Title:

Isolation and characterization of nine microsatellite loci of *Terapon jarbua* (Forsskål, 1775) from Socotra Island (Gulf of Aden) using multiplex PCR.

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Abstract

Ten polymorphic microsatellite loci were identified and characterized from 22 individuals of *Terapon jarbua* (Forsskål, 1775) from Socotra Island (Gulf of Aden, Yemen). Microsatellite polymorphism was tested, revealing 4 to 19 alleles per locus. The observed heterozygosity values ranged from 0.318 to 0.909. Nine loci out of ten conformed to Hardy-Weinberg Equilibrium. They did not show evidence for null alleles and linkage disequilibrium. These loci will be used in an ongoing study of the population structure of this species; associated with a study assessing habitat connectivity based on otolith microchemistry of *T. jarbua*. Results are expected to inform estuarine conservation efforts on Socotra Island and in the Gulf of Aden region.

Terapon jarbua (Terapontidae, Perciformes) inhabits marine and brackish waters of the Indo-Pacific, from the Red Sea and East coast of Africa to Samoa. Its juveniles have been observed to thrive even in coastal freshwater courses. The type locality of the species is Jeddah, Saudi Arabia, and the type series is deposited in the Zoological Museum of Copenhagen (Klausewitz & Nielsen 1965, Nielsen 1974). Although advances have been made in the taxonomy and biology of the Terapontidae (Vari 1978, Whitfield & Blaber 1978, Miu 1990), no in-depth studies of the reproductive ecology, life history strategy and population structure of *T. jarbua* have been conducted as of yet. The microsatellite markers presented herein are therefore the first developed for this species, and will be especially supportive to further genetic studies of the species.

DNA libraries for *Terapon jarbua* enriched for microsatellite sequences containing AAC, ATG, CATC and TAGA repeat motifs were constructed by Genetic Identification Services following the method described by Jones et al. (2002). Resulting recombinant clones were selected at random and sequenced on an Applied Biosystems™ 377 DNA Sequencer, using Amersham's DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Biosciences P/N US81050). Initial polymerase chain reaction (PCR) primers were designed for flanking regions of microsatellite containing sequences using DESIGNER PCR v1.03 (Research Genetics Inc.).

Genomic DNA was extracted from muscle tissue, preserved in 95% ethanol of 22 individuals collected in March 2007 by seine net at Matief Estuary (12° 26' 48.5'' N and 54° 18' 17.6'' E) on Socotra Island (Fig. 1). Extractions were performed using AcroPrep™ 96 well Filter Plates (1mL) with 1μm Glass Fiber media (PALL® 5051), following the extraction protocol for DNA barcoding by Ivanova et al. (2006). PCRs conducted on a GeneAmp PCR system 9700 (Applied Biosystems™) were optimized for each primer individually on four randomly selected samples. After optimisation, PCR reactions were multiplexed in a total reaction volume of 10μL, using 5μL of Master-mix, 1μL of Solution Q both from the Qiagen Multiplex PCR kit, 1μL of multiplexed primer-mix (Table 1) containing specific primers and the labelled universal primers 6Fam-TAGTCGACGACCGTTA, Yakima Yellow YY-TCGGATAGCTAGTCGT, and Dargonfly Orange DO-CTGGCCGTCGTTTTAC (Chang et al. 2004) in order to avoid the expenses of using specific fluorescent primers, 1μL of template

DNA (30-50 ng.µL^{-1}) and 2µL of water. Touchdown PCR conditions consisted of an initial denaturing step at 95°C (15') followed by 11 cycles at 94°C (30''), 63-53°C (1'30'') and 72°C (1'), followed by 25 cycles at 94°C (30''), 53°C (1'30'') and 72°C (1'). A final elongation step at 60°C (30 min) ended the PCR. 3µL of PCR products were added to 12µL of formamide and 0.2µL of a 50-500bp size standard (GeneScan™-500 LIZ™) to visualise microsatellite alleles using an ABI 3130 Genetic Analyzer (Applied Biosystems™). Alleles were then scored using GeneMapper® Software v4.0 (Applied Biosystems™). The number of alleles and the observed and expected heterozygosity values were calculated using GENETIX v4 (Belkhir 2004); deviation from the Hardy–Weinberg equilibrium (Fisher's exact test) and linkage disequilibrium (Fisher's exact test) among loci were tested using GENEPOP v4 (Raymond & Rousset 1995, Rousset 2008). Both tests were corrected for multiple simultaneous tests by calculating the q-value of each test which measures the minimum *false discovery rate (FDR)* that is incurred when calling that test significant. The bootstrap method was chosen as recommended by the authors for a limited number of p-values (Storey 2002). The q-values were calculated using the R package QVALUE (www.r-project.org/, Ihaka & Gentleman 1996, Storey 2002, Storey & Tibshirani 2003, Storey 2003, Storey et al. 2004). This correction was preferred over the commonly used sequential Bonferroni correction (Rice 1989) following Moran (2003). Null allele frequencies were calculated based on Brookfield (1996) using the program MICRO-CHECKER (Van Oosterhout et al. 2004).

Ten out of 12 loci were reliably amplified and found to be polymorphic for *T. jarbua* (Table 2). The number of alleles per locus ranged from 4 to 19, with observed and expected heterozygosity values varying respectively from 0.318 to 0.909, and from 0.328 to 0.941. Exact tests after correction indicated that one locus (B107) deviated significantly from Hardy-Weinberg proportions (q-value < 0.01). Exact test for linkage disequilibrium yielded five weakly significant p-values ($p\text{-value} < 0.05$) out of 45 pair wise comparisons; none of which were ultimately found to be significant following the FDR correction ($q\text{-value} > 0.05$). No locus showed evidence for a null allele. Therefore, nine of the ten markers presented in this study can be applied in studying the genetic structure of *T. jarbua* populations. Such studies are expected to be instrumental in future estuary conservation and management efforts (1) in Yemen including the Socotra Archipelago, representing a UNESCO World Heritage (2008), which is a treasure of marine biodiversity of regional and global importance (Zajonz & Krupp 2006), and (2) in the wider Indian Ocean.

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Figure 1: Map of Socotra Archipelago and sampling site location (Matief)

Table1: Multiplex Primer-mix

Multiplex PCR 1	Multiplex PCR 2
5μL R C102 primer, 100nM	5μL R A4 primer, 100nM
5μL R C108 primer, 100nM	5μL R B107 primer, 100nM
5μL R C3 primer, 100nM	5μL R D108 primer, 100nM
5μL F C102 primer + univ. ext.,10nM	5μL F A4 primer + univ. ext., 10nM
5μL F C108 primer + univ. ext., 10nM	5μL F B107 primer + univ. ext., 10nM
5μL F C3 primer + univ. ext., 10nM	5μL F D108 primer + univ. ext., 10nM
5μL 6FAM-univ. primer, 100nM	5μL 6FAM-univ. primer, 100nM
5μL YY-univ. primer, 100nM	5μL YY-univ. primer, 100nM
5μL DO-univ. primer, 100nM	5μL DO-univ. primer, 100nM
117μL H_2O	117μL H_2O
150 μL	150μL

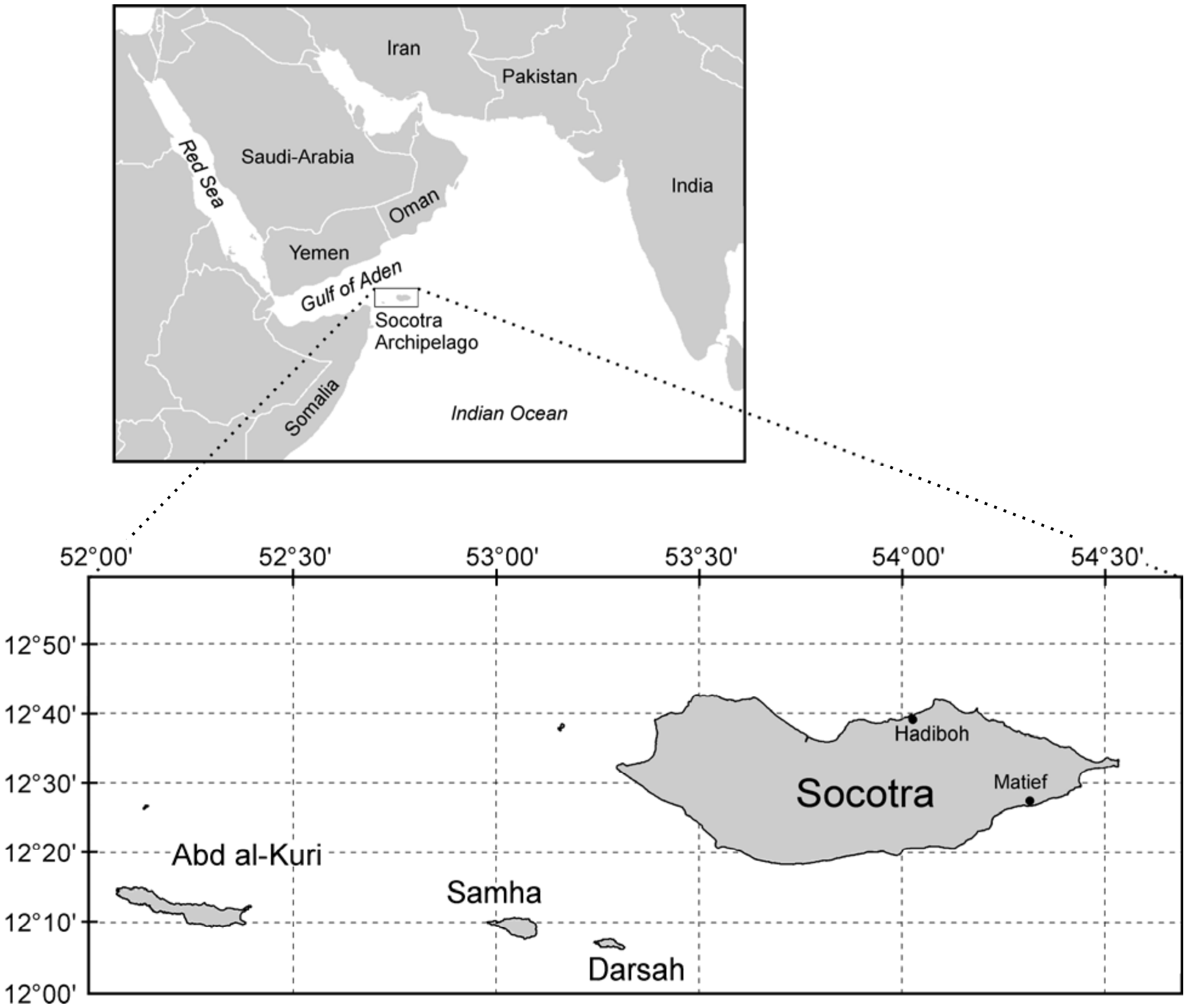
Multiplex PCR 3	Multiplex PCR 4
5μL R B103 primer, 100nM	5μL R B106 primer, 100nM
5uL R C103 primer, 100nM	5μL R C105 primer, 100nM
5μL R D102 primer, 100nM	5μL R D3 primer, 100nM
5μL F B103 primer + univ. ext., 10nM	5μL F B106 primer + univ. ext., 10nM
5μL F C103 primer + univ. ext., 10nM	5μL F C105 primer + univ. ext., 10nM
5μL F D102 primer + univ. ext., 10nM	5μL F D3 primer + univ. ext., 10nM
5μL 6FAM-univ. primer, 100nM	5μL 6FAM-univ. primer, 100nM
5μL YY-univ. primer, 100nM	-
5μL DO-univ. primer, 100nM	7,5μL DO-univ. primer, 100nM
117μL H_2O	119,5μL H_2O
150μL (Total volume)	150μL (Total volume)

Primer and universal extension association is shown in Table 2

Table 2: Primer sequences for 10 microsatellite loci and allele statistics in one population (N = 22) of *Terapon jarbua*

Loci	EMBL #	Repeat motif	5' Universal Extension	Primers 5' → 3'	*i*	A	R	H_E	H_O	P_{HW}	Q_{HW}
C102	FR719958	$\mathrm{(CTAT)_{13}(CCAT)_{28}(CTAT)_{2}}$	F: CTGGCCGTCGTTTTAC	GTCTCCCTCCCTCATGTCTG	1	19	171-257	0.938	0.909	0.0662	0.0513
			R:	TTGCCACAGTGGACCTGTAG							
C108	FR719959	$\mathrm{(ATCC)_{9}ATTT(ATCC)_{2}}$	F: TCGGATAGCTAGTCGT	CCATCCATTCATCCATCTAC	1	7	274-336	0.763	0.727	0.2318	0.0773
			R:	GCTTTGGAGTATTTTGCAGTT							
C3	FR719960	$\mathrm{(CATC)_{7}}$	F: TAGTCGACGACCGTTA	CATAATGAGCGAGGTCAGAT	1	6	278-305	0.791	0.727	0.0770	0.0513
			R:	ATCACGGAGGTTCTAAGAGTC							
A4	FR719961	$\mathrm{(AAC)_{14}}$	F: TCGGATAGCTAGTCGT	ACCTGCCTACTACAGCCTCAG	2	5	262-271	0.711	0.636	0.2146	0.0773
			R:	CACTCCACTTGCCCATTTC							
B107	FR719962	$\mathrm{(CAT)CAA(CAT)_{7}}$	F: CTGGCCGTCGTTTTAC	CCAAGTTCCTGATGCTAAAAG	2	10	205-231	0.868	0.773	0.003*	0.0008*
			R:	AGACGATGATGGGATTATTTG							
B103	FR719963	$\mathrm{(CAT)_{2}CTT(CAT)_{9}CAC(CAT)}$	F: TCGGATAGCTAGTCGT	GGGCTGTAACAGTATGCAATG	3	4	215-227	0.669	0.727	0.5839	0.1557
			R:	ATGCAGCACCTTCAGAGTTTA							
C103	FR719964	$\mathrm{(TCCA)_{8}}$	F: CTGGCCGTCGTTTTAC	CTTTCAATAGCCAGGACTACC	3	4	183-198	0.665	0.545	0.1209	0.0645
			R:	TCTTCCACACTGAGACTGCT							
B106	FR719965	$\mathrm{(CAT)_{2}CA(CAT)_{4}CAC(CAT)}$	F: CTGGCCGTCGTTTTAC	AGAGGAGGACCACATAAACAC	4	11	112-159	0.859	0.727	0.0540	0.0513
			R:	TTCCACCAGATGAGAGGAG							
C105	FR719966	$\mathrm{(ATCC)_{5}CTCC(ATCC)_{12}}$	F: CTGGCCGTCGTTTTAC	AGCTTTGTGAGGCTAATACCAG	4	6	242-314	0.328	0.318	0.5475	0.1557
			R:	AAGTCTTCTTCAACCCTGTGAG							
D3	FR719967	$\mathrm{(TCTA)_{10}}$	F: TAGTCGACGACCGTTA	CAGTCCAGTAATGTCGTTTGT	4	19	283-349	0.941	0.909	0.2256	0.0773
			R:	AGTGTTAGACAGGAGCACATG							

i, multiplex PCR index (Table 1); A, allele nb; R, size range; H_E, expected heterozygosity; H_O, observed heterozygosity; P_{HW}, p-value of HW exact test; Q_{HW}, q-value of HW exact test; *, Significant deviation from HWE.


Iran
Pakistan
Saudi-Arabia
Red Sea
India
Oman
Yemen
Gulf of Aden
Socotra Archipelago
Somalia
Indian Ocean
52°00'
52°30'
53°00'
53°30'
54°00'
54°30'
12°50'
12°40'
12°30'
12°20'
12°10'
12°00'
Hadiboh
Socotra
Matief
Abd al-Kuri
Samha
Darsah