames.
- Okoshi K, Mori K, Nomura T, 1987. Characteristics of shell chamber formation between the two local races in the Japanese oyster, *Crassostrea gigas*. *Aquaculture* 67: 313–320.

- Ozaki H, Fujio Y, 1985. Genetic differentiation in geographical populations of the Pacific oyster (*Crassostrea gigas*) around Japan. *Tohoku J Agric Res* 36: 49–61.
- Page RDM, 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12: 357–358.
- Park BH, Park MS, Kim BY, Hur SB, Kim SJ, 1989. Culture of the Pacific Oyster (*Crassostrea gigas*) in the Republic of Korea. FAO, Rome.
- Park MS, Lim HJ, Jo Q, Yoo JS, Jeon MJ, 1999a. Assessment of reproductive health in the wild seed oysters, *Crassostrea gigas*, from two locations in Korea. *J Shellfish Res* 18: 445–450.
- Park MS, Lyu HY, Lee TS, 1999b. Investigation on the cause of bad natural seed collection of the Pacific oyster, *Crassostrea gigas*: relationships between the conditions of mother shell and the viability of the released eggs and larvae based on the pathological and embryological survey. *J Korean Fish Soc* 32: 62–67.
- Pollock DD, Eisen JA, Doggett NA, Cummings MP, 2000. A case for evolutionary genomics and the comprehensive examination of sequence biodiversity. *Mol Biol Evol* 17: 1776–1788.
- Rana K, Immink A, 2001. Trends in Global Aquaculture Production: 1984–1996. FAO, Rome.
- <http://www.fao.org/DOCREP/FIELD/006/AD742E/AD742E00.HTM>.
- Ross HA, Lento GM, Dalebout ML, Goode M, Ewing G, McLaren P, et al., 2003. DNA surveillance: web-based molecular identification of whales, dolphins, and porpoises. *J Hered* 94: 111–114.
- Saitou N, Nei M, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425.
- Sato M, Nagashima K, 2001. Molecular characterization of a mitochondrial DNA segment from the Japanese scallop (*Patinopecten yessoensis*): demonstration of a region showing sequence polymorphism in the population. *Mar Biotechnol* 3: 370–379.
- Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680.
- Zbawicka M, Skibinski DOF, Wenne R, 2003. Doubly uniparental transmission of mitochondrial DNA length variants in the mussel *Mytilus trossulus*. *Mar Biol* 142: 455–460.