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PRIMER NOTE

Isolation and characterization of eight microsatellite loci for the Neotropical freshwater catfish *Pimelodella chagresi* (Teleostei: Pimelodidae)

ANDREW A. MOESER*† and ELDREDGE BERMINGHAM*†

*Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Panama, †Department of Biology, McGill University, Montréal, Québec H3A 2K6, Canada

Abstract

We report eight $(CA)_{10–35}$ unlinked microsatellite loci from the Neotropical freshwater catfish, *Pimelodella chagresi* (Siluriformes: Pimelodidae). These loci were characterized with 23 individuals collected in Panama. Number of alleles per locus varied from 7 to 23 (mean = 12.9) and observed heterozygosity ranged from 0.522 to 0.909 (mean = 0.732). These loci will be used to investigate the existence of cryptic species within the *P. chagresi* clade, and to study fine-scale population structure.

Keywords: freshwater fish, microsatellites, Neotropical

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Pimelodella chagresi is a freshwater catfish whose distribution stretches from northwestern South America into lower Central America (Panama and Costa Rica). Phylogeographical studies based on mitochondrial DNA (mtDNA) sequence and restriction fragment length polymorphism data have documented the existence of substantial population genetic structure within *P. chagresi* (Bermingham & Martin 1998; Martin & Bermingham 2000). Co-existing clades within Panamanian drainages exhibit as much as 16% sequence divergence in their mtDNA ATPase, 6/8, suggesting that *P. chagresi* may comprise a cryptic species complex (Martin & Bermingham 2000). Nonetheless, the syntopic distribution of the two principal mtDNA haplotype clades in some Panamanian rivers and their similar morphology suggest that the different lineages of *P. chagresi* may be ecologically equivalent, and raises the question of whether or not they are reproductively isolated from one another. The microsatellite loci described here will be used to determine whether there is ongoing or recent gene flow between the sympatric *Pimelodella* lineages. Additionally, the loci will allow for investigation of population subdivision within watersheds, and comparison of the historical demography of *P. chagresi* between watersheds.

Correspondence: Andrew Moeser, Redpath Museum (Department of Biology), McGill University, 859 Sherbrooke St. W., Montréal, Québec, H3A 2K6, Canada. Fax: (514) 398 3185; E-mail: andrew.moeser@mail.mcgill.ca

Microsatellite loci were cloned and sequenced from a microsatellite-enriched library to isolate DNA fragments containing dinucleotide (CA) repeats. Genomic DNA was extracted from the gills of one *P. chagresi* individual by standard phenol-chloroform extraction (Sambrook *et al*. 1989), except dialysis purification was used instead of ethanol precipitation. The extracted DNA was digested with *Sau*3AI (Promega), size-selected for fragments 400–1000 bp in length, and ligated to *Sau*3AI linkers in equimolar concentrations of linker and genomic fragment. The ligated genomic DNA was amplified by polymerase chain reaction (PCR) in a 100-μL reaction including 10 μL of 10 × PCR buffer (500 mM KCl; 100 mM Tris-HCl pH 8.3), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 μM *Sau*-L-A primer, 2.5 U *Taq* polymerase (Perkin Elmer), and 15 μL of the ligation product (approximately 40 ng DNA/linkers). PCR was performed on an MJ Research PTC 200 thermocycler under the following conditions: 3 min initial denaturation at 94 °C; 25 cycles of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C, and a final extension of 72 °C for 10 min. This PCR product was separately hybridized to a 5′ biotinylated $(AC)_{12}$ probe, and target sequences were captured with VECTREX Avidin D matrix.

The microsatellite-enriched DNA fragments were amplified as described previously, and cloned with the TOPO TA Cloning Kit for sequencing (Invitrogen Corp.). Resultant colonies (necessarily 'positive' due to cloning protocol) were screened for the presence of microsatellites by

Table 1 Eight polymorphic microsatellite loci isolated from the Neotropical catfish, *Pimelodella chagresi*, with primer sequences and characteristics of each locus (bp, base pair; T_a, annealing temperature). Size range, allele number, observed (H_O) and expected (H_E) heterozygosity are reported from a Chagres River population in Panama (n = 23)

Locus	Repeat	Primer sequences (5′–3′)	Size range (bp)	T_a (°C)	No. alleles	H_O	H_E	GenBank Accession no.
PC58	$(CA)_{15}$	F: CGATTACTTTCCTACTCCAGC R: CCAAGCAGGACACCGTTCG‡	197–217	56	11	0.783	0.876	AY833372
PC75	$(CA)_{22}$	F: CTGTTGACATAAGCGGGTTG† R: TGTTCTGTCTGTAACAAAGACCTG	175–205	58	14	0.714	0.907	AY833373
PC87	$(TG)_{13}$	F: AAGCCCTGGTACCCACAAG† R: GTCAGCGCTTTTAGGCTTTG	238–284	56	8	0.522	0.759	AY833374
PC90	$(CA)_{20}$	F: CCCAGTGGGCTCATTAACAC R: CACACAGTCCGGATTAGAGG†	119–201	56	23	0.909	0.946	AY833375
PC92	$(CA)_{35}$	F: CACACGGAAAACAAACATGC† R: GGGGAAACACTGAACATCTAAGTC	311–391	58	21	0.913	0.944	AY833376
*PC2**	$(TG)_{10}$	F: **CACGACGTTGTAAAACGAC**-TCTATGTGCGTGCTGGTTTC R: GGAATGTCAGGATGCATGTC	215–237	58	8	0.636	0.808	AY833370
*PC17**	$(CA)_{21}$	F: **CACGACGTTGTAAAACGAC**-GCCTGCCAGGTAAATCTGAA R: AGGTATGCGGAACACTGACC	234–270	56	11	0.522‡	0.888	AY833371
*PC97**	$(CA)_{14}$	F: **CACGACGTTGTAAAACGAC**-GTTGGTTTGAGGTCGGTTTG R: GGAACAGTGAGAGCGGAGAC	263–277	56	7	0.650	0.678	AY833377
Mean Values					12.9	0.732	0.851	

*Locus amplified with M 13 tailed forward primer (M 13 sequence in bold type); †Dye-labelled primer; ‡Significant departure from Hardy–Weinberg equilibrium.

sequencing the DNA inserts on a Perkin Elmer ABI 377 automated sequencer. The procedure involved PCR amplification using M13 universal primers in a 10-μL reaction (with the same reaction conditions reported previously). For each clone, a forward and a reverse cycle sequencing reaction were performed using M13 universal primers, following the procedure recommended by Applied Biosystems Incorporated. Each clone was sequenced in two directions. One hundred fifty recombinant clones were obtained in total from the library; 74 randomly selected clones were sequenced, and 23 clones contained arrays of at least 10 uninterrupted repeats. Primers were designed for 17 of these loci that had sufficient flanking regions using OLIGO version 4.06 (Rychlik 1992) and PRIMER3 (Rozen & Skaletsky 1996) software.

Loci were assayed with 23 *P. chagresi* individuals from the Chagres River of Panama. All loci were PCR-amplified under the following conditions: 3 min initial denaturation at 94 °C; 35 cycles of 45 s at 94 °C, 45 s at annealing temperature (Table 1), and 1 min at 72 °C, and a final extension of 72 °C for 3 min. Out of the 17 loci assayed, eight amplified polymorphic loci in the size range expected from the original (cloned) microsatellite sequence (Table 1). The other nine loci were discarded because of poor PCR performance (nonamplification or nonspecific amplification).

For five loci (Table 1), one of the primers was labelled with a fluorescent dye (HEX, TET, or 6-FAM; Integrated DNA Technology), and each PCR reaction (10 μL) contained 1 μL 10 × PCR buffer, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, 0.2 U *Taq* DNA polymerase (Qiagen), 0.4 μM of each primer, and 10–30 ng DNA. The other three loci (Table 1) were amplified with a forward primer containing an M-13 tail at the 5′ end of the oligonucleotide; these loci were visualized by the addition of a dye-labelled M-13 primer to the PCR reaction mix (Oetting *et al*. 1995). These three loci were amplified with single-step PCR using the conditions and reactants described previously except with different primer concentrations: 0.1 μM M-13 tailed forward primer, 0.4 μM reverse primer, 0.5 μM labelled M-13 primer. Amplified products were run through a 6% polyacrylamide gel on an MJ Genesys Base station sequencer and analysed with CARTOGRAPHER version 1.2.6 software (MJ Research). Amplified products were sized by comparison with an internal size-standard (GENESCAN ROX-500).

Allelic diversity and average observed and expected heterozygosity were computed with GENETIX version 4.04 (Belkhir *et al*. 2002) using data from the Chagres population. The number of alleles per locus varied from 7 to 23 (mean = 12.9) and observed heterozygosity ranged from 0.522 to 0.909 (mean = 0.732). GENEPOP version 3.3 (Raymond & Rousset 1995) was used to assess Hardy–Weinberg

equilibrium (HWE) and genotypic linkage disequilibrium. No linkage disequilibrium was observed among the eight loci. Using the Markov chain method of the exact test, only locus PC17 was found to deviate significantly from HWE predictions following a sequential Bonferroni correction. Although this result could be caused by the presence of null alleles, there were no nonamplifying individuals at locus *PC17* in the population sampled.

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