Open Journal of Genetics, 2014, 4, 355-361
Published Online September 2014 in SciRes. http://www.scirp.org/journal/ojgen
http://dx.doi.org/10.4236/ojgen.2014.45032

Isolation and Characterization of 26 Microsatellite Loci for the Shortfin Silverside Fish *Chirostoma humboldtianum* Valenciennes 1835 (Atherinopsidae: Menidiinae) Derived from Next Generation Sequencing and Their Cross-Amplification in Central Mexican Atherinopsids

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Received 10 June 2014; revised 9 July 2014; accepted 8 August 2014

Abstract

The endemic silverside fish *C. humboldtianum* is of great ichtyologycal, economical and cultural relevance in central Mexico and it has been suggested that it is among a group of other "peces blancos", the most ancestral species. Here we characterized a set of 26 microsatellite loci from the species in order to further assess population and phylogeographic issues that aid in evaluating their highly impacted populations. From 58 primer pairs tested on specimens from Villa Victoria dam (Rio Balsas Basin), 26 loci positively amplified on the species and cross-primed on specimens of the closely related and threatened *Poblana alchichica*, *C. promelas* and *C. riojai*. Loci resolved medium and high genetic variation (mean N_A = 14.3, SD = 9.38; mean H_O & H_E = 0.47, SD = 0.32 and

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How to cite this paper: de los Angeles, B.-S.I., *et al*. (2014) Isolation and Characterization of 26 Microsatellite Loci for the Shortfin Silverside Fish *Chirostoma humboldtianum* Valenciennes 1835 (Atherinopsidae: Menidiinae) Derived from Next Generation Sequencing and Their Cross-Amplification in Central Mexican Atherinopsids. *Open Journal of Genetics*, **4**, 355-361. http://dx.doi.org/10.4236/ojgen.2014.45032

0.58, SD = 0.32, respectively) and it is expected that these polymorphic loci are also useful in studing threatened atherinopsid species.

Keywords

Endemic, Polymorphism, Villa Victoria, *Poblana alchichica*, *C. promelas*, *C. riojai*

1. Introduction

Up until the first half of the 20th century, atherinopsids were the main fishing product of the inland waters of Mexico and counted among the top scale fish species. The representative endemic genera of Atherinopsidae in Central Mexico are *Chirostoma* and *Poblana*, locally recognized as "charales" and "peces blancos" (silversides), *C. humboldtianum* included. The species is of great economic importance, because it has been used for decades as food by humans inhabiting the shores of Pátzcuaro and Chapala lakes and reservoirs in the States of Michoacán and Mexico. Although it is true that atherinopsids are of great interest as a substantial screed in epicontinental fisheries capture, it is also true that the development and growth of artisanal fisheries in these regions lack of scientific basis and are rather based on the needs to supply high quality animal protein in short term. The species is composed by two morphotypes, clearly segregated into western and eastern populations and characterized by having differences in size, and in the mean values for median lateral and predorsal scales [1] [2]; andrecognized as the hypothetical ancestral form of the "humboldtianum group" [1] [3]. During the last decades the populations of the species have been reduced or extirpated due to the introduction of non-native fish to the western basins [1] [2] [4]. Similarly, habitat loss, pollution and overfishing have played important roles in the decline and extinction of local populations of related species (*C. riojai*, *C. promelas*, *Poblana alchichica*) listed in the Official Mexican Standard NOM-059-Semarnat in 2010 and in the IUCN Red List.

A recent study on the population genetics of the species and based on sequences of the mitochondrial control region, described the species as highly differentiated and with intermediate levels of diversity [5]; and although the authors proposed the conservations of all the populations studied, they also pointed out the possible evolutionary stochastic effects and the limitation of defining conservation units only on the bases of mtDNA. Therefore, eight poymorphic microsatellite loci were developed [6] based on an enrichment method [7] in order to further assess and confirm the diversity and structure of the species. Furthermore and with the aim of developing a larger set of genetic codominant markers available to study the silversides of México, a recently diverged group of fishes [8] [9], here we characterized 26 additional polymorphic loci for the species and using Next Generation Sequencing and succesfully cross-amplified them in closely related species with the aim of their use in assessing further population genetics and phylogeographic issues that can aid in the conservation and management of atherinopsids.

2. Materials and Methods

2.1. Microsatellite Isolation

Genomic DNA was extracted from muscle tissue of one individual of *C. humboldtinuam* using a salt extraction protocol [10] and sent to the Georgia Genomics Facility (University of Georgia, Athens, USA) for sequencing. The specimen was deposited in the Colección Nacional de Peces Dulceacuícolas, Escuela Nacional de Ciencias Biológicas-IPN (ENCB-IPN-P6423). DNA was sheared using a Covaris S2, and Illumina adapters were ligated on using methods derived from [11] but using adapters equivalent to Illumina TruSeq with 10 nt indexes [12]. Libraries were pooled and run on the Illumina HiSeq 2000 (paired-end 100 reads). Resulting fastq files were demultiplexed, and reads were filtered and run through the PALfinder pipeline for microsatellite identification and primer design [13]. Over 10 million reads at 100 bp were obtained, covering approximately 1.3^{12} bases and resolving 3× genome coverage. Detected reads with microsatellites were 108, 228, of which 0.16% presented primers. Further primer sorting was as follow: 1) all loci with primers that were found more than once were deleted, particularly most of the really long (>500 bp) and short dinucleotide repeats (<24 bp); 2) removal of the longest repeats. Suitable primer design was possible in 1153 pairs with repeats of mid-length. Fifty eight pairs of primers were selected on the bases of the following parameters: a) length (18 - 30 bp), b) G-C content (40% -

60%), c) melting temperature (55˚C - 65˚C), d) 3'-stability, e) avoid hairpin or dimer formation and f) no self-priming, and tested for polymorphism.

2.2. Sampling

Mexican government kindly issued permit number DGOPA.07343.310810.4128 to conduct this research. Organisms from Villa Victoria were obtained from commercial artisanal catches. *C. riojai* and *P. alchichica* specimens were kindly donated by Gerardo Figueroa Lucero and Héctor Espinoza, respectively. Gill lamella tissue samples from *C. promelas*, *C. sphyraena*, *C. grandocule* and *C. jordani* were obtained from the Tissue collection of the Laboratorio de Genética y Biología Molecular, Planta Experimental de Producción Acuícola, Universidad Autónoma Metropolitana Iztapalapa.

2.3. DNA Extraction, PCR Amplification and Analysis

Total DNA was isolated as previously described from 30 *C. humboldtianum* from Villa Victoria Dam, State of Mexico (19˚26'N, 100˚00'W) and 22 specimens of *Chirostoma* and *Poblanaalchichica* (**Table 1**) to test cross-amplification.

Table 1. Cross-amplification of 26 microsatellite loci of *Chirostoma humboldtianum* in five species of *Chirostoma* and one *Poblana* species. Sample size in parenthesis.

	C. jordani Xochimilco Channels (N = 4)	*C. sphyraena* Chapala lake (N = 4)	*C. riojai* Ignacio Ramírez (N = 4)	*C. grandocule* Pátzcuaro Lake (N = 4)	*C. promelas* Chapala Lake (N = 2)	*P. alchichica* Alchichica, Puebla (N = 4)
Chum07	++	++	++	++	++	++
Chum10	+	+	+	++	-	-
Chum11	+	+	++	++	++	++
Chum12	+	++	++	++	++	++
Chum14	+	++	++	++	-	++
Chum15	++	++	-	++	-	-
Chum18	++	++	++	++	++	++
Chum27	+	++	++	++	++	++
Chum30	++	++	++	+	-	++
Chum33	++	++	++	++	++	++
Chum39	++	++	++	++	++	++
Chum44	++	++	++	++	+	++
Chum48	++	++	++	++	+	++
Chum53	++	++	++	++	++	++
Chum55	++	-	++	++	+	++
Chum56	++	+	++	++	++	++
Chum57	++	++	++	++	++	++
Chum58	++	++	++	++	+	+
Chum59	++	+	++	++	-	++
Chum60	++	++	++	++	++	++
Chum62	++	++	++	++	++	++
Chum63	++	++	++	++	++	++
Chum65	++	++	++	++	+	++
Chum68	+	++	++	++	+	++
Chum69	++	++	++	++	++	++
Chum70	++	+	++	-	-	++
++	77%	77%	92%	92%	54%	88%
+	23%	19%	4%	4%	23%	4%
Proportion Polymorphic Loci	77%	88%	84%	88%	50%	63%

For *C. promelas* ++ = 2 genotypes recorded; + = 1 genotype; for *C. jordani*, *C. sphyraena*, *C. riojai*, *C. grandocule*and *P. alchichica* ++ = 3 - 4 genotype products recorded; + = 1 - 2 genotypes; - = no amplification success when n = 4 or n = 2.

PCR were performed in 10 µl reaction, containing 40 ng DNA, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2 mM dNTPs, 0.2 µM each primer, 2.5 mM $MgCl_2$, and 0.25 - 0.35 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Cycling conditions included: 95˚C 5 min, 35 cycles of 30 s at 94˚C, 30 s at the locus-specific annealing temperature (T_m) (**Table 2**), 40 s at 72˚C, and a final 72˚C extension for 15 min and using an iCycler (BIORAD Laboratories, Hercules, CA, USA). PCR products were visualized by silver staining in 6% polyacrylamide gels (7.5 M urea). Allele sizes were determined using 10 bp ladder (Invitrogen). Cross-species PCR amplifications required a reduction on the stringency of reactions on some loci, carried out lowering 1 or 2 grades the T_m.

Table 2. Primer sequences and characteristics of 26 microsatellite amplified in 30 individuals of *Chirostoma humboldtianum* (Ch) from Villa Victoria dam.

Locus	Genbank accession number	PCR primers sequence[i]	SSR motif	T_m (˚C)	Allelic range (Ch)	N_A	H_O	H_E	Allelic range all samples	N_A all samples
Chum07	KM099040	F: GGCCAGATGTGGATCTCTTAGC R: CAGTGTGTCTGAGATGGAGCG	$(TTC)_{27}(AC)_{26}$	55	127 - 139	2	0.519	0.475	127 - 149	9
Chum10	KM099041	F: TTAATAGGGGAGATCGAGGCG R: TTCCTCAAGAAAAGTCACCAGC	$(TTCC)_{28}$	58	144 - 156	4	0.444	0.474	144 - 156	4
Chum11	KM099042	F: CATGCACAGCCTTAAAAGACG R: AAAGTTCTGACTCACCCACGC	AC(40) AC(68)	58	111 - 167	14	0.462	0.856*	111 - 183	21
Chum12	KM099043	F: TGACCCCGGATAAACTGTGG R: CATGAGGATTCCCAAGGGC	$(ATGG)_{44}(ATGG)_{44}$	58	125 - 185	10	0.815	0.869	117 - 185	14
Chum14	KM099044	F: TCATTGTCAGTGTGTATGTGCG R: TAAACTGAGTGGGAAAGGGC	$(AC)_{42}(AC)_{30}$	52	118 - 168	19	0.815	0.208	118 - 168	21
Chum15	KM099045	F: GAGCACTGGGAGATAATGAACC R: AACTCCTAGAATGTGCATCACC	$(ATT)_{72}$	55	149 - 212	15	0.778	0.073	149 - 253	20
Chum18	KM099046	F: CCTCCTTTCCTCCTGTTTTCC R: GATCCGAAGCTCTTAAAGCCC	$(TCC)_{27}(TCC)_{30}$	60	116 - 137	4	0.074	0.561*	107 - 180	11
Chum27	KM099047	F: TGTGGAAGATGGAGATGTGG R: ACATACTATTAAGTAAAACTGAC ATTGAGC	$(AT)_{42}(AT)_{48}$	52	106 - 156	15	0.231	0.936*	106 - 156	18
Chum30	KM099048	F: GAAGGAGTGATGGAGCAGGG R: CAGTCCTGTTTCTGATCCGC	$(TCC)_{24}(TCC)_{24}(TCC)_{24}$	60	115 - 147	6	0.400	0.108*	105 - 147	12
Chum33	KM099050	F: CTCCTGCCTCACCTTCATCC R: TGTTCATGCTGTCAGTGACCC	$(TCC)_{24}$	64	131 - 139	2	0.000	0.845	119 - 140	7
Chum39	KM099051	F: ACGTCAGGGTAAGGGTTGGC R: GTACCGGCCCAAAGTTTACG	$(AC)_{40}$	64	156 - 172	8	0.259	0.944*	150 - 172	11
Chum44	KM099053	F: AGGGAGGTCTGAAGTCGTGC R: TTGTTGCCAAACGTCTGAGG	$(AAAG)_{48}$	62	160 - 270	21	0.889	0.927	158 - 328	30
Chum48	KM099054	F: TTCTAGTTCCTCCTGTTAGCATCG R: AGGAACCAGTGCCCAGACC	$(TTCC)_{32}$	61	159 - 191	3	0.037	0.583	155 - 219	11
Chum53	KM099055	F: AAACTACCCGAAGCGAGACG R: TGTCTGCCGATAAACAGTTTCC	$(ATGG)_{24}$	60	274 - 274	1	0.000	-	128 - 274	3
Chum55	KM099056	F: AGGGCAGCGCATTTTACG R: AGAGTGCTCAGAGGCAAAAGG	$(AAAG)_{24}$	57	322 - 354	10	0.846	0.802	322 - 418	17
Chum56	KM099057	F: TGAGATCCCAGCTCAGTCC R: GAAAGGACAGCGGTCAGC	$(ACTG)_{24}$	57	146 - 158	4	0.333	0.300	142 - 166	6

Continued

Chum57	KM099058	F: CTGATAACAAGCGTGGGAGG R: TTTAATCTGAGGGGAAGAGATGG	$(AAAT)_{24}$	61	148 - 160	4	0.481	0.535	148 - 160	4
Chum58	KM099059	F: CAGAGGAAACGGAGAGTGTAGC R: CAGCGAAAACAGATACATAAAG ACC	$(ATCT)_{24}$	61	253 - 363	22	0.926	0.942	253 - 363	22
Chum59	KM099060	F: GGCACTCAAACGAACACAAGC R: CGGCCGGTGTCCAATAGC	$(ATAC)_{24}$	60	115 - 139	4	0.154	0.215	115 - 143	6
Chum60	KM099061	F: CTTTGCACCATGACTGACTGG R: ATGTTCTTGTACACCAACGACCC	$(ATGG)_{28}$	61	265 - 301	10	0.889	0.855	237 - 321	21
Chum62	KM099062	F: TTCCAAGGCTTCATTGTAAGG R: CGACAGGAAAGTGGAAAAGG	$(AAAG)_{28}$	57	289 - 361	16	0.846	0.937	289 - 401	29
Chum63	KM099063	F: CACTCCTCCTATGTCCCTCC R: GCTGTGGACTGAAGTTTAGATGC	$(AAAT)_{28}$	58	228 - 256	5	0.115	0.184	228 - 256	6
Chum65	KM099064	F: TCCCAGTGCTGCAGAAAGC R: GGGCCCTAAAAGCTGTCACC	$(AAAG)_{32}$	61	183 - 199	4	0.519	0.476	175 - 199	5
Chum68	KM099065	F: GCTTTGCTGCAACGGACC R: CTGCCAGCTAGCAAGTTACCC	$(ATCT)_{36}$	58	234 - 386	25	0.846	0.968*	234 - 406	39
Chum69	KM099066	F: GCATGAAGCAGAACATTAAAACC R: CAGAGTTCAGTCAAAGGAGAGC	$(AAAT)_{40}$	58	206 - 310	12	0.630	0.783	202 - 330	21
Chum70	KM099067	F: GGATTAGTCGAACAAATCAAACC C R: CCAACATCGGTTGTGAAACG	$(ATAC)_{48}$	58	107 - 123	3	0.111	0.234	107 - 127	5

*Significant deviation from HWE ($p < 0.05$); AN = Accession number; ‡Forward primers were 5' labeled with the M13 sequence (5'TGTAAAACGA CGGCCAGT), for further fluorescence detection.

Potential presence of null alleles was assessed in Free N_A [14]. Alleles per locus (N_A) and observed (H_O) and expected (H_E) heterozygosities were calculated using deviations from Hardy-Weinberg equilibrium, linkage disequilibrium was estimated and tested using GENEPOP 4.0.10 [15] under default parameters. Multiple hypothesis tests p values were adjusted by the False Discovery Rate (FDR) method [16]. In order to gain information on the possible utility of these loci in the closely related species assayed, the percentage of polymorphism per species (%) was obtained by accounting only those loci that resolved more than one allele per locus and wihtin each species.

3. Results and Discussion

Among 58 primers tested, 26 microsatellites screened successfully, 25 resolved as polymorphic and Chum53 was monomorphic in specimens from Villa Victoria. Mean number of alleles in the polymorphic loci was 14.3, SD = 9.38, a mean number 2× higher than the resolved earlier for the species (see [6]; 6.4, SD = 2.5), but similar to those characterized closely related species [17] [18]; and H_O and H_E 0.47, SD = 0.32 and 0.58, SD = 0.32, respectively. These later values are similar to those previously reported (see [6]; 0.61, SD = 0.19 and 0.63, SD = 0.12, for H_O and H_E, respectively). Linkage disequilibrium was detected for a pair of loci within specimens from the population (Chum15 and Chum39, $p = 0.00$). Null allele frequency estimates were negligible for half of the loci, moderate (>0.05 and <0.20) for 12 loci (Chum11, Chum14, Chum18, Chum30, Chum33, Chum39, Chum48, Chum59, Chum63, Chum68, Chum69 and Chum70) and high at one locus (Chum27 = 0.357). Significant deviation from HWE was detected in six loci ($p < 0.05$, **Table 2**). This deviation might result from the heterozygote deficiency found in some loci (Chum11, 18, 27, 30 & 39), which in turn might be caused by endogamy as has been earlier suggested for the species [5] and in other species of the genus [19] [20].

Cross-PCR amplifications of 22 individuals of *Chirostoma* and *Poblana* resulted in 16 polymorphic loci and the remaining 10 loci resolved genotypes in most of the species (see **Table 1**). For instance Chum 07, 18, 33, 39, 53, 57, 60, 62, 63 and 69 resolved genotypes in 3 or 4 individuals (++) of each of the six screened species, excepted for *C. promelas*, for which the number of specimens analyzed was two; Chum 12, 27, 44, 48, 56 and 65, rendered genotypes (++) in five of the species and genotypes (+) in one species per locus. Chum 11, 58 and 68, resolved genotypes (++) in four species and (+) in two species; Chum 14, 30, 55 and 59, also resolved genotypes (++) in four species and (+) in another species, however these loci did not amplified neither in *C. promelas* or *C. sphyraena*; the remaining loci amplified genotypes (++ or +) in one to three species and did not amplified in other two or three species. Although eight microsatellite loci have been recently developed for the species (see [6]), these 26 new microsatellite loci are the first to be tested in cross-amplification between the Mesa Central Atherinopsids and will be helpful tools for genetic population and phylogeographic studies of *C. humboldtianum*, as well as in paternal assignments for culture purposes, studies all that can aid in developing a conservation plan for this important species, which although is among the group known as "pecesblancos" the species with the widest distribution (see [19]), some of its populations have been reduced or extirpated due to habitat loss, pollution and overfishing (see [19]). These markers will also aid in investigating the integrity of stocks/populations for management and conservation of related endangered species.

Acknowledgements

Funding for this research was provided by Consejo Nacional de Ciencia y Tecnología-CB-2009-01-130220 and UAM.147.07.03/147.09.01 granted to IDLABS. This work was completed by Irene D. L. A. Barriga-Sosa during her sabbatical leave in CCA-UAA.

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