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Development and validation of microsatellite markers in a protandrous fish species *Eleutheronema tetradactylum* (Shaw, 1804) through cross-species amplification

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ABSTRACT

The four fingered threadfin *Eleutheronema tetradactylum* (Shaw, 1804) is a prioritised species for mariculture in India. Their demand in the domestic markets is rapidly growing. Genetic stock structure analysis of fish populations is an important aspect from fisheries management perspective. The present study was conducted to develop microsatellite primers through cross-priming to elucidate the genetic structure of *E. tetradactylum*. A total of 13 polymorphic microsatellite markers were developed from the resource species, Pacific salmon *Polydactylus sexfilis.* The observed mean and the effective number of alleles were found to be 11.962 and 6.927 respectively. The mean of observed heterozygosity (Ho) and expected heterozygosity (He) values obtained were 0.784 and 0.798 respectively. These new microsatellite markers can be used as effective tools for studying genetic disparity as well as for elucidating evolutionary relationships among *E. tetradactylum* populations.

Keywords: Cross-priming, *Eleutheronema tetradactylum,* Indian waters, Polymorphic microsatellites

Introduction

The four fingered threadfin *Eleutheronema tetradactylum* (Shaw, 1804) (Family: Polynemidae), commonly known as Indian salmon is a fast growing predatory fish species. This neretic-pelagic-species mainly distributed in the Indo-West Pacific, typically occur in shallow, turbid inshore waters, often in large numbers (Mukhopadhyay *et al.,* 1995). They are protandrous hermaphrodites (Shihab *et al.,* 2017). Indian salmon is a highly valued table fish and their market demand is increasing (Hena *et al.,* 2011). Knowledge of genetic stock structure is essential for sustainable management of the fishery and conservation of the species (Welch *et al.,* 2002). *E. tetradactylum* is subjected to extreme exploitation in south-east Asian countries and in northern Australia (Thirumaraiselvi and Thangaraj, 2015).

Development of molecular markers coupled with the use of advanced statistical tools aid in determining variances and similarities between stocks and individuals (Okumus, 2003). Microsatellites are neutral markers which can be effectively used to evaluate genetic variation and population structure in different populations (Schlotterer *et al.,* 1997). These are extremely polymorphic and are perfect markers for forensic studies, parentage assignment, conservation and population genetic studies (Jarne and Lagoda, 1996). Co-dominant inheritance and simple assay strategies make microsatellites good molecular markers for genotyping (Abdul-Muneer, 2014). As development of species-specific microsatellite markers is time consuming and expensive, cross-species amplification is used as a cost-effective method (Moore *et al.,* 1991; Primmer *et al.,* 1996). Population genetic studies in many fish species have been successfully carried out using cross-species amplification (Umino *et al.,* 2013; Kathirvelpandian *et al.,* 2014; Mohitha *et al.,* 2015). The present study, attempted to develop and validate microsatellite markers through cross-priming method, which could be helpful in revealing the genetic structure of different stocks of *E. tetradactylum*.

Materials and methods

Sample collection and DNA isolation

Individuals of *E. tetradactylum* were collected from four geographical locations along the coastal belts of India, *i.e.,* Gujarat, Kerala, Tamil Nadu and Andhra Pradesh during October 2014 to June 2016 (Table 1). A total of 20 individuals were collected from each location and preserved in 95% ethanol. The total genomic DNA was isolated from the muscle tissues/fin clips using DNeasy blood and tissue kit (Qiagen). DNA concentration was assessed using BioSpectrometer®basic (Eppendorf).

Table 1. Sampling sites with geographical coordinates

Sampling location	No. of indiviuals	Geographical coordinates
Gujarat (Veraval)	20	20°54'00.0"N; 70°22'12.0"E
Kerala (Cochin)	20	9°58'12.0"N; 76°16'48.0"E
Tamil Nadu (Mandapam)	20	13°03'45.3"N; 80°17'17.0"E
Andhra Pradesh (Vishakhapatnam)	20	17°40'00.4"N; 83°17'24.8"E

Identification of polymorphic microsatellite markers and polymerase chain reaction

GenBank data were used for collecting microsatellite information from a related species. Twenty five microsatellite primers from Pacific threadfin, *Polydactylus sexfilis* were used for cross priming (Pan and Yang, 2010; Wang *et al.,* 2010). PCR was carried out following standard conditions using EmeraldAmp GT PCR Master Mix (Takara) with 20 pmol of each primer and 20 ng of genomic DNA. The amplification conditions were: 95°C for 5 min followed by 34 cycles at 94°C for 30 s, annealing at 51-58°C for 30 s, extension at 72°C for 30 s followed by final extension at 72°C for 10 min. After the amplification, 10 µl of the PCR products were electrophoresed on 8% non-denaturing polyacrylamide (19:1, acrylamide: bis-acrylamide) gels. The gels were silver stained (Silver Staining Kit, Amersham Biosciences) to visualise microsatellite loci and allele patterns with standard DNA ladder (pBR 322/ MspI digest). In order to optimise the annealing temperature, cross-priming standardisation was done with the samples collected from different locations. Amplified 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.

Data analysis

The number of alleles per locus (Na), effective number of alleles (Ne), observed heterozygosity (Ho) and expected heterozygosity (He) were calculated using GenAlex version 6.5 (Peakall and Smouse, 2012). The polymorphic information content (PIC) for each locus was calculated according to Nagy *et al.* (2012). Hardy-Weinberg Equilibrium deviations were tested with exact p values being estimated using the Markov chain algorithm with 10,000 dememorisation steps, 100 batches and 1,000 iterations. Also, the data was checked for genotype linkage disequilibrium between pairs of loci in a population based on null hypothesis (genotypes at one locus is independent of genotypes at other loci) and the inbreeding coefficient (F_{IS}) was estimated through the estimator of Weir and Cockerham (1984) using GENEPOP version 4.1.1 (Rousset, 2008). The significant criteria were adjusted for the number of simultaneous tests using sequential Bonferroni technique (Rice, 1989). MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.,* 2004) was used for testing the presence of null alleles.

Results and discussion

The success rate of developing specific markers through cross-priming is related to factors like evolutionary divergence between the resource and the target species (Galbusera *et al.,* 2000). Cross-species amplification has been effectively carried out in many fish species (Gopalakrishnan *et al.,* 2004; Ma *et al.,* 2011; Mohitha *et al.,* 2014). We screened a total of 25 polymorphic primers selected from the resource species, *P. sexfilis* (Wang *et al.,* 2010; Pan and Yang, 2010) and 13 primers generated successful amplicons in *E. tetradactylum*. Microsatellite pattern of locus Pse82 (bp 150) in *E. tetradactylum* is shown in Fig. 1. DNA sequencing confirmed that all the 13 microsatellite loci contain repeat sequences. The percentage of cross-amplification was 52% and the remaining 48% either failed or feebly amplified. In the present study, the optimum annealing temperature was found to be 51 to 58°C which differed from earlier reports on *P. sexfilis* (Pan and Yang, 2010; Wang *et al.,* 2010). Zardoya *et al.* (1996) and Galbusera *et al.* (2000) studied the requirement of PCR condition standardisation in cross-amplification tests. All the 13 amplified loci contain perfect dinucleotide repeats (Table 2). However, the type of repeat motif in the resource species and *E. tetradactylum* differed in some loci. This may be due to the enormously fast rates of repeat evolution that may differ among loci, keeping the highly conservative flanking regions unchanged as reported by Zardoya *et al.* (1996).

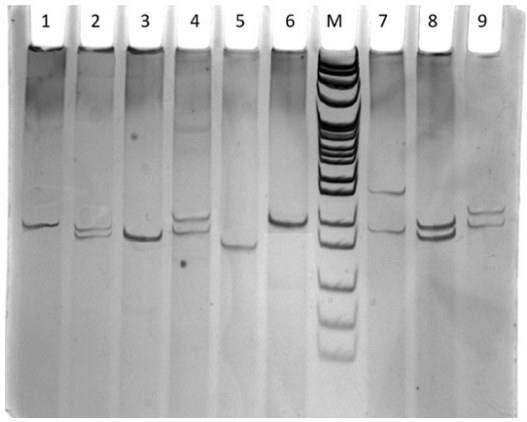

Fig. 1. Microsatellite pattern of locus Pse82 (bp 150) in *E. tetradactylum*
Lanes 1 to 9 - Amplified samples; M - Marker

Table 2. Primers used for amplifying 13 microsatellite loci in Indian Salmon, *Eleutheronema tetradactylum*

Locus	Repeat Motif (5'-3')	Primer sequences (5'-3')	Tm (^{O}C)	Size (bp)	GenBank Accession No.
Pse8	(AC)n	F: AGTGCCCGTGCAACCATACC R: GACTTGGGGTTCAATGTCGT	51	286-320	MH623030
Pse9	(AC)n	F: GCGTCTCCTCCAAACTCAT R: -CCGTTGTTGTGCTGAAAATC	56	180-218	MH623031
Pse24	(GT)n	F: CAGACATTCCTCCCTCACAA R: ATGCCTCCAGCAAAACTCAA	52	120-144	MH623032
Pse27	(AC)n	F: TGACATATTGCGTGGGATTG R: AATGGTCACCTGCTGGGAAG	58	184-200	MH623033
Pse34	(GA)n	F: TGAAACCGAAGCCGAGACCA R: TCACTACCTGTTGACCTTTA	56	200-220	MH623034
Pse35	(CT)n	F: TTGAGACTGCCCAACTCTAT R: TAAAGGCTGTGGATGAGTGC	51	190-240	MH623035
Pse82	(CA)n	F: TGAAAGGCTCAACAAGTA R: ATCAGCAGCAGAATCTATG	52	100-154	MH623036
Ptd11	(CA)n	F: AAGATCCTCGTGCCACCTCA R: GTTATTTAGAGTTGTCACCG	53	98-126	MH623037
Ptd15	(AT)n	F: GCACCCACAAACATGCTCAAAT R:TGTGACGGTTTCTCCATTG	54	120-150	MH623038
Ptd16	(CA)n	F: CGCAATGGAGAAACCGTCA R: GATGTTCACCTTGGCTCTC	52	100-124	MH623039
Ptd20	(TG)n	F: AAAGTCTCCCAACAGATGAT R: ATGCCAATTACAAGAGTCGA	54	192-210	MH623040
Ptd57	(GT)n	F: AAAAGGCTGTGAGTGAATGA R: GACCTGGTGCTTTATTACTT	56	186-206	MH623041
Ptd84	(TG)n	F: TGTCAGTCAGTCGACGGTG R: CGTAGGAACAGACGGAGCA	53	202-222	MH623042

MICROCHECKER test showed lack of 'null allele' and 'large allele' drop out and all other loci were included in the analysis. No linkage disequilibrium was observed in between any pair of polymorphic loci for any population ($p>0.05$). So it was assumed that allelic differentiation at microsatellite loci could be measured independently across 13 loci for all the four populations. All the primers used in the present study were highly polymorphic and the PIC values ranged from 0.658 (Pse 8) to 0.986 (Ptd 57) (Table 3). The observed mean and effective number

Table 3. Parameters of genetic variability for each microsatellite loci in *E. tetradactylum* samples from four locations

Locations		Pse 8	Pse 9	Pse 24	Pse 27	Pse 34	Pse 35	Pse 82	Ptd11	Ptd15	Ptd16	Ptd20	Ptd57	Ptd84
Gujarat	Na	4	9	12	13	20	15	9	8	11	19	12	16	15
	Ne	1.363	5.970	7.018	5.797	14.286	10.390	3.493	3.828	6.612	8.421	2.462	7.018	8.081
	Ho	0.800	0.900	0.850	1.000	0.900	0.950	0.550	0.750	0.650	0.900	0.550	0.600	0.900
	He	0.766	0.833	0.858	0.828	0.830	0.904	0.614	0.739	0.849	0.881	0.594	0.658	0.876
Kerala	Na	4	8	12	14	14	15	8	7	10	21	6	7	14
	Ne	1.606	5.556	7.407	5.096	18.605	9.091	3.524	3.828	5.926	16.327	3.333	2.204	6.838
	Ho	0.550	0.850	0.800	0.900	0.800	0.950	0.600	0.650	0.700	0.800	0.900	0.600	0.900
	He	0.578	0.820	0.865	0.804	0.946	0.890	0.616	0.739	0.831	0.939	0.700	0.546	0.854
Tamil Nadu	Na	2	12	11	17	14	15	10	5	10	17	6	12	13
	Ne	1.406	5.755	7.143	11.765	10.390	12.121	3.704	4.301	6.061	7.018	1.626	7.339	7.143
	Ho	0.450	0.550	0.850	0.850	0.750	0.850	0.600	0.950	0.850	0.900	0.550	0.950	0.900
	He	0.489	0.826	0.860	0.915	0.904	0.918	0.730	0.768	0.835	0.858	0.585	0.864	0.860
Andhra Pradesh	Na	5	8	11	22	20	11	9	8	10	16	11	19	11
	Ne	1.231	5.755	7.692	13.793	17.778	6.504	4.678	5.263	5.926	10.256	2.139	11.594	7.767
	Ho	0.700	0.700	0.850	0.850	0.950	0.950	0.700	1.000	0.800	0.900	0.500	0.650	0.950
	He	0.688	0.826	0.870	0.928	0.944	0.846	0.786	0.810	0.831	0.903	0.533	0.914	0.871
PIC		0.658	0.899	0.965	0.952	0.899	0.945	0.854	0.799	0.982	0.907	0.954	0.986	0.856

of alleles were found to be 11.962 and 6.927 respectively. Allelic patterns across populations of *E. tetradactylum* are represented in Fig. 2. The mean of observed heterozygosity (Ho) and expected heterozygosity (He) values were found to be 0.784 and 0.798 respectively. Significant deviation from Hardy-Weinberg Equilibrium (HWE) ($p<0.001$), demonstrating heterozygote deficiency was noticed at some of the loci in the sampled populations. The measure of heterozygote deficiency (F_{IS}) was also calculated and positive F_{IS} values were found in all the loci except for Pse 8, Pse 27, Ptd 11, Ptd 20 and Ptd 84. Positive F_{IS} values were found in most of the loci showing heterozygote deficiency. Similar results have been reported in many species of marine fish (Kathirvelpandian *et al.,* 2014). When a pairwise F_{ST} analysis ($p<0.001$ after sequential Bonferroni correction) was performed, no significant differentiation was observed between populations (F_{ST}: 0.006-0.015). So the primers developed from this study could be effectively used for understanding population structure of the species.

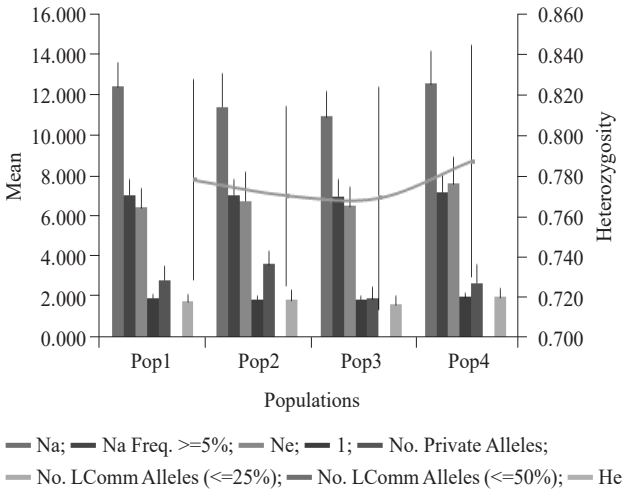

Fig. 2. Allelic patterns across populations of *Eleutheronema tetradactylum*

This work was an exhaustive attempt to test the transferability of markers from *P. sexfilis* to *E. tetradactylum* and validated that the regions flanking these microsatellites are conserved enough to permit the locus amplification. The loci developed in the target species can be employed for better conservation and management of the target species. The results obtained from the current study justify the process of cross-amplification, in saving time, effort and money. The 13 polymorphic loci obtained from this study can be used to measure the gene flow, genetic diversity and to study the population structure of *E. tetradactylum*.

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