

Full Length Research Paper

Genetic diversity of small yellow croaker *Larimichthys polyactis* revealed by AFLP markers

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The population genetic structure of small yellow croaker *Larimichthys polyactis* in the Yellow Sea and East China Sea was analyzed using amplified fragment length polymorphism (AFLP). A total of 304 loci were detected by four primer combinations among 53 individuals collected from four locations representing three stocks of small yellow croaker, 231 of which were polymorphic (75.99%). The proportion of polymorphic loci and Nei's genetic distances for four populations ranged from 58.11% to 69.43% and from 0.1076 to 0.1331. AMOVA analysis and pairwise F_{ST} revealed significant genetic differentiation among four samples, supporting separate stocks in this species and endemic branch tribes in South Yellow Sea stock. The UPGMA tree also revealed the significant geographic structure in this species. Pattern of isolation by distance was observed in this species, indicating that significant genetic differentiation among localities of small yellow croaker was mainly due to the geographic distance. Besides geographic distance, the migratory behavior might be another factor, which influences the genetic structure of this species.

Key words: *Larimichthys polyactis*, small yellow croaker, genetic structure, AFLP.

INTRODUCTION

Understanding fish stock structure is an important component of successful and sustainable long-term management and has attracted considerable interest, because of a fundamental interest in biotic evolution (Tudela et al., 1999). Determination of population genetic structure provides essential information to underpin resource recovery and to aid in delineating and monitoring populations for fishery management. Failure to detect population units can lead to local over-fishing and ultimately to severe declines (Zhang et al., 2006). Molecular genetic techniques offer the ability to identify and delineate fish stock structure where it may not be apparent from phenotypic or behavioural characteristics. Such techniques have been used successfully to understand the structure of marine fish species (Liu et al., 2006; Han et al., 2008a).

The small yellow croaker, *Larimichthys polyactis* (Bleeker), is a benthopelagic fish species of family Sciaenidae that inhabits coastal waters and estuaries (Zhu

et al., 1963). Extensively distributed in the Bohai Sea, Yellow Sea and the East China Sea, *L. polyactis* has supported a major fishery for decades in China and Korea. Global capture production for *L. polyactis* reached 320 thousand metric tons in 2000 (Seikai National Fisheries Research Institute, 2001). After extensive commercial exploitation, the biomass of small yellow croaker continuously declined, and is now considered over-exploited (Yan et al., 2006). Therefore, measures are being taken by the Chinese government and scientific community to restore its resource and prevent against future over-exploitation.

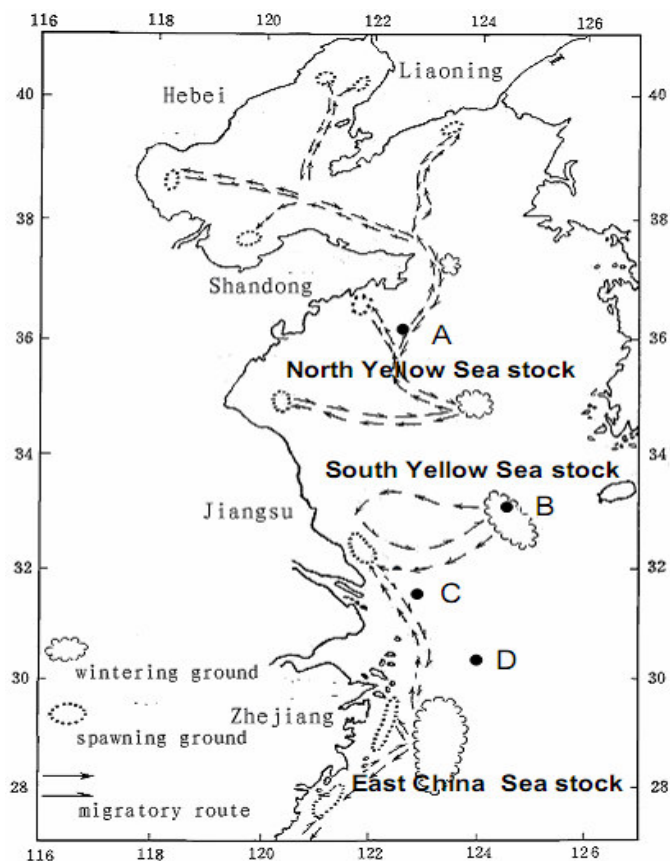
The small yellow croaker spawned pelagic eggs and breeding grounds of the species always located in estuaries and coastal waters. The species spawned eggs in East China Sea from March to April, but in May in Yellow Sea and Gulf of Bohai Sea (Lin, 1962; Liu, 1962; Seikai National Fisheries Research Institute, 2001). In previous studies, Liu (1962) and Ikeda (1964) believed that three wild stocks or tribes existed throughout the range of *L. polyactis* based on the investigation of this species' routes of breeding migration and morphological data.

Understanding genetic structure is important for the management and conservation of exploited species. However, whether these stocks are independent biological units

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Table 1. Parameters of genetic diversity for populations of small yellow croaker.

| Locations | n | Date of collection | Coordinates | Number of loci | Number of polymorphic loci | Proportion of Polymorphic loci (%) | Nei's genetic diversity |
|-----------|----|--------------------|---------------------|----------------|----------------------------|------------------------------------|-------------------------|
| A | 18 | May 2005 | 122°30'E 36°10'N | 265 | 184 | 69.43 | 0.1331 |
| B | 13 | May 2005 | 124°30'E 33°01'N | 250 | 165 | 66.00 | 0.1243 |
| C | 12 | April 2005 | 122°50'E 31°40'N | 222 | 129 | 58.11 | 0.1076 |
| D | 10 | April 2005 | 124°00'E 30°30'N | 236 | 151 | 63.98 | 0.1166 |

**Figure 1.** Sample sites and migration routes for small yellow croaker. Three fishery stocks were also shown in the map. The migration routes for small yellow croaker were following Liu (1962).

has not been investigated genetically until recently.

Amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995) is a PCR-based multilocus fingerprinting technique that combines the strengths and overcomes the weaknesses of PCR-RFLP and RAPD-PCR. AFLP analysis has been used for indirect examination of levels of genetic diversity in several species (Liu et al., 2005; Chen et al., 2005; Kim et al., 2007). The major strengths of the AFLP method include simultaneous screening of a large number of polymorphic loci, high reproducibility due to high stringency of PCR and relative

cost effectiveness (Liu and Cordes, 2004). Moreover, it does not require any prior molecular information about sequences under investigation and is thus especially applicable to species in which the genome sequences are not well characterized, like small yellow croaker. For any species, the success of conservation programs and the creation of effective management policies depend on determining the levels of genetic divergence within and between populations and developing strategies to maintain genetic diversity. Because small yellow croaker is an important commercial fish species, confirmation of whether or not it is a single stock in the East China Sea and Yellow Sea or whether different genetic stocks occur in over its wide distribution would represent a great contribution to conservation policies and population management. The objectives of this study are determination of genetic diversity and intraspecific population differentiation of small yellow croaker in the East China Sea and Yellow Sea using AFLP analysis for which no data are available at present.

MATERIALS AND METHOD

Sampling

Fifty three individuals of small yellow croaker were collected from four geographic locations (A to D) from the East China Sea and Yellow Sea from April 2005 to May 2005 (Figure 1 and Table 1) by the Yellow Sea Fisheries Research Institute through trawl net surveys. According to Liu et al. (1962), location A belongs to North Yellow Sea stock, B and C belong to the South Yellow Sea stock; while D belongs to East China Sea stock. The whole fish was frozen and shipped to the Ocean University of China. Muscle samples were obtained and preserved in 95% ethanol or frozen for DNA extraction after specimen identification and morphological measure.

AFLP analysis

Genomic DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method (Sambrook et al., 1989). DNA was subsequently resuspended in 100 μ L of TE buffer (10 mmol/L Tris-Cl, 1 mmol/L EDTA, PH = 8.0). Procedures of AFLP were essentially based on Vos et al. (1995) and Wang et al. (2000). PCR products were run on 6.0% denaturing polyacrylamide gel electrophoresis (PAGE) for 2.5 h at 50°C on the Sequi-Gen GT Sequencing Cell (Bio-Rad, USA), and finally detected

Table 2. Adaptor and primer sequences used in AFLP analysis.

| Primer adapters | Sequence |
|--------------------------------|--|
| <i>Eco</i> RI-adapter | 5'-CTCGTAGACTGCGTACC-3' 5'-AATTGGTACGCAGTCTAC-3' |
| <i>Mse</i> I-adapter | 5'-GACGTGAGTCCTGAG-3' 5'-TACTCAGGACTCAT-3' |
| Pre-amplification primer | |
| <i>Eco</i> RI | 5'-GACTGCGTACCAATTC-3' |
| <i>Mse</i> I | 5'-GATGAGTCCTGAGTAA-3' |
| Selective amplification primer | |
| E-ACT/M-CAA | 5'-GACTGCGTACCAATTC-3' 5'-GATGAGTCCTGAGTAACAA-3' |
| E-ACG/M-CAC | 5'-GACTGCGTACCAATTCACG-3' 5'-GATGAGTCCTGAGTAACAC-3' |
| E-ACT/M-CTT | 5'-GACTGCGTACCAATTC-3' 5'-GATGAGTCCTGAGTAAC-3' |
| E-ACG/M-CAA | 5'-GACTGCGTACCAATTCACG-3' 5'-GATGAGTCCTGAGTAACAA-3' |

using the silver staining technique modified from Merrill et al. (1979). Sequences of AFLP adapters and primers are listed in Table 2. Four primer combinations (E-ACT/M-CAA, E-ACG/M-CAC, E-ACT/M-CTT and E-ACG/M-CAA) were chosen for AFLP analysis (Table 2).

Data analysis

AFLP bands were scored for presence (1) or absent (0) and transformed into 0/1 binary character matrix. Proportion of polymorphic loci, Nei's genetic diversity and Shannon diversity index were calculated by POPGEN. Similarity indices were calculated using the formula $S = 2N_{ab}/(N_a + N_b)$, genetic distances between individuals were computed using the formula $D = -\ln S$ (Nei and Li, 1979). Genetic relationships among populations were estimated by constructing UPGMA tree based on Nei's genetic distance (Nei, 1978) in Mega 3.0. Population structure of small yellow croaker was investigated using the molecular variance software package (AMOVA) and *F*-statistics in ARLEQUIN 2.000. To test the isolation by distance (Slatkin, 1993), pairwise values of $F_{ST}/(1-F_{ST})$ were plotted against geographical distance (one-dimensional stepping-stone model) between sample sites of small yellow croaker. The strength and significance of the relationship between genetic distances and geographic distances was assessed using reduced major axis regression and Mantel tests.

RESULTS

A total of 304 loci were detected by the four primer combinations, 231 of which were polymorphic (75.99%, Table 3). The average number of bands scored per primer pair was 76, ranging from 57 to 104. The number of polymorphic loci amplified by each primer combination over all populations ranged from 42 to 83, with the average of 57.75 polymorphic loci per primer combination (Table 3).

The population with the highest proportion of polymorphic

loci (69.43%) was population A, whereas that with the lowest value was population C, in which the proportion of polymorphic loci and number of polymorphic loci was 58.11% and 129, respectively. The population with the highest Nei's genetic diversity was also A population, with a value of 0.1331, the lowest Nei's genetic diversity was found in C population, only with a value of 0.1076 (Table 1).

According to ANOVA analysis, overall genetic differentiation among small yellow croaker from the four populations was small but significant ($F_{ST} = 0.0422$ and $P < 0.001$), suggesting significant genetic differentiation among localities. Moreover, pairwise F_{ST} values among populations were also significant ($P < 0.05$), ranging from 0.0303 to 0.0675 (Table 4). These analyses indicated that several distinct populations of small yellow croaker existed in the East China Sea and Yellow Sea. Additionally pairwise F_{ST} analysis indicated the largest genetic difference among populations existed in locations A and D ($F_{ST} = 0.0675$, $P < 0.01$), whereas the difference between B and C was the smallest ($F_{ST} = 0.0303$, $P < 0.01$). Nei's genetic distance analysis also suggested that populations A and D were the most different genetically ($D = 0.0229$), whereas the most similar populations were B and C ($D = 0.0093$) (Table 4). Further, cluster analysis placed populations B, C and D in a group, while population A as another group (Figure 2). Among sample sites for small yellow croaker, a Mantel test indicated a significant relationship ($P = 0.007$, $r = 0.88$) between $F_{ST}/(1-F_{ST})$ and geographic distance indicating isolation by distance (Figure 3), with geographic distance explaining 88% of the variation in genetic differentiation for species.

DISCUSSION

Analysis of genetic diversity and population differentiation is essential for genetic research of various organisms. Marine fish generally show low levels of genetic differentiation among geographic regions due to higher dispersal potential during planktonic egg, larval, or adult history stages coupled with an absence of physical barriers to movement between ocean basins or adjacent continental margins (Hewitt, 2000). However, the present study provided the molecular evidence for the existence of separate small yellow croaker stocks in the East China Sea and Yellow Sea, as the F_{ST} values among four populations were all significant ($P < 0.001$). The AMOVA analysis also supported the significant differentiation among populations of small yellow croaker. The causes of differentiation in marine organisms are not well understood. In marine environments, the geographic structure of populations may be affected by local conditions and species life history. A plausible explanation might be that the gene exchange among populations was limited by some factors such as oceanographic characteristics, geographic distance, and species life history.

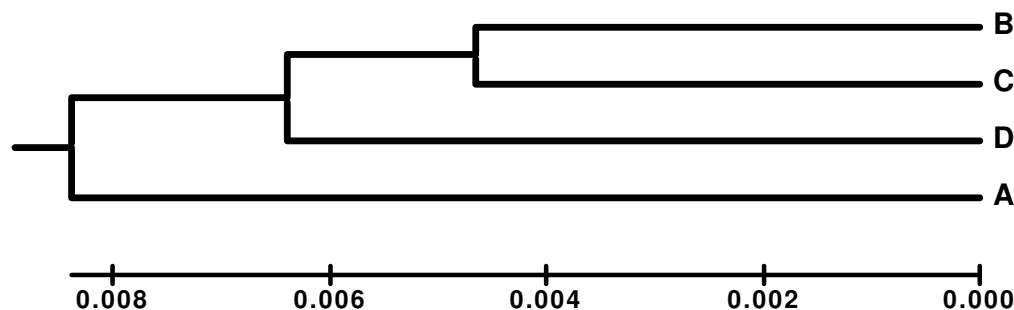
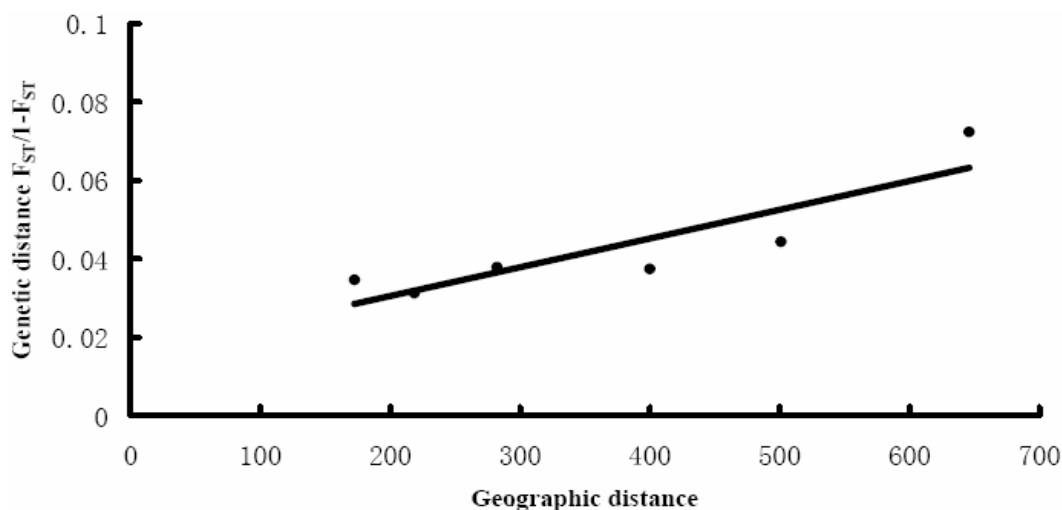
Many marine organisms have pelagic larvae that can potentially interconnect distant populations through dispersal on ocean currents (Liu et al., 2007). The ocean current be-

Table 3. Number of bands generated by primer combinations.

| | E-ACT/M-CAA | E-ACG/M-CAC | E-ACT/M-CTT | E-ACG/M-CAA | Total |
|--------------------------------|-------------|-------------|-------------|-------------|--------|
| Number of loci | 104 | 57 | 73 | 70 | 304 |
| Number of polymorphic loci | 83 | 42 | 47 | 59 | 231 |
| Proportion of polymorphic loci | 79.81% | 73.68% | 64.38% | 84.29% | 75.99% |

Table 4. Nei's genetic distance (above) and pairwise F_{ST} (below) between populations.

| | A | B | C | D |
|---|---------|---------|---------|--------|
| A | | 0.0128 | 0.0146 | 0.0229 |
| B | 0.0361* | | 0.0093 | 0.0139 |
| C | 0.0425* | 0.0303* | | 0.0117 |
| D | 0.0675* | 0.0365* | 0.0335* | |

*Significant $P < 0.05$.**Figure 2.** UPGMA cluster analysis based on Nei's genetic distances among four populations.**Figure 3.** Plot of pairwise estimates of $F_{ST}/(1 - F_{ST})$ and geographic distance between samples of small yellow croaker.

tween the Yellow and the East China seas consists of inflow from the East China Sea to the Yellow Sea along the west coast of Korea (Yellow Sea Warm Current), and outflow of water from the Yellow Sea to the East China Sea along the China coast (China Coastal Current) (Li, 2000).

Previous studies have revealed that the ocean currents in the East China Sea and Yellow Sea facilitate the dispersal of marine larvae among distant populations (Yu et al., 2005; Han et al., 2008b). So the ocean currents might be not responsible for the significant genetic differentiation in small

yellow croaker.

Generally, in our study more geographically related populations were more genetically similar due to gene flow among populations. The UPGMA cluster analysis based on the Nei's genetic distance indicated the samples from the A were obvious separated from other sites, while populations B, C and D were clustered in a group. The pairwise F_{ST} analysis also indicated that a significant difference existed in population A and other three subpopulations ($P < 0.001$). Therefore, the population A was more different genetically from other populations. To further investigate the correlation between the geographic distance and genetic distance, the isolation by distance test (Slatkin, 1993) was conducted in this study. The plot of $F_{ST}/(1-F_{ST})$ and geographic distance revealed a strong pattern of isolation by distance in this species, indicating that population subdivision among localities of small yellow croaker was mainly due to the geographic distance (88%). Marine environments are often seen as open habitats in which isolation by distance is the main mechanism that may promote genetic differentiation among populations. Patterns of isolation by distance are often established over long periods through equilibrium between gene flow and drift (Slatkin 1993). Isolation by distance in small yellow croaker indicated this species is at genetic equilibrium under dispersal and genetic drift.

Besides geographic distance, the migratory behavior including the different migration routes and different over-wintering grounds, and different mating period in the East China Sea and Yellow Sea might also be responsible for the significant genetic differentiation among populations of small yellow croaker. In previous study, three stocks (North Yellow Sea stock, South Yellow Sea stock and East China Sea stock) were identified throughout the range of small yellow croaker based on the species breeding migration routes and over-wintering grounds (Liu, 1962). The over-wintering ground for North Yellow Sea stock is located in 33°30'-35°30'N, 127°00'-125°00'E; in March, the adults migrate from the over-wintering ground north to the Haizhou Bay, Laizhou Bay, Bohai Bay for spawning. For South Yellow Sea stock, the over-wintering ground is located in 32°00'-34°00'N, 123°00'-126°00'E; the adults migrate from this over-wintering ground west to Yangtze Rivers estuary for spawning in March, the migration distance is no longer 300 sea miles. The southern coastal waters of Zhejiang Province is the over-wintering ground for the East China Sea stock, the Zhoushan archipelago is the main spawning areas for this stock (Table 1; Seikai National Fisheries Research Institute, 2001). Our results were partly consistent with this previous study, supporting at least three stocks in small Yellow croaker. However, the significant genetic differentiation between locations B and C might supported that there were endemic branch tribes of *L. polyactis* in South Yellow Sea stock. Hu et al. (2005) reported that there were four endemic branch tribes of *L. polyactis* in the South Yellow Sea stock, supporting our results. These migration behaviors might predispose small

yellow croaker to genetic structuring along its geographical distribution, although the species showed strong larval dispersal ability.

Genetic analysis of fish species in the East China Sea and Yellow Sea is still few in number, which can be used for comparison to our present study. Genetic study on the *Nibea albiflora* revealed no significant genetic structure in China coastal waters by mtDNA and AFLP markers, suggesting high gene flow among populations (Han et al., 2006; Han et al., 2008b). The Ocean currents in the East China Sea and Yellow Sea were responsible for the lack of genetic structure in *N. albiflora*. A study on the redlip mullet, *Chelon haematocheilus* in the Northwestern Pacific, found no significant genetic structure between the Yellow and the East China seas, although there were significant genetic differences between the three marginal seas of Northwestern Pacific (the Sea of Japan, East China Sea and South China Sea) (Liu et al., 2007). Recent range expansion and insufficient time to attain migration-drift equilibrium were reasons for the lack of phylogeographical structure in redlip mullet. However, similar to our study, AFLP analysis of Japanese Spanish mackerel *Scomberomorus niphonius* in China coastal waters also revealed significant genetic differentiation among populations in the East China Sea and Yellow Sea (Shui et al., 2008). The explanation for significant genetic differentiation in this species might result from migration behavior. MtDNA analysis of white croaker *Pennahia argentata* in the East China Sea and Yellow Sea revealed two geographic groups, with the similar geographic distribution of small yellow croaker (Han et al., 2008c). This geographic structure of white croaker was partly due to the biological characteristics of this species.

Although sample size from each geographic site in this study was limited, specimens were collected from different geographic stocks. This might be sufficient to generate the preliminary data on genetic diversity and population differentiation of small yellow croaker in the East China Sea and Yellow Sea. Moritz (1994) proposed the concept of a management unit (MU), which was defined as a conservation unit that had statistically significant divergence in allele frequencies (nuclear or mitochondrial). Therefore, the small yellow croaker in the Yellow Sea and East China Sea should be considered to be at least four management units, which provides a guideline for further effective conservation and management of the species and would help greatly in understand.

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