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Stock Characterization of the Fleshy Prawn (*Penaeus chinensis*) in the Yellow Sea by Intraspecific Sequence Variation of the Cytochrome *c* Oxidase Subunit I Gene

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To determine the amount of genetic variation among populations of *Penaeus chinensis* (Osbeck) in the Yellow Sea, 342 bp region of the mitochondrial cytochrome *c* oxidase subunit I gene was amplified and sequenced. Six haplotypes, which differ by from one to four nucleotide sustitutions, were detected from 34 individuals of 4 populations examined. Mean sequence divergence between pairs of haplotypes was 0.68 %. Most individuals from 4 populations were shared by the most common genotype. This genotype was distributed evenly in the Korean and Chinese populations. This result is in accordance with findings observed using RFLPs analysis of mtDNA (Hwang et al., 1997). Therefore, it is suggested that *P. chinensis* should be treated as one unit stock in the Yellow Sea.

Key words : *Penaeus chinensis*, mitochondrial DNA, cytochrome *c* oxidase subunit I gene, stock discrimination

Introduction

The fleshy prawn, *Penaeus chinensis* (Osbeck), is distributed in the western Pacific, particularly along the northeastern coast of Asia. This species is of considerable commercial importance in the Yellow Sea, East China Sea and Korean Bight, where it is trawled because of its large size and interests for aquaculture (Holthuis, 1980). Concerning the stock problems for fisheries management, it has been reported that two distinct groups of *P. chinensis* exist in the Yellow Sea following its spawning grounds. Furthermore, Mako and Shojima (1969) divided *P. chinensis* in the Yellow Sea into three groups; Pohai Bay first group, Pohai Bay second group which includes the group originated from the northern Yellow Sea, and the west coast Korean group based upon tagging data. Later, Kim (1970) reported that another group may exist along the southern coast of Korea based on the morphometric differences inhabiting the southern and western part of Korea and on differences in their migration route. However, Hwang et al. (1997) have shown that there are no distinct genetic differences between Korean and Chinese populations based on the restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA). They treated Yellow Sea *P. chinensis* as one unit based on genetic stock concepts. Therefore, to clarify the gene exchange among populations of Yellow Sea *P. chinensis*, more detailed analysis other than the methodology of RFLPs is required.

RFLPs of mtDNA have been used intensively for many fisheries species to discriminate the genetic stocks (Bentzen et al., 1988; Mulligan and Chapman, 1989; Brown, 1989; Brown and Paynter, 1991). However, RFLPs analysis of mtDNA also has some drawbacks. Detection of the fragments smaller than 100 bp is difficult by ethidium bromide staining and DNA assessed in indirect way. Therefore, recently, direct sequence analysis has been introduced for population structure analysis. Following the development of molecular biology techniques, accumulating sequence data were available in many fisheries species. Population variation study using partial mtDNA or nuclear DNA sequence was reported in many species (Beckenbach et al., 1990; McVeigh et al., 1991; Waldman and Wirgin, 1994).

Up to 94% of mtDNA molecules encode 37 functional RNA. Among these genes, selected mtDNA sequence of interest, usually the cytochrome *b* gene, cytochrome *c* oxidase I (COI) gene, or the control region may be used for population genetics and molecular phylogeny. These sequences have been described across many taxa (Crozier et al., 1989; Waldman and Wirgin, 1994). For population comparison of the *P. chinensis*, COI gene was selected because it is moderate conservative and sequenced in various invertebrate taxa (Clary and Wolstenholme, 1985; Crozier et al., 1989; Palumbi and Benzie, 1991). Therefore, purpose of this study is to delineate the stock problems of *P. chinensis* in the Yellow Sea using a intraspecific sequence variation of COI gene in mitochondrial genome.

Materials and Methods

1) Collection of specimens and total DNA extraction

The fleshy prawn were collected from April to May of 1995 from two sites of Korean coast (Daechon and Narodo) and two sites of Chinese coast (Pohai and Qingdao) in the Yellow Sea. Samples were frozen immediately in the field by dry ice and stored in a freezer (−70℃) until analysis. Total genomic DNA extractions from 34 individuals were carried out by a modification of the methods of Lee and Taylor (1990), as described by Song (1994).

2) Primers and PCR amplification

Total nucleic acid preparations were used as templates for the partial amplification of the mtDNA gene coding COI molecule using Polymerase Chain Reaction (PCR) as described by Mullis et al. (1986) and Saiki et al. (1988). Oligonucleotide primers were designed based on the mtDNA sequences of *Drosophila yakuba* (Clary and Wolstenholme, 1985) and *P. stylirostris* (Palumbi and Benzie, 1991). The primers used in this study are listed in Table 1.

The PCR mixtures were consisted of 200μM of each deoxy nucleotide triphosphate, 50 mM KCl, 2 mM $MgCl_2$, 0.1 % Triton X-100, 10 mM Tris-HCl, pH 8.3, 4 pM of each primer and 1 unit of *Taq* polymerase (Promega Co.), respectively. Approximately 100 ng of total DNA was used as a starting template and the reaction volume for PCR was usually 50μl. Each cycle of PCR consisted of denaturation for 1 min at 94℃, primer annealing for 1 min at 45℃ and extension 1 min 30 sec at 72℃. This cycle was repeated 30 times with DNA thermal cycler (Techne Co.). The extension time of the latter 15 cycles was increased 5 sec for every cycle.

3) Cloning of PCR product

Amplified DNA from each individuals by PCR was electrophoresed on 1 % low melting agarose (American Biorganics Co.) gel to remove the remaining primers and non-specific products. The target band was excised and DNA was eluted using GeneClean II kit (Bio 101 Co.) according to manufacturer's directions.

Table 1. The list of primers used in this study

Primers	Sequences
PCR Primers	
Forward	5′-GAAGCWTTYGGWACWYTHGGKATN-3′
Reverse	5′-TGRGCAATWCCTGCRAARATWCCN-3′
Sequencing Primers	
pUC/M13 Forward	5′-GTTTTCCCAGTCACGACGTTGTAA-3′
pUC/M13 Reverse	5′-CACACAGGAAACAGCTATGAC-3′

* W=A,T; Y=C,T; R=A,G; K=G,T; H=A,T,C; N=A,G,T,C

The eluted DNA fragments were ligated with pGEM-T vector (Promega Co.).

The ligation was carried out in 10μl of mixture consisted of 50 ng pGEM-T vector, about 15 ng of insert DNA, 1 unit of ligase and 1/10th volume of 10X ligation buffer. This mixture was incubated at 15℃ overnight and transformed to DH5α or JM109 competent cells. The 4 μl of ligation mixture was mixed with 100 μl of competent cells and left on ice for 30 min. This mixture was then heat-shocked for 45 sec at 42℃ and kept on ice for 2 min. Then they were grown in 1 ml LB media for 1 hr at 37℃. The transformed cells were applied on LB-agar plates containing 100 μg/ml ampicillin, 40 μg/ml X-gal, and 40 μg/ml IPTG. Usually 5~10 white colonies were selected from each plate and cultured in 3 mL of LB media containing 100 μg /ml ampicillin at 37℃ overnight.

Clones carrying the target fragment were selected and cultured in 25 ml LB media containing 100 μg/ml ampicillin at 37℃ for 5~10 hrs. The cultured bacterial cells were harvested by centrifugation at 6,000×g for 20 min at 4℃ when the optical density was 1.0~1.5 at 600 nm wavelength. The plasmid DNA of harvested cells was isolated by using QIAGEN midiprep kit (QIAGEN Co.) according to the supplier's recommendation. The resulting plasmid DNA was rehydrated in 50 μl sterile distilled water and stored at −20 ℃ until sequencing.

4) Sequencing

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1(2287)                                                                  60
ATT TAT GCT ATA CTC GCT ATT GGA GTT CTA GGA TTT GTA GTT TGA GCA CAT CAT ATA TTT
      66  69                                                            120
ACA GTA GGT ATG GAT GTT GAT ACC CGT GCT TAC TTT ACA TCT GCT ACA ATA ATT ATT GCT
                                                                        180
GTT CCC ACA GGA AAT AAA ATT TTT AGC TGA ATA GGA ACC CTC CAC GGG ACA CAG CTA AAT
                192                     213             225 228         240
TAT AGC CCT TCT TTA ATT TGA GCC CTA GGA TTT GTA TTT TTA TTC ACA GTG GGG GGA CTC
                253                                                     300
ACA GGA GTT GTT TTA GCT AAC TCA TCA ATT GAT ATT ATC CTA CAT GAT ACT TAT TAT GTA
                                                        342
GTG GCC CAT TTC CAT TAT GTT CTT TCT ATA GGA GCA GTA TTT
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Fig. 1. The most common haplotype sequence of the cytochrome *c* oxidase subunit I gene from the mtDNA of *P. chinensis*. The variable sites observed are indicated by underlines. Number in parentheses indicates the position of *Drosophila yakuba* (Clary and Wolstenholme, 1985).

Table 2. Partial sequence variation revealed in 342 bp of the COI gene in the mtDNA among 4 populations of *P. chinensis* in the Yellow Sea. Dots represent positions that are identical to the top sequence. The most below line is amino acids translated from DNA. Numbers in parentheses indicate the sample size of each population

Sequences								Daechon (8)	Narodo (9)	Pohai (7)	Qingdao (10)
66	69	192	213	225	228	253	Position				
GTA	GGT	TCT	TTT	TTC	ACA	TTA	Type I	6	8	6	7
. . T	. . .	. . .	. . .	. . .	. . .	. . .	Type II	1	0	0	1
. . .	. . G	. . .	. . .	. . .	. . T	. . .	Type III	0	1	1	0
. . .	. . .	. . C	. . .	. . .	. . T	. . .	Type IV	0	0	0	1
. . .	. . .	. . .	. . C	. . T	. . .	. . .	Type V	1	0	0	0
. . .	. . .	. . .	. . .	. . .	. . .	. . C	Type VI	0	0	0	1
Val	Gly	Ser	Phe	Phe	Thr	Leu	Amino Acids				

The sequencing reaction was performed by using Silver Sequence (Promega Co.) DNA sequencing system, which is a non-radioactive DNA sequencing system that employs a silver staining method for band detection. This sequencing was according to the dideoxy termination reaction (Sanger, 1981) using pUC /M13 forward and reverse primers (Table 1).

Results and Discussion

1) Intraspecific sequence variation

To get the direct information about mtDNA of *P. chinensis*, 342 bp region of COI gene was sequenced. Thirty four individuals from 4 populations of this species in the Yellow Sea were analyzed. Six haplotypes were found in the partial COI gene. These genotypes differed from one another at one to four positions. The most common haplotype sequence examined from 4 populations is shown in Fig. 1. Seven of 34 individuals and total of 7 nucleotide sites showed variation. These variants are shown in Table 2. However, all substitutions are silent changes that do not alter amino acid sequence. All of the 7 variant sites is not phylogenetically informative. Four of the 7 variant sites are transitions and others are transversions. All transitions are pyrimidine transition whereas high proportions of purine transition were observed in fish species (Beckenbach et al., 1990; Carr and Marshall, 1991). The relatively high ratio of transitions to transversions is observed elsewhere as a reflection of differences in mutation rates (Kocher et al., 1989; Carr and Marshall, 1991). All of the base substitutions occurred in the third position of codon, except on the type VI where the substitution occurred in the first position of codon. In this case, however, amino acid was also not changed.

The amount of variation among haplotypes is averaged to 0.68 %. Percent Sequence divergence between type I and type II and those of type I and type VI shown lowermost variation with 0.29 %. While on the other, variation between type IV and type V shown the highest value with 1.17 %. This level of divergence among genotypes is slightly higher than what has been estimated using restriction endonucleases (0.44 %, Hwang et al., 1997).

At least six lineages of mtDNA were observed in this study based on actual sequence data, whereas three clonal lines were detected by means of restriction fragment analysis in Hwang et al. (1997). These results suggest that possibility of underestimation of variant could be caused in restriction enzyme analysis because of its limitation of recognition sequences.

There is moderate intraspecific variation in *P. chinensis* in the COI gene when compared to fish (McVeigh et al., 1991; Hartely et al., 1992) and sea urchin (Vawter and Brown, 1986).

2) Stock discrimination of *P. chinenesis* in the Yellow Sea

As shown in Table 2, type I is predominant in all populations. Among 34 individuals examined, 27 prawns were identical in sequence for COI fragment analyzed and distributed evenly in 4 populations. More than 79 % of the fleshy prawn exhibited a common genotype, whereas none of the 5 variant genotypes was found in more than 6 % of the samples. The Daechon and Qingdao populations share Type II which has only one variant site on the position 66. Two variant nucleotide sites were observed in type III. This haplotype was occurred in the Narodo and Pohai populations. Type IV revealed one variant site on the position 192 and it was limited to the Qingdao population. Type V, which occurred only in the Daechon group, showed 2 variant nucleotide sites on the position 213 and 228. Type VI was only observed in the Qingdao population. This result can be interpreted that there was a single population of *P. chinensis* in the Yellow Sea.

This study is in accordance with findings observed using RFLPs of mtDNA in Hwang et al. (1997). They assumed that dispersal on planktonic larval stage

and migration ability of adult prawn make sufficient genetic exchange among populations to preclude substantial mtDNA differentiation. Mixing in the wintering ground is also presumed one reason of homogeneous genetic structure. Because of weak geographic partitioning and strong vagility, it is more common for marine species to have lower intraspecific mtDNA sequence diversities than terrestrial species (Ovenden, 1990; Waldman and Wirgin, 1994). However, Carr and Marshall (1991) found substantial mtDNA variability and evidence of two stocks of Atlantic cod (*Gadus morhua*) in the Northern Atlantic. They showed that genotype proportions differed significantly between western and eastern Atlantic populations : the majority genotype of western populations was present in a minority of eastern cod. Despite the assumed homogenizing effect of the oceans, reproductive barriers do exist in the marine environment (Ovenden, 1990).

In this study, it is apparent that *P. chinensis* populations in the Yellow Sea are from single homogeneous population having little variation. Therefore, it may be reasonable that this species be treated as one unit stock in the Yellow Sea.

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