ONLINE RESOURCES

Characterization of microsatellite markers in silver pomfret, *Pampus argenteus* (Perciformes: Stromateidae) through cross-species amplification and population genetic applications

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[Mohitha C., Joy L., Divya P. R., Gopalakrishnan A., Basheer V. S., Koya M. and Jena J. K. 2014 Characterization of microsatellite markers in silver pomfret, *Pampus argenteus* (Perciformes: Stromateidae) through cross-species amplification and population genetic applications. *J. Genet*. **93**, e89-e93. Online only: http://www.ias.ac.in/jgenet/93/OnlineResources/e89.pdf]

Introduction

Pampus argenteus (Euphrasen 1788) is one of the commercially important marine food fish in India, having high value and demand in both domestic and export market. Pomfrets in Indian waters showed a steady decline in catch due to capture of undersized, juvenile fishes. Baseline information on the genetic stock structure is important for conservation and sustainable harvest of this species. Fish stock structure and genetic variation can be evaluated/identified through microsatellite molecular markers. An alternative for finding polymorphic microsatellites is cross-species amplification between genetically closely related species.

Silver pomfret, *P. argenteus* (Euphrasen 1788) (Perciformes: Stromateidae) is widely distributed throughout the Indo-west Pacific region: from the Persian Gulf to Indonesia, Japan, west Korea and southwest Korea, and eastern parts of China (Zhao *et al.* 2011). Pomfrets are schooling, pelagic, medium-sized fishes inhabiting shallow waters (Fischer and Bianchi 1984). They are highly relished table fish and demand high value in domestic and export market. The species attain a maximum size of about 60 cm (Fischer and Bianchi 1984). Population genetic analysis has proven to be the best tool for evaluating genetic divergence, and for obtaining information about the conservation genetics of a species (Crandall *et al.* 1999). Microsatellites are considered as one of the best markers for population genetic analysis (Weber and May 1989).

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Microsatellites contain short-repeated DNA sequences that give short arrays at each locus and act as codominant markers. These markers are abundant and dispersed relatively evenly throughout eukaryotic genomes (Tóth *et al.* 2000). Codominant inheritance and fast and easy assay strategies of microsatellites adds to their acceptance as good molecular markers for genotype (Weber and May 1989).

Developing species-specific microsatellites is a time-consuming procedure. Fortunately, flanking region similarities between closely related species mean that considerable effort can be saved by using conserved regions for polymerase chain reaction (PCR) primer design (Sun and Kirkpatrick 1996). An alternative for finding polymorphic microsatellites is cross-species amplification between genetically closely related species that require less time to develop. Number of attempts have been made to study the cross-species amplification of microsatellite loci in fishes (Scribner *et al*. 1996; Gopalakrishnan *et al*. 2004; Lal *et al*. 2004). In this study, we developed seven microsatellite markers through cross-species amplification from *P. cinereus* which can be helpful in revealing the genetic structure among the stocks of *P. argenteus.*

Materials and methods

Specimens of silver pomfret were obtained using commercial trawlers from Arabian Sea 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 including Kolkata, West Bengal (21°53′46.23″N, 88°05′29.65″E) in the north (45 from each location).

Keywords. silver pomfret; microsatellites; cross-priming; population genetic structure.

Sampling was done between October 2011 and June 2013, and the average length and weight of the individuals were 17 cm and 340 g, respectively. Specimens were transported to the laboratory on ice and preserved in molecular grade ethanol. Total genomic DNA was isolated from muscle tissue/fins using a salting out method following Sambrook *et al*. (1989).

PCR amplification was conducted in 25 μL volume containing 2.5 μL of 10 × PCR buffer (100mM Tris, pH 8.8, 500 mM KCl, 25 mM $MgCl_2$, 0.8% (v/v) (Fermentas, Burlington, Canada) and 1.5 U of *Taq* DNA polymerase (Fermentas), 200 μM of each dNTPs (dATPs, dCTPs, dGTP and dTTPs) (Fermentas), 20 pmol of each primer, 6-FAM labelled (Wei *et al*. 2009) and 20 ng of genomic DNA. The amplification conditions were 95°C for 7 min followed by 34 cycles at 94°C for 30 s, annealing temperature for 30 s and 72°C for 30 s, with a final extension of 72°C for 20 min. PCR products were stored at 4°C. After amplification, 8 μL of PCR products were electrophoresed on nondenaturing polyacrylamide (19:1, acrylamide: bis-acrylamide) gels (size 10 × 10.5 cm, Amersham Bioscience, Piscataway, USA). The gel concentration was optimized according to allele size for better resolution. Electrophoresis was done at 4°C with 1 × TBE buffer for 4.30 h at 150 V. The gels were silver stained (Silver Staining, Amersham Biosciences) to visualize microsatellite loci and allele patterns; and DNA ladder (*Msp*I cut p^{BR} 322 DNA) was run in every gel. Cross-priming standardization was done with 10 individuals for getting optimum annealing temperature. The primers having amplified products were again evaluated with more number of individuals ($n = 45$). The PCR products were genotyped on an ABI 3730 sequencer (Applied Biosystems, Carlsbad, USA) and analysed with GeneMapper ver. 4.0 software (Applied Biosystems).

The number of alleles and allele frequencies at each locus were calculated using Genetix (Belkhir *et al.* 1997). The mean number of alleles per locus; observed and expected heterozygosities (H_o and H_e) and percentage of polymorphic loci for overall and each population were calculated with GenePop ver. 3.3 (Raymond and Rousset 1995). Polymorphism information content (PIC) was calculated using the PIC calculator (Nagy *et al*. 2012). Exact *P* tests for conformity to Hardy–Weinberg equilibrium (HWE) (probability and score test) were performed by the Markov Chain method using GenePop ver. 3.3 (Raymond and Rousset 1995) with parameters, dememorization = 1000; batches = 10 and iterations = 100. *F* statistics (F_{IS} and F_{ST}): the coefficient of genetic differentiation (F_{ST}) and the inbreeding coefficient (F_{IS}) were estimated through estimator of Weir and Cockerham (1984) using GenePop. Pairwise F_{ST} was calculated using FSTAT ver. 2.9.3.2 (Goudet 1993).

An analysis of molecular variance (AMOVA) to examine the amount of genetic variation partitioned within and among populations was carried out using GenAIEx ver. 6.5 (Peakall and Smouse 2012). By using the software FigTree ver. 1.4.0, an UPGMA dendrogram was constructed based on Nei (1987) unbiased genetic distance estimates to depict the genetic relationships among the populations of *P. argenteus*. Presence of null alleles was tested to rule out false homozygotes. The expected frequency of null alleles was calculated using Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004) and all the genotypes of the loci showing deviation from HWE were tested for null alleles.

Results and discussion

Of the 10 heterologous primer pairs tested, seven primers successfully amplified and showed homologous loci in *P. argenteus*. The optimum annealing temperature (T_a°C) observed in *P. argenteus* differed from that reported in the *P. cinereus* for respective primer pair. The repeat motif, size range and annealing temperature obtained in case of *P. argenteus* are provided in table 1. Sequencing of amplified products revealed the presence of repeat motifs (accession nos: JX87221–JX87227). All seven amplified loci were perfect in nature and contained dinucleotide repeat sequence.

The parameters of genetic variation at each locus and over all loci differed between the samples from three locations (table 2). Observed and expected heterozygosities ranged from 0.2222 to 0.9333 and 0.3176 to 0.9680. The probability test revealed that the observed allele frequencies in all the loci did not show significant deviation ($P<0.05$) from HWE. Usmani *et al*. (2003) reported the same in some of the catfishes. The average observed heterozygosity and expected heterozygosity of all populations were 0.7513 and 0.8725, respectively. PIC values ranged from 0.7339 to 0.9625. The total number of alleles per locus ranged from 20 (PAP 119) to 44 (PAP 72) and the allele size ranged from 94 to 308 bp.

Pairwise F_{ST} analysis showed significant values (table 3). The coefficient of genetic differentiation value ranged from 0.05418 (between Kerala and West Bengal) to 0.06198 (between Gujarat and West Bengal). Samples from more geographically distant locations had significant differences in values for F_{ST}. Other samples from closer geographic distances had smaller or insignificant F_{ST} values. The values ranged from −0.038 for the locus PAP85 to +0.327 for locus PAP 119. In most of the loci, the value of F_{IS} was found to deviate significantly from zero, indicating a deficiency of heterozygotes.

The AMOVA based on microsatellite data also indicated significant genetic differentiation among *P. argenteus* populations (overall $F_{ST} = 0.416$; $P<0.0001$). This analysis showed 81% variation inside populations. The percentage of variation among populations (6%) and among regions (13%) was low but significant. The genetic differentiation exhibited through pairwise F_{ST} was in concordance with the results of AMOVA analysis. The genetic differentiation may be explained with low level of migration among the populations of both the coasts of India.

Nei's measure of genetic distance (1987) between Gujarat and West Bengal populations were the highest (0.438),

Table 1. Characteristics of amplified microsatellite loci in *P. argenteus.*

Locus	Accession number	Repeat motif	Primer sequences	T_m (°C)	Size range
PAP15-2	JX87221	$(AC)_n$ $(TG)_n$	F: GCA AGC CTC TAA TTC ACT CC R: CTG CCT CTG TTT CTT CCT G	57	181–219
PAP72	JX87222	$(GT)_n$	F: ACA CCC TAA ACA TGT CAG CAT C R: CAC AGC AGG AAT CAC TCA AAT A	53	204–308
PAP85	JX87223	$(AC)_n$ $(CA)_n$	F: CGC ACA AAT CTC CAC CTA R: ATA CAG AGA CAG GGG AAG CCA A	50	93–147
PAP106	JX87224	$(GT)_n$ $(TG)_n$	F: ATT CCA AAA CCG TGG CTA T R: GCA GAC ACC ATC CCA GAC T	52	237–275
PAP119	JX87225	$(TG)_n$	F: CCC TCT ATC CTT CAA ACC CT R: TGA CTC TCA CCT CTG CCA TC	56	234–270
PAP189	JX87226	$(GT)_n$	F: ATT CAA TAA CAA CTC CAC C R: TGT CTC ACC ACT CTT CAG C	56	138–190
PAP230	JX87227	$(AG)_n$ $(TG)_n$ $(GT)_n$	F: CCG TCC TCT TCC CTG TAA R: GCC AAG CAA GCC TCT AAT	57	153–183

Table 2. Parameters of genetic variability for each microsatellite loci in *P. argenteus* samples from three locations.

Locus	Location	No. of alleles at each locus	H_o	H_e	PIC for each loci
PAP15-2	Gujarat	28	0.84444	0.95506	0.9482
	Kerala	25	0.91111	0.92559	
	West Bengal	24	0.62222	0.87266	
PAP72	Gujarat	27	0.86667	0.95431	0.9625
	Kerala	34	0.71111	0.96804	
	West Bengal	25	0.73333	0.93233	
PAP85	Gujarat	15	0.80000	0.84769	0.8466
	Kerala	15	0.93333	0.84419	
	West Bengal	17	0.82222	0.83620	
PAP106	Gujarat	26	0.93333	0.93858	0.9357
	Kerala	21	0.93333	0.93683	
	West Bengal	21	0.82222	0.92834	
PAP119	Gujarat	15	0.91111	0.87840	0.9201
	Kerala	14	0.48889	0.92060	
	West Bengal	12	0.42222	0.90787	
PAP189	Gujarat	21	0.64444	0.84494	0.9165
	Kerala	26	0.75556	0.92959	
	West Bengal	17	0.71111	0.91685	
PAP230	Gujarat	13	0.75556	0.76829	0.7339
	Kerala	21	0.93333	0.90062	
	West Bengal	6	0.22222	0.31760	

Table 3. Pairwise Fisher's F_{ST} (θ) between different populations of *P. argenteus* using microsatellite markers (significance level $P < 0.05$).

Population	Gujarat	Kerala	West Bengal
Gujarat	0.00000	0.02866	0.06198
Kerala	0.02866	0.00000	0.05418
West Bengal	0.06198	0.05418	0.00000

Table 4. Genetic distances among populations of based on *P. argenteus* Nei's (1987) similarity coefficient.

Population	Gujarat	Kerala	West Bengal
Gujarat	–		
Kerala	0.286	–	
West Bengal	0.438	0.312	–

while that between Gujarat and Kerala populations were the lowest (0.286) (table 4). The dendrogram based on Nei's measure of genetic distance (1987) showed two major clusters: Gujarat and Kerala populations both were in one clade, and West Bengal population in the other clade (figure 1).

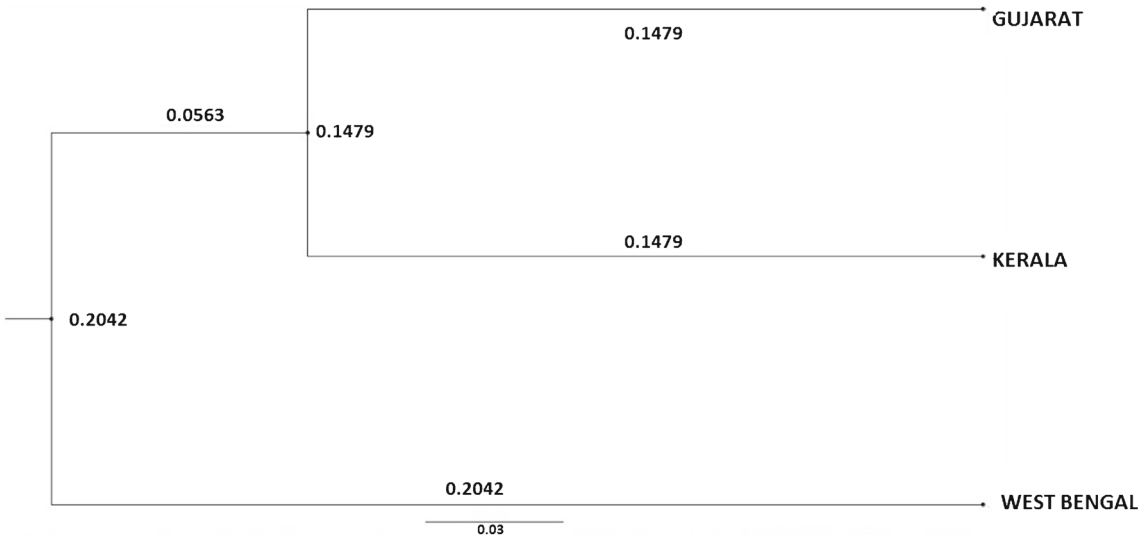

Figure 1. Dendrogram of genetic diversity among *Pampus argenteus* populations based on Nei's (1987) similarity coefficient and UPGMA method. The scale bar at the bottom represents 0.03 units of genetic distance.

Many microsatellite loci are extremely fast rate of repeats evolution and quite conservative in their flanking regions. For population genetic analysis, cross-species amplification has been a useful tool to identify set of markers without developing specific primers for each species. Primers developed for a species by this method have been effectively tested for cross-species amplification on its related species (Gopalakrishnan *et al*. 2004; Lal *et al*. 2004; Ma *et al*. 2011). From this study, it is inferred that the availability of the cross polymorphic loci presented here will be helpful for identifying the stock structure and diversity assessment of *P. argenteus* and other related species.

The optimum annealing temperature to get scorable band in *P. argenteus* differed from that reported for the respective primer pair in the resource species. All microsatellite sequences were perfect and the base pair falls within the range similar to the loci developed in *P. cinereus*. In this study genetic variation of *P. argenteus* was examined in three different locations using seven polymorphic microsatellite loci, which indicated the level of genetic diversity in number of alleles and heterozygosity and thus gives a clear idea about the genetic connectivity between these three populations. F_{ST} values can be used to determine the degree of genetic differentiation among populations of *P. argenteus*.

According to Wright (1978), there are four guidelines for interpreting F_{ST} values: 0–0.05 for little genetic differentiation, 0.05–0.15 for moderate genetic differentiation, 0.15–0.25 for large genetic differentiation and above 0.25 for very large genetic differentiation. The pairwise F_{ST} values between populations from east coast and west coasts of India, i.e. between West Bengal and Gujarat (0.06198) and West Bengal and Kerala (0.05418) were significantly different from zero ($P<0.0001$). This suggests significant but moderate level of genetic differentiation among the populations of this species along both coasts of India. The higher rate of mutation and polymorphism of DNA markers result in high level of population differentiation (Rousset and Raymond 1995). The genetic distance between West Bengal and Gujarat (0.06198) was the highest which may be in concordance with geographic distance. The populations of Kerala and Gujarat (west coast of India) coast lie in the same cluster in the dendrogram computed using Nei's measure of genetic distance (1987).

The various estimates provided strong evidence that different genetic stocks exist among three populations of *P. argenteus* distributed along Indian waters. Excess homozygosity observed through positive F_{IS} values in the genetic analysis among these three populations gives a hint that silver pomfret population along Indian waters is overexploited.

In conclusion, the present study developed seven polymorphic microsatellite loci that can be utilized for detecting genetic diversity in populations of *P. argenteus*. This study offered the basement for regulation of wild caught fisheries to protect natural populations and will be useful for stock enhancement programmes of this important fishery resource along Indian waters. Based on the present results, there are two stocks of silver pomfret in Indian waters (i) Gujarat and Kerala; and (ii) West Bengal, and the two stocks need separate management strategies for sustainable fisheries development.

Acknowledgements

The authors acknowledge DBT (Department of Biotechnology) for providing support and facilities for carrying out the above work. The technical help rendered by Raj Kumar is duly acknowledged.

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Received 14 February 2014, in revised form 19 March 2014; accepted 15 April 2014
Published online: 19 August 2014