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Identification and characterisation of microsatellite markers in narrow barred Spanish mackerel *Scomberomorous commerson* (Lacepede, 1800)

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ABSTRACT

Narrow barred Spanish mackerel, *Scomberomorus commerson* (Lacepede 1800) is one of the commercially important fish in Indian waters. For sustainable management of the species, it is necessary to understand the genetic stock structure of the species. Accurate delineation of genetic stock structure can be achieved using nuclear microsatellite markers. A study was carried out to identify and validate polymorphic microsatellite markers in *S. commerson* using information available from closely related fish species. The study could identify 10 polymorphic microsatellite loci which can be effectively utilised for identifying genetic stock structure of the species. Validation of the identified loci was also done by testing the loci in natural populations of the species. The findings of the present study will be useful in devising stock-specific management measures for conservation and sustainable utilisation of this commercially important fish species in Indian waters.

Keywords: Cross species amplification, Genetic diversity, Polymorphic microsatellite markers, Population genetic structure, *Scomberomorus commerson*

Introduction

Narrow barred Spanish mackerel, *Scomberomorus commerson* (Lacepede 1800), (Family: Scombridae) is one of the commercially important fish species in Indian waters having high demand in the domestic and the export markets. It is an epipelagic species, distributed widely across Red Sea, Indo-West Pacific and the Mediterranean Sea (Eschmeyer and Fricke, 2016). *S. commerson* forms an important fishery in Indian waters with a total catch of 39,362 t (CMFRI, 2015). Considering overexploitation and increased fishing pressure on populations over its distribution range in the Indian Ocean, this species is categorised as "Near Threatened" in the IUCN Redlist assessment (Collette *et al.*, 2011). Considerable uncertainty remains regarding the stock structure and the total catch of *S. commerson* in the Indian Ocean region. Currently, no information on quantitative stock assessment is available for this species for the entire Indian Ocean, except for the report on preliminary stock status by Indian Ocean Tuna Commission (IOTC, 2013).

Stock structure data is vital for devising any stock specific management for sustainable utilisation and conservation of fish species. Genetic stock structure can be efficiently accessed through nuclear polymorphic microsatellite markers (Wright and Bentzen, 1994). Microsatellites are short tandemly arrayed repeats of 1-6 nucleotides that occur at high frequency in the nuclear genomes of most taxa. These repeat regions are flanked by regions of non-repetitive unique DNA sequences (Tautz, 1989). Developing polymorphic microsatellites for a particular species is costly and time consuming. Fortunately, flanking region similarities between closely related species reduces considerable effort for developing polymorphic microsatellite markers for population genetic analysis (cross-species amplification). This cost-effective alternative for developing polymorphic microsatellite markers is comparatively easier and has been successfully carried out in many fish species for population studies (Lal *et al.*, 2004; Mohitha *et al.*, 2014; Kathirvelpandian *et al.*, 2014). In the present study, development of 11 microsatellite markers in *S. commerson* through cross-species amplification was carried out from closely related species such as *Scomber australasicus, Scomber japonicus, Scomberomorus cavalla* and *Rastrelliger kanagurta*. The study also attempted to validate the utility of microsatellite markers in revealing the population genetic structure of commercially important fish species.

Materials and methods

Sample collection and DNA isolation

Tissue samples of *S. commerson* were taken from specimens collected from landing centers of Vishakhapatnam in Andhra Pradesh and Chennai in Tamil Nadu on the east coast of India and Kochi in Kerala and Veraval in Gujarat on the west coast of India, during December 2013 to September 2014 (Table 1). Twenty tissue samples each were collected from all the sampling locations and preserved in 95% ethyl alcohol. DNA isolation was carried out following Miller *et al.* (1988) with minor modifications.

Table 1. Sampling sites with geographical co-ordinates

Location	Geographical co-ordinates	Sample size
Vishakhapatnam (Andhra Pradesh)	17.6883° N, 83.2186° E	20
Chennai (Tamil Nadu)	13.0839° N, 80.2700° E	20
Kochi (Kerala)	9.9312° N, 76.2673° E	20
Veraval (Gujarat)	20.9000° N, 70.3700° E	20

Identification of polymorphic microsatellite markers

Microsatellite information of the closely related species were collected from GenBank (National Centre for Biotechnology Information - NCBI; www.ncbi.nlm.nih.gov) and other published resources. For cross-species amplification, a total of 28 microsatellite primers (Table 2) comprising 10 from *S. australasicus* (Tang *et al*., 2009), 8 from *S. japonicus* (Cha *et al.*, 2010), 4 from *S. cavalla* (Broughton *et al.*, 2002) and 6 from *R. kanagurta* (Candy *et al.*, 2013, unpublished) were used.

PCR amplification

PCR amplifications were carried out following standard protocol in 25 µl reactions containing 2.5 µl of 10 × PCR buffer (100 mM 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 (dATP, dCTP, dGTP and dTTP) (Fermentas), 20 pmol of each primer and 20 ng of genomic DNA. The amplification conditions were: 95^{O}C for 5 min followed by 34 cycles at 94^{O}C for 30 sec, annealing (Table 3) for 30 sec and 72^{O}C for 30 sec, with final extension of 72^{O}C for 10 min. PCR products were stored at 4^{O}C. After amplification, 8 µl of PCR products were electrophoresed on 8% non-denaturing polyacrylamide (19:1, acrylamide: bis-acrylamide) gels (size 10×10.5 cm, Amersham Bioscience, Piscataway, USA). The gels were silver stained (Silver Staining kit, Amersham Biosciences) to visualise microsatellite loci and allele patterns using standard DNA ladder (pBR 322/MspI digest). Cross-priming standardisation was carried out with the samples collected from different locations for optimising annealing temperature. The PCR products of polymorphic microsatellite loci were sequenced in ABi 3730 DNA sequencer (Applied Biosystems) in the sequencing facility to confirm the occurrence of the repeat units. Genotyping of polymorphic microsatellite loci was carried out manually.

Table 2. Details of microsatellite primers used for cross-priming in *S. commerson*

Resource species	Locus code	Accession no.	References
Scomber australasicus	Sa42	EF494258	Tang *et al.* (2009)
	Sa2068	EF494259	
	Sa2344	EF494261	
	Sa2599	EF494262	
	Sa2657	EF494263	
	Sa2683	EF494264	
	Sa2769	EF494265	
	Sa2770	EF494266	
	Sa2873	EF494267	
	Sa3337	EF494268	
Scomber japonicus	KSj3	EF109782	Cha *et al.* (2010)
	KSj4	EF109783	
	KSj12	EF109784	
	KSj14	EF109785	
	KSj18	EF109786	
	KSj22	EF109787	
	KSj26	EF109789	
	KSj27	EF109789	
Scomberomorous cavalla	Sca37		Broughton *et al.* (2002)
	Sca44		
	Sca8		
	Sca30		
Rastrelliger kanagurta	Raka36		Candy *et al.* (2013)
	Raka45		
	Raka26		
	Raka46		
	Raka1		
	Raka2		

Data analysis

The parameters like number of alleles per locus (N_A), observed (Ho) and expected (He) heterozygosities and exact P-tests for conformity to Hardy-Weinberg Equilibrium (probability and score test) using Markov Chain method were estimated using GENEPOP version 3.3d (Raymond and Rousset, 1998) with parameters, dememorisation = 1000; batches = 10 and iterations = 100; and based upon a null hypothesis of random union of gametes. The significant criteria were adjusted for the number of simultaneous tests using sequential Bonferroni technique (Rice, 1989). 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 Hardy-Weinberg equilibrium were tested for presence of null alleles. The inbreeding coefficient (F_{IS}) was estimated through estimator of Weir and Cockerham (1984) using GENEPOP version 3.3 d (Raymond and Rousset, 1998). An analysis of molecular variance (AMOVA) was also carried out to examine the amount of genetic variation partitioned within and among populations using Gen Alex version 6.5 (Peakall and Smouse, 2012). Samples collected from east coast and west coast were considered as separate groups for analysis.

Results and discussion

Cross-species amplification can be used as an ideal tool to identify a set of markers without developing specific primers for each species studied, which helps in saving money and time (Galbusera *et al.*, 2000). Flanking region similarities or conserved regions between closely related species helps to reduce the effort required for designing primers in other marine fish species (Kathirvelpandian *et al.*, 2014; Mohitha *et al.*, 2014). The success of cross-species amplification is higher for species with long generation time, mixed or out crossing breeding system and smaller genome size compared to the resource species (Barbara *et al*., 2007). A total of 28 microsatellite primer pairs were tested for cross-species amplification and out of these, 11 primers were successfully amplified in *S. commerson*. The percentage of amplification rate was 39.2%. Out of the 11 primers, *Raka 36* was found to be monomorphic, and was consequently excluded from the analysis. The characteristics of the developed primers are given in Table 3. The optimum annealing temperature was different in *S. commerson* from that reported in the resource species.

Criteria for cross-amplification success are amplification of band in the expected size range and presence of polymorphism. The primers which got amplified in the present study were in the same range of resource species. The degree of polymorphism was determined at each locus. The range of annealing temperature was from 48^{O}C to 59^{O}C, similar to earlier studies (Gopalakrishnan *et al.*, 2004; Mohitha *et al*., 2014). The repeat motif, size range and annealing temperature obtained in *S. commerson* are given in Table 3. Sequence information of the PCR products confirmed the microsatellite repeat motifs and the sequences were submitted to Genbank (Table 3). Most of the loci (9 loci) were perfect dinucleotide repeats. Remaining were tetra and trinucleotide repeats.

Table 3. Characteristics of selected microsatellite loci in *S. commerson*

Locus	Repeat motif in resource species	Primer sequences	*S. commerson*			
			Annealing temperature Ta (°C)	Repeat motif	Size range (bp)	Accession no.
Sa 2769 S. australasicus	$(AC)_{18}$	F-TTTTGCATTTTAAGCAGCTCAGT R-GTGGTGGACACACACAGATTCAT	56	(AC)9	221-259	KP120688
Sa 2657 S. australasicus	$(CA)_{16}$	F-TGTCAGAGATGTAGCACATACGG R-AGCATTATCTGGTGCTGTAAGGA	56	(CG)8 (AG)6	240-328	KP120686
Sa 2068 S. australasicus	$(GGA)_{9}$	F-CAAGACATGACAGTAGGACATTGAC R-AGATTGGGAGTTTGTAGGGGTAATA	56	(AG)8	146-176	KP120684
Sa2770 S. australasicus	$(CA)_{13}(CCT)_{3}$	F-AGAAATGAAAAGGGCTTTAAGGA R-ACTGAGCTGCTTAAAATGCAAAA	56	(AC)15 (CCT)4	195-285	KP120689
S ca44 S. cavella	(CTCG)2 CTAT $(CTGT)_{5}$	F-ATGGCCAAATGGCACATAATCA R-GGGCAGCTCCATGGGTCTGAGT	58	(TCTG)8	169-175	KP120692
Sca-37 S. cavella	$(TG)_{8}$ AG $(TG)_{4}$ AG $(TG)4_{3}$	F-GCG CCGTGACTTTTTATTGCTC R-CAACAATTAGTCGCAGCCCTAG	58	(GT)10	154- 168	KP120692
Raka -36 R. kanagurta	$(AGTG)_{10}$	F-TGTGTCTACACAGACAGAGGG R-TAATCACTCTCGCTCGCTCG	58	(AC)9	101-153	KP120683
Sa2873 S. australasicus	$(CA)_{17}$	F- TCACACTGTGCAATAATCACTCC R- TATTTGAGCAGCCTCAAGAAGAG	59	(TC)11	224-312	KP120690
Sa2683 S. australasicus	$(TG)_{17}(GA)_{15}$	F-CTGAGACACAGTGATGTTTGTCC R- TGCATATAGCACGAAAAAGTCAT	52	(GT)8 (TC)8	223-297	KP120687
Sa2344 *S. australasicus*	(GT)51	F-CACAAAAGCTGCTTAACACACTCT R- TCACACTCAGCAAAATGAAGTTTC	48	(TG)20	118-180	KP120684
KSj-26 S. japonicus	$(GT)_{13}$AT $(GT)_{3}$	F-GGAGCATTTGACAACACTTAC R-AGTCAGTTTTTGGTGGATGAG	55	(GT)7	174-196	KP120682

The number of alleles per locus ranged from 9 (Sa 2873) to 26 (Sa 2769) with a mean of 15.5. The allele size was between 138 bp (Sa 2657) and 300 bp (KSj 26). High microsatellite allele variation observed in the study was comparable with previous findings in marine fishes including Atlantic cod (8-46 alleles per locus) (Bentzen *et al.*, 1996), silver pomfret (13-17 alleles per locus) (Archangi *et al.*, 2013) and Atlantic salmon (5-41 alleles per locus; Nikolic *et al.*, 2009). The expected and observed heterozygosities per locus and the estimated Nm (gene flow) ranged from 0.441 (Sa2068) to 0.875 (Sa2769); 0.175 (Sa2873) to 0.875 (Sa2769) and 0.859 (Sa 2683) to 4.636 (Sa 2770) respectively (Table 4). The observed allele frequencies in most of the loci did not show significant deviation (p>0.05) from Hardy-Weinberg equilibrium after sequential Bonferroni correction, which was similar to results in earlier studies (Kathirvelpandian *et al.*, 2014; Mohitha *et al.*, 2014). Wright's (1978) fixation index (F_{IS}) is a measure of heterozygote deficiency or excess (inbreeding co-efficient) and the values of F_{IS} for all populations ranged from -0.150 for the locus Sa 2657 to + 0.694 for locus Sa 2873. In most of the loci, the value of F_{IS} was found to deviate significantly from zero, indicating a deficiency of heterozygotes. The presence of null alleles was checked using different algorithms in Micro Checker software and the estimated null allele frequency at all loci was not significant indicating the absence of null alleles.

Table 5. F-Statistics and estimates of Nm over all populations for each locus in *S. commerson*

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
SA2873	0.694	0.745	0.168	1.235
KSj26	0.691	0.743	0.169	1.225
Sa2344	0.358	0.49	0.206	0.966
Sa2683	0.188	0.371	0.225	0.859
Sa2769	0	0.068	0.068	3.415
SCA44	-0.012	0.15	0.16	1.312
SCA37	0.4	0.472	0.12	1.835
SA2657	-0.15	-0.053	0.084	2.72
SA2068	0.207	0.318	0.141	1.528
SA2770	0.264	0.302	0.051	4.636
Mean	0.264	0.361	0.139	1.973
SE	0.089	0.084	0.018	0.391

Table 4. Summary of observed and expected number of alleles and heterozygosity statistics of 10 microsatellite loci in *S.commerson*

		Sa 2873	KSj 26	Sa 2344	Sa 2683	Sa 2769	Sca 44	Sca 37	Sa 2657	Sa 2068	Sa 2770
Na	Mean	5.25	5.5	7	7	12.75	5.75	8.75	6.25	4	6.75
	SE	0.479	1.19	1.354	1.472	0.946	0.479	1.25	0.25	1.08	0.854
Ne	Mean	2.513	3.468	4.252	4.121	9.013	4.043	5.225	3.875	2.226	4.568
	SE	0.411	0.703	0.89	1.442	1.497	0.51	0.971	0.565	0.529	1.003
Ho	Mean	0.175	0.20	0.45	0.55	0.875	0.75	0.475	0.825	0.35	0.55
	SE	0.025	0.082	0.065	0.132	0.048	0.087	0.131	0.118	0.155	0.119
He	Mean	0.571	0.646	0.701	0.678	0.875	0.741	0.791	0.718	0.441	0.748
	SE	0.065	0.11	0.102	0.082	0.029	0.031	0.032	0.056	0.16	0.053

Deviations from Hardy-Weinberg Equilibrium are often reported in marine fishes (Scribner *et al.*, 1996; Selkoe and Toonen 2006). The F_{ST} values of each loci for all populations ranged from 0.051 (Sa 2770) to 0.225 (Sa 2683) with a mean value of 0.139 (Table 5).

The mode of developing microsatellite markers through cross-species amplification was tested and validated in the current study. The results obtained from the present study authenticate that the microsatellite markers obtained through cross priming from closely related species are apt for population genetic analysis in *S. commerson*. In future, these primers can be effectively utilised for the stock identification of *S. commerson* for the successful management of the species.

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