SHORT COMMUNICATION

Mitochondrial ATPase 6/8 genes to infer the population genetic structure of silver pomfret fish *Pampus argenteus* along the Indian waters

P. R. Divya¹, A. Gopalakrishnan², V. S. Basheer¹, Raja Swaminathan¹, C. Mohitha¹, Linu Joy¹, Raj Kumar¹, P. Manoj³, and J. K. Jena⁴

¹National Bureau of Fish Genetic Resources Cochin Unit, CMFRI Campus, Cochin, India, ²Central Marine Fisheries Research Institute, Cochin, India, ³Rajiv Gandhi Centre for Biotechnology, Trivandrum, India, and ⁴National Bureau of Fish Genetic Resources, Lucknow, India

Abstract

Silver pomfret, *Pampus argenteus* is an economically important seafood species. The fishery resource of pomfret in Indian waters shows a dwindling catch since the last few years and the pomfrets caught were mostly undersized which calls for immediate attempts for management of resources. An accurate definition of population structure is important for management of this species. The genetic stock structure of *P. argenteus* distributed along Indian coast was identified using analysis of 842 bp of complete ATPase 6/8 genes of mitochondrial DNA. Altogether, 83 silver pomfret (*P. argenteus*) collected from 4 locations along Indian coast (Gujarat, Kerala, Tamil Nadu and West Bengal) were sequenced. Twenty four haplotypes were identified among 83 individuals with haplotype diversity (0.87) and nucleotide diversity (0.0025). The significant pair-wise FST and AMOVA values, between samples from West Bengal (east coast) and other locations along the west coast (Gujarat and Kerala) indicated the occurrence of distinct population structure in silver pomfret along the coast.

Introduction

Silver pomfret (*Pampus argenteus*, family Stromateidae) is a high valued marine food fish inhabiting pelagic waters. It has an extensive geographical distribution from the East China Sea to Southeast Asia, Indian Ocean, Arabian Gulf and the North Sea (Davis & Wheeler, 1985; Froese & Pauly, 2011). The species play an important role in the fisheries of Kuwait, Iran, China, India, Korea, Malaysia, Thailand and Japan. It attains a maximum size of about 60 cm (Fischer & Bianchi, 1984). Along the Indian coast, silver pomfret landings were about 28,000t, during 2009–10, which formed about 0.9% of the marine fish landings in India (CMFRI, 2011). The landings are mainly from Gujarat and Maharashra coasts along the north west and Orissa and West Bengal on the north east coast. The fishery resource of pomfrets in Indian waters showed a steady decline in catch since 1990s and the pomfrets caught were mostly undersized. In India, silver pomfret fishery faces both recruitment as well as growth overfishing. The Central Marine Fisheries Research Institute, India has recommended a size restriction of minimum legal weight of 300 g for the pomfrets exported from India to curb the growth overfishing. Restriction of dolnet was suggested to minimize recruitment overfishing. Across the nations, including India, attempts to breed the species in captivity, culture them in marine cages and ranching of this species are in progress.

Despite the importance of this species as a valuable marine resource, little is known about genetic diversity and population structure of silver pomfret in the Indian waters. Failure to detect population units coupled with local overfishing will ultimately lead to decline in populations. In order to devise adequate conservation and management strategies for a declining species, it is important to investigate its population history, genetic diversity and geographical partitioning throughout its natural distribution range. These features may be directly assessed through genetically controlled markers (Hutching, 2000). mtDNA marker, ATPase gene is comparatively fast evolving and is extremely useful in assessing population structure and levels of connectivity in fish species (McGlashan & Hughes, 2001; Ovenden & Street, 2003). For the effective management of the silver pomfret species along the Indian coast, the genetic stock of *P. argenteus* distributed along Indian coast (including Arabian Sea and Bay of Bengal) was investigated using complete sequence information of ATPase 6/8 genes.

Materials and methods

Specimens of silver pomfrets were collected from four different geographical locations along the coastal waters of India. Fresh wild samples (*N*= 83) were obtained from commercial trawl landings from Arabian Sea (*N*= 43) including Veraval, Gujarat (20°53′N 73°26′E) in the north and Cochin, Kerala (9°59′05.80″N, 76°10′28.62″E) towards south and from Bay of Bengal (*N*= 40) including Kolkata, West Bengal (21°53′46.23″N, 88°05′29.65″E) in the north and Chennai, Tamil Nadu (13°22′41.38″N, 80°17′57.27″E) towards south. Sampling procedures were performed at actual site of collection and
samples were transported to the laboratory in 95% ethyl alcohol until used for genomic DNA extraction. Total genomic DNA was isolated from muscle tissue/fins using a salting out method following Sambrook et al. (1989). To analyze the yield and quality, extracted DNA was electrophoresed through a 0.8% agarose gel containing ethidium bromide (5 µg/ml) and final DNA concentration was estimated by optical density (OD) reading using spectrophotometer (SPECORD 205, Analytik, Jena, UK) set at 260 nm. The extracted DNA samples were mostly concentrated; therefore, samples were diluted with sterile double distilled water to reach appropriate concentrations (20 ng/µl) for PCR reactions.

The complete ATPase 6 and 8 genes were amplified by PCR (Applied Biosystems, Carlsbad, CA) using universal Primers ATPL82L8331: 5'-AAAGCRTYRGCCTTTAAGC-3' and COH1H9236: 5'-GTTAGTGTGTCAKGGGCTTGRTC-3' (http://nmg.si.edu/bermlab.htm). Amplification were conducted in 25 µl volume containing 2.5 µl of 10X PCR buffer (100 mM Tris, pH 8.8, 500 mM KCl, 25 mM MgCl2, 0.8%(v/v) (Fermentas), and 1.5 units of Taq DNA polymerase (Fermentas), 200 µM of each dNTPs (dATPs, dCTPs, dGTP, dTTPs) (Fermentas), 20 pmol of each primer and 20 ng of genomic DNA. The thermal profile used to amplify ATPase gene consisted of an initial denaturation of 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were stored at 4 °C. For each sample, 3 µl of PCR product were electrophoresed through 1.5% agarose gels following ethidium bromide staining, and visualized under UV illumination in the Gel-Doc system (BIO-RAD, Molecular Imager, Gel Doc™ XR, Stockholm, Sweden). Molecular weights were determined using StepUp™ 100 bp DNA markers (Genetyx™, Bangalore, India). Products were labeled using the Big Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems Inc.) and sequenced bidirectionally using ABI 3730 capillary sequencer (Applied Biosystems Inc.) following the manufacturer's instructions.

Once amplification and sequencing of the mtDNA ATPase region were completed, subsequent analyses were conducted to access the genetic diversity of silver pomfret in these regions. DNA sequences were aligned and edited using BioEdit version 5.0.9 (Carlsbad, CA) (Hall, 1999). Sequence polymorphism was analysed, genetic divergence values within and between populations were estimated, and a neighbor joining (NJ) tree was constructed for all the haplotypes according to Kimura 2-parameter (K2P) model using PHYLIP ver 3.7a (Felsenstein, 2009) and MEGA version 5 (Tokyo, Japan) (Tamura et al., 2011). Robustness of the inferred tree was evaluated using bootstrap analysis on 10,000 replications. Nucleotide diversity (π) and haplotype diversity (h) were estimated using DnaSP5.0 (Barcelona, Spain) (Librado & Rozas, 2009). Population structure was evaluated using the analysis of molecular variance (AMOVA) model in the Arlequin ver 3.5 software (Bern, Switzerland) (Excoffier & Lischer, 2010). To evaluate the demographic history of the P. argenteus, the neutrality Tajima’s D statistic, Fu’s FS and mismatch distribution analysis was run using Arlequin 3.5 software. The genetic identity of silver pomfret from Kolkata and other three locations from Indian waters was also investigated using mitochondrial cytochrome oxidase 1 sequence data.

**Result**

**Characteristics of ATPase 6/8 genes**

Complete ATPase 6/8 gene of 842 bp was sequenced for 83 silver pomfret samples. Out of a total of 842 bp of mitochondrial gene amplified, 168 bp fragments were of ATPase 8 and 684 bp of ATPase 6, with an overlapping region of 10 bp from 159–168. ATG was the start codon in ATP 6/8 genes. TAA was the stop codon in ATP 8 genes and incomplete stop codon of TA was found in ATPase 6 genes. A total of 24 haplotypes were identified among 83 individuals of 4 populations. Of the 842 bp in the mitochondrial sequences, 57 sites (6.7%) were variable among individuals. Among the 57 polymorphic sites observed, 7 were singleton variable sites and 50 were parsimony-informative. The pattern of nucleotide substitution was biased in favour of transitions over transversions in variable sites, including 18 transitions and 3 transversion changes. The overall transition/transversion bias is $R = 5.392$. As expected, most of the changes occurred at the third codon, resulting in always synonymous substitutions. The A/T base contents were higher than the C/G base contents among the sequences examined and the mean number of nucleotide composition in the species was $A = 29\%$, $T = 31\%$, $C = 27\%$, and $G = 13\%$. Estimates of the genetic divergence (P-distance) among haplotypes, based on Kimura 2 parameter ranged between 0–0.057.

Samples from all four locations selected for this study were characterized by low values of nucleotide diversity (0.0025). Number of polymorphic sites, nucleotide and haplotype diversities for different populations estimated using DnaSP 5.0 software is shown in Table 1. Mean haplotype diversity ($H_d$) was found to be 0.87 with variance 0.0004 $\pm$ 0.023. Greatest genetic diversity with higher number of haplotype and nucleotide diversities was observed for samples from west coast (Arabian Sea) compared to east coast (Bay of Bengal). Sixteen haplotypes were specific to Arabian Sea and 6 specific to Bay of Bengal. Six were specific to Kerala, 8 to Gujarat, 2 to Tamil Nadu and the remaining 4 were unique to West Bengal. Mean number of polymorphic loci was estimated to be seven. All haplotypes representing ATPase gene were submitted to the GenBank (Accession No. JX 293025–293034) and (JX 460972–JX 460982, JX 944218–944220).

**Population genetic diversity and genetic divergence**

Analysis of molecular variance of silver pomfret mtDNA ATPase6/8 sequences in four different regions of the Indian sea was attempted using Arlequin ver 3.5 (Table 2). The significant pairwise $F_{ST}$ values and the AMOVA values between samples from West Bengal and other locations indicated the occurrence of distinct population structure in silver pomfret between north east (West Bengal) and remaining populations. Pairwise $F_{ST}$ using Arlequin 3.5 Software is given in Table 3. AMOVA within four

<table>
<thead>
<tr>
<th>Collection location</th>
<th>Sample number</th>
<th>No. of polymorphic sites</th>
<th>Haplotype ($h$)</th>
<th>Haplotype diversity ($H_d$)</th>
<th>Nucleotide diversity ($\pi$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kolkata (West Bengal)</td>
<td>25</td>
<td>3</td>
<td>4</td>
<td>0.23</td>
<td>0.0003</td>
</tr>
<tr>
<td>Veraval (Gujarat)</td>
<td>18</td>
<td>13</td>
<td>10</td>
<td>0.93</td>
<td>0.004</td>
</tr>
<tr>
<td>Cochin (Kerala)</td>
<td>25</td>
<td>10</td>
<td>7</td>
<td>0.87</td>
<td>0.002</td>
</tr>
<tr>
<td>Chennai (Tamil Nadu)</td>
<td>15</td>
<td>5</td>
<td>3</td>
<td>0.45</td>
<td>0.002</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>8.7</td>
<td>0.0025</td>
<td></td>
</tr>
</tbody>
</table>
populations revealed that out of total variations only 7% was contributed due to variation within population, however, 92% was attributed to difference among populations and population structuring revealed high and significant \( F_{ST} \) values of 0.92 at \( p < 0.001 \). The historical demographic expansion in \( P. \) argenteus populations examined by \( D \) test of Tajima revealed significant negative values (−0.28−1.73), indicating a history of genetic bottleneck followed with subsequent population expansion. The demographic expansions for the four silver pomfret populations along Indian coast were estimated using Tajima’s \( D \), Fu’s \( Fs \) and mismatch distribution using Arlequin 3.5 software. Tajima’s \( D \) and Fu’s \( Fs \), corresponding \( F \) values and mismatch distribution parameter estimates are indicated in Table 4.

Phylogeographic analysis

The phylogeographic analyses conducted using the NJ method revealed two lineages indicating the distribution of four populations (Figure 1). Lineage I is the major lineage that contains specimens in all three sampling regions (Gujarat, Kerala and Tamil Nadu) and is supported with a bootstrap value more than 53%. In contrast, lineage II is a minor lineage that is supported with a bootstrap value 99%. Interestingly, West Bengal population didn’t share any haplotypes with other population indicating that West Bengal stock can be genetically distinct.

The haplotype network (Figure 2) based on statistical parsimony clearly showed that all individuals from West Bengal clustered into one clade, whereas samples from Gujarat, Kerala and Tamil Nadu formed a unit cluster. As the samples from Kolkata showed a distinct clade without any haplotype sharing from other regions, the genetic identity of specimens collected from Kolkata were doubtful. Silver pomfret collected from Gujarat, Kerala Tamil Nadu and Kolkata (West Bengal) were checked with taxonomic keys and were found to be morphologically identical. Hence to rule out the possibility of cryptic speciation among the samples collected from Kolkata, the genetic identity of five individuals each of \( P. \) argenteus collected from all the four locations were revealed through amplification of cytochrome oxidase I gene. Mitochondrial cytochrome oxidase I gene (COI) is reasonably well conserved within species and appears to possess a greater phylogenetic signal than any other mitochondrial gene and the evolution of this gene is rapid enough to allow discrimination of closely allied species (Hebert et al., 2003). All haplotypes representing COI gene were submitted to the Gen Bank (Accession No. KF 373001–KF 373012). The samples collected from Kolkata revealed average pairwise genetic distance of 1.22% with samples collected from other localities of Indian waters. Each haplotype was connected with others by 1 to 4 mutational steps. Population specific haplotypes was found in all the populations. Maximum number of population specific haplotypes was found in samples of Gujarat, followed by Kerala.

Discussion

Population genetic analysis have been considered to be the best tool for evaluating genetic divergence and for obtaining information on the conservation genetics of a species (Crandall et al., 1999). Silver pomfrets being a commercially important species and as the fishery is in a dwindling stage along Indian coast, a better understanding of fish population is important for its effective fisheries management. Fishery biologists use the stock concepts as a basis to manage commercially important marine organisms. In the present study, ATPas 6/8 gene sequences were used to investigate the genetic diversity and population structure of \( P. \) argenteus population in the Arabian Sea and Bay of Bengal.

Low pairwise \( F_{ST} \) values was reported for samples from Gujarat, Kerala and Tamil Nadu indicating low genetic differentiation between silver pomfret populations; however statistically significant levels of genetic structuring were found when the samples from these areas where compared to samples from West Bengal (North east coast of India \( p < 0.001 \)). Mostly, lower levels of genetic differentiation are reported in marine fishes due to higher dispersal potential during planktonic egg, larval or adult by life history stages, coupled with an absence of physical barrier to movement between ocean basins. The continuously changing coastal current pattern also may result in exchange of larvae along the Indian coast, resulting in low population genetic differentiation (Mandal et al., 2012). Sun et al., (2012a, b) found high genetic divergence between pomfrets from Mumbai, India (Arabian Sea) and Ngao, Thailand (Bay of Bengal) and concluded that the mainland of India blocks genetic exchange between Bay of Bengal and Arabian Sea. However, our studies revealed that Tamil Nadu samples (Bay of Bengal) was genetically closer to specimens from Cochin, Kerala (Arabian Sea), and specimens from Kolkata, West Bengal (Bay of Bengal) formed a genetically distinct cluster. NJ tree analysis revealed two lineages among the

Table 2. Analysis of molecular variance of silver pomfret mtDNA ATPase region sequences in four different regions of the Indian sea using Arlequin. *(\( p < 0.001 \)).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>Percentage of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>3</td>
<td>767.2</td>
<td>12.46 Va</td>
<td>92.97</td>
</tr>
<tr>
<td>Within populations</td>
<td>79</td>
<td>74.5</td>
<td>0.94 Vb</td>
<td>7.03</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>828.329</td>
<td>13.41</td>
<td></td>
</tr>
<tr>
<td>Fixation Index</td>
<td></td>
<td></td>
<td></td>
<td>FST : 0.9296*</td>
</tr>
</tbody>
</table>

Table 3. Pairwise \( F_{ST} \) between four populations of \( P. \) argenteus at *(\( p < 0.001 \)).

<table>
<thead>
<tr>
<th>Location</th>
<th>West Bengal</th>
<th>Gujarat</th>
<th>Kerala</th>
<th>Tamil Nadu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.96*</td>
<td>0.97*</td>
<td>0.98*</td>
</tr>
</tbody>
</table>

Table 4. Tajima’s \( D \) and Fu’s \( Fs \) tests, corresponding \( p \) value and mismatched distribution parameter estimates for \( P. \) argenteus population.

<table>
<thead>
<tr>
<th>Location</th>
<th>Tajima’s ( D )</th>
<th>Fu’s ( Fs )</th>
<th>Mismatch distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( D )</td>
<td>( Fs )</td>
<td>Tau 0.0 0.1 0.3 SSD 0.0026 0.42 0.34</td>
</tr>
<tr>
<td>Kolkata</td>
<td>−1.73</td>
<td>0.02</td>
<td>−3.4 0.0 0.03 109 0.0119 0.34 0.05</td>
</tr>
<tr>
<td>Veraval</td>
<td>−0.35</td>
<td>0.02</td>
<td>−2.0 0.01 2.3 1.23 109 0.0119 0.34 0.05</td>
</tr>
<tr>
<td>Cochin</td>
<td>−0.28</td>
<td>0.02</td>
<td>−2.6 0.02 3.0 0.03 9.1 0.0049 0.57 0.02</td>
</tr>
<tr>
<td>Chennai</td>
<td>−0.84</td>
<td>0.01</td>
<td>−2.0 0.01 1.0 0.00 9999 0.2983 0.01 0.34</td>
</tr>
</tbody>
</table>

\( Tau \) – units of mutation time; \( 0_0 \) – 0 before population growth; \( 0_1 \) – 0 after population growth; SSD – sum of square deviations; \( Rg \) – Rangedness index.
northern Bay of Bengal region may be playing an influential role (et al., 2012). Such strikingly varied oceanographic features in the profile of Bay of Bengal from that of Arabian Sea (Jaswal, 2009). Low mean sea surface temperature, presence of freshwater received from the peninsular rivers, weak winds and the continental shelf pattern differentiates four regions of silver pomfret populations. None of the haplotypes from West Bengal coasts were shared by haplotypes from other regions. The recirculation cells noted by Durand et al. (2009) at the Western boundary of Bay of Bengal may be the cause for this genetic differentiation, at least in part. Low mean sea surface temperature, presence of freshwater received from the peninsular rivers, weak winds and the continental shelf pattern differentiates the profile of Bay of Bengal from that of Arabian Sea (Jaswal et al., 2012). Such strikingly varied oceanographic features in the northern Bay of Bengal region may be playing an influential role for the large scale differences in the genetic diversity of silver pomfret populations of West Bengal coast. However, this hypothesis can be proved only by a correlative study of these factors with larval/adult migration pattern of this fish. The average genetic divergence in silver pomfret using ATPase 6/8 genes was within the range 0–0.57 and this exceeds the usual intra specific range and it has to be suspected that P. argenteus distributed in the Indo-West Pacific region might have diverged at the population/sub species level.

Haplotype diversity for the entire population was high. The origin of new haplotypes as observed from high haplotype diversity is possible as ATPase 6/8 gene is reported to have high mutation rate of 1.3% per million years (My) (Sun et al., 2012a).

It needs special mention that populations along west coast are genetically more diverse than east coast as evidenced by more number of haplotypes. Similar results were reported in pomfrets by earlier workers (Sun et al., 2012a). A negative Tajma’s D as observed in this study signifies an excess of low frequency polymorphisms indicating population size expansion after a bottleneck (Peng et al., 2009). Based on different combinations of haplotype diversity (h) and nucleotide diversity (π) magnitudes of mtDNA sequence, marine fishes can be classified into four categories defined by Grant & Bowen (1998). According to their reports, small nucleotide (π<0.5) and smaller haplotype (h<0.5) diversity (category I), which was found in pomfrets from east coast hints recent population bottle neck or founder event by single or few mitochondrial lineages. Similarly, small nucleotide (π<0.5) and larger haplotype (h>0.5) diversity (category II), hints population bottle neck followed by rapid growth and accumulation of mutations which was found in pomfret samples from west coast. Similar pattern of nucleotide and haplotype diversities were noted in marine species like bill fishes (h = 0.68–0.85, π = 0.0018), Spanish sardines and haddocks (h = 0.79–0.98, π = 0.0029) (Grant & Bowen, 1998). High haplotype diversity (Hd) and low nucleotide diversity (π) values indicates a population bottleneck followed by rapid population growth and accumulation of mutations, which is consistent with the result of mismatch distribution analysis and neutrality tests. Although little is known about the evolutionary history of these silver pomfret fishes from Indian coast, their genetic architecture indicates periods of low effective population size among samples within recent millennia. Hence greater attention should be made for conservation and sustainable exploitation of this species.

P. argenteus populations in Indian waters are being exploited at a higher level than the optimum (Ghosh et al., 2009). The length at first capture (Lc) for pomfret was found to be 8.20 cm, which was very low when compared to the length at first maturity in silver pomfrets (Lm) of 27.5 cm indicating that majority of them were caught before they matured and spawned at least once in their life. Maximum Sustainable Yield being lower than annual catch and a higher exploitation ratio also suggests that the stock is under more fishing pressure warranting immediate decrease in fishing effort for the optimal management of this species (Boopendranath et al., 2012). Intense fishing in certain habitats cause the elimination of distinct, locally adapted stocks, resulting in loss of diversity and the adaptive potential of that species (Menezes & Parulekar, 1998). Hence, responsible fishing regimes are required in silver pomfret resource management along the Indian waters, so as to avoid extinction of a regional population. Hatchery development and stock identified rehabilitation programme also will help to conserve this dwindling resource. Hatchery production of silver pomfret fries are successful (James & Almatar, 2007).

Based on the present results, it seems intuitive that the wild populations and brood stocks of (i) Gujarat, Kerala, Tamil Nadu and (ii) West Bengal coasts of India should be managed
separately. From a resource conservation and management perspective, the conclusion of the study also strongly indicates the need for adoption of stock specific rehabilitation programmes for *P. argenteus* from Indian waters.

**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. We gratefully acknowledge the financial support by the Department of Biotechnology, Government of India, New Delhi and facilities provided by Indian Council of Agricultural Research, New Delhi.

**References**


demographic history of *Pampus argenteus* in the Indo-West Pacific
inferred from mitochondrial cytochrome b sequences. Biochem Syst
Ecol 43:54–63.
(*Pampus argenteus*) in the Arabian Sea, Bay of Bengal and South
China Sea as indicated by mitochondrial COI gene sequences. J Appl
Ichthyol 1–5.
MEGA5: Molecular Evolutionary Genetics Analysis using Maximum
Likelihood, Evolutionary Distance, and Maximum Parsimony