Notes

Microsatellite Loci for the Threatened Pearl Darter and Cross Amplification in Channel and Coal Darters

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Abstract

The Pearl Darter *Percina aurora* is an imperiled species of fish currently only found in the Pascagoula River drainage in southern Mississippi. We tested 60 microsatellite loci identified by Illumina pair-ended sequencing. Forty of these loci were polymorphic in Pearl Darters with a mean number of alleles per locus of 11.5 and mean observed and expected heterozygosity values of 0.818 and 0.805, respectively. Eleven to 17 of these loci were also polymorphic in the closely related Channel Darter *P. copelandi* and Coal Darter *P. brevicauda*. These loci should prove useful in genetic studies associated with informing future management decisions for Pearl Darters and conservation efforts in other species of darter.

Keywords: *Percina aurora*; *Percina copelandi*; *Percina brevicauda*; microsatellite loci

Citation: Kreiser BR, Clark SR, Schaefer JF. 2021. Microsatellite loci for the threatened Pearl Darter and cross amplification in Channel and Coal Darters. *Journal of Fish and Wildlife Management* 12(1):257–262; e1944-687X. https://doi.org/10.3996/JFWM-21-008

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Introduction

The Pearl Darter *Percina aurora* was formally described in 1994 on the basis of specimens from the Pearl and Pascagoula River drainages in southeastern Louisiana and southern Mississippi (Suttkus et al. 1994), and is currently listed as endangered in the state of Mississippi (Mississippi Natural Heritage Program 2016) and threatened by the U.S. Fish and Wildlife Service (USFWS 2017), as provided by statute under the Endangered Species Act (ESA 1973, as amended). While the Pearl Darter was historically known from the Pearl River system, recent sampling efforts and an environmental DNA survey (Schaefer and Mickle 2011; Wagner et al. 2017) have failed to detect them in this drainage. In contrast, populations in the Pascagoula River drainage appear moderately stable, although substantial variability has been observed across years (Clark et al. 2018).

The Pearl Darter is a member of the subgenus *Cottogaster*, which also contains the Channel Darter *P. copelandi* and the Coal Darter *P. brevicauda* (Suttkus et al. 1994; Near et al. 2011). The Coal Darter has a restricted range with populations only being found above the Fall Line in the Black Warrior, Cahaba, and Coosa Rivers of the Mobile River basin (Boschung and Mayden 2004). The Channel Darter has a much broader distribution with disjunct populations found across the Mississippi River basin and Great Lakes. While this broad distribution has led to a designation of globally secure, locally the Channel Darter is listed as critically imperiled, imperiled, or vulnerable in 13 states or provinces (NatureServe 2021). The Channel Darter may also contain cryptic taxonomic diversity based on the morphological and
genetic distinctiveness of populations from the Ouachita Highlands (Suttkus et al. 1994).

Our primary objective was to identify polymorphic microsatellite loci for Pearl Darters to assist in recovery efforts, including delimiting population genetic structure and managing hatchery stocks. Our second objective was to test the utility of these loci in the Channel Darter and Coal Darter. Here, we describe 40 polymorphic microsatellite loci for Pearl Darters and identify which of these are also polymorphic in Coal and Channel darters.

Methods

We obtained tissue from a Pearl Darter from the Mississippi Museum of Natural Science (MMNS) tissue collection (accession number MMNS 190; Chickasawhay River near Stonewall, MS). We extracted total genomic DNA with a DNeasy Tissue Kit (Qiagen Inc.), and checked for quality on a 0.8% agarose gel stained with ethidium bromide prior to sending the sample to the Savannah River Ecology Laboratory Molecular Ecology Laboratory. They prepared an Illumina paired-end shotgun library following the methods of Nunziata et al. (2013) and then used PAL_FINDER v0.02.03 (Castoe et al. 2012) to identify reads containing di-, tri-, tetra-, penta-, and hexanucleotide microsatellite motifs from the approximately 11 million total reads. Loci with two or more reads were removed, and Primer3 (Rozen and Skaletsky 2000) was subsequently used to design primers for the 724 microsatellite-bearing loci that remained.

We tested 60 loci, representing 1 hexanucleotide, 3 pentanucleotide, 39 tetranucleotide, and 17 trinucleotide repeat motifs, for amplification quality and variability. The initial screening used five individuals from the MMNS tissue collection (MMNS 208; Chickasawhay River near Waynesboro, MS). Amplifications were conducted in a total volume of 12.5 μL using 1X standard Taq buffer (New England Biolabs), 0.2 mM dNTPs, 2 mM MgCl₂, 0.25 units of Taq polymerase (New England Biolabs), 0.16 μM of the M13 tailed forward primer (Boutin-Ganache et al. 2001), 0.16 μM of the reverse primer, 0.006 μM of the labeled M13 primer (Eurofins), 10-40 ng of template DNA, and water to the final volume. PCR cycling conditions consisted of an initial denaturing step of 94°C for 2 min followed by 35 cycles of 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C. A final elongation step of 10 min at 72°C ended the cycle. Loci that were not resolved were tried again under different reaction conditions including varying MgCl₂ concentrations (1.5 and 3 mM) and annealing temperature (54 or 58°C). We visualized microsatellite alleles on acrylamide gels using a LICOR 4300 DNA analyzer and scored allele sizes with GeneProfiler, ver. 4.05 (LICOR Co.). As a size standard, we used amplified fragments of the Lambda phage following methods our laboratory adapted from Wang et al. (2010).

Of the 60 loci tested, 40 (Table 1) were well resolved after optimization attempts and were further tested on 34 individuals from the Chickasawhay River (Table S1, Supplemental Material) using the conditions described above. Genotypic frequencies for each locus were tested for Hardy-Weinberg equilibrium and linkage disequilibrium using the R package ‘genepop’ (Rousset et al. 2020) in R, version 3.6.3 (R Core Team 2020). We used a sequential Bonferroni correction (Rice 1989) to adjust statistical significance over multiple comparisons for a total alpha of 0.05. The number of alleles (N_a), observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated for each locus with GenALEx 6.501 (Peakall and Smouse 2012). Lastly, we used MICROCHECKER 2.2.3 (van Oosterhout et al. 2004) to test for potential scoring errors, allelic dropout, and null alleles. We also tested the 60 loci using the original reaction conditions in the Coal Darter (MMNS 875), the Channel Darter from a site east of the Mississippi River (MMNS 167), and the Channel Darter from a site in the Ouachita Highlands (MMNS 667). We did not test for Hardy-Weinberg equilibrium or linkage disequilibrium for these species given the small sample sizes per site (Table S1, Supplemental Material).

Results and Discussion

We found that 40 loci were polymorphic in Pearl Darters using the conditions we tested (Table 1; Table S2, Supplemental Material). The number of alleles per locus ranged from 4–31 with a mean of 11.5 and standard error (SE) of 0.91. Observed and expected heterozygosity values ranged from 0.235–0.939 and 0.287–0.949, with means of 0.818 (SE = 0.02) and 0.805 (SE = 0.02). Five loci were not in HWE (Paur014, Paur019, Paur032, Paur057 and Paur058) after a sequential Bonferroni correction. MICROCHECKER suggested these loci possessed null alleles as fewer heterozygotes were observed than predicted. We also found that four pairs of loci exhibited significant linkage disequilibrium (Paur002-Paur018, Paur014-Paur018, Paur005-Paur020, and Paur017-Paur042). Seventeen to 18 of these loci produced successful amplifications in Coal and Channel Darters (Table 2). In the Coal Darter, 17 loci amplified, although only 11 of these were polymorphic with 2–7 alleles per locus (average = 4.1; SE = 0.62). The Channel Darter from the eastern portion of its range was polymorphic at seventeen loci with 2–13 alleles per locus (average = 6.7; SE = 1.15) while the Channel Darter from the Ouachita Highlands was polymorphic at sixteen loci with 2–13 alleles per locus (average = 7.5; SE = 1.15). The microsatellite loci characterized by this project provide an important set of tools that will allow us to better understand the biology of the Pearl Darter and inform our management decisions through the assessment of genetic connectivity among populations, measuring levels of genetic diversity, testing for genetic bottlenecks and kinship studies. These loci should also prove useful in similar studies of the closely related species we tested.

Supplemental Material

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Table 1. Microsatellite loci characterized from the Pearl Darter *Percina aura* by Illumina paired-end sequencing in 2020. The summary statistics are reported for individuals (n = 34) from the Chickasawhay River. For each locus, the repeat motif, primer sequences (forward [F] and reverse [R] listed 5’ to 3’), the number of alleles (N_A) with size range in base pairs (bp; including the 19 base pairs from the M13 tail added to the forward primer), observed heterozygosity (H_O), and expected heterozygosity (H_E) is reported. The annealing temperature (T_A °C) for each locus is also indicated. Locus *Paur026* was only successfully amplified in Channel and Coal Darter, but is listed here to provide the primer sequences. Sequence data for these loci can be found in the NCBI Sequence Read Archive listed under BioProject accession PRJNA688648 and BioSample number SAMN17184339 (https://www.ncbi.nlm.nih.gov/bioproject).

| Locus   | Repeat motif | Primer sequence (5’–3’) | T_A (°C) | N_A (size [bp]) | H_O     | H_E     |
|---------|--------------|--------------------------|----------|-----------------|---------|---------|
| Paur002 | (TTCTC)      | F: CTTGACCTCAGCAGTGGCC   | 56       | 9 (240–280)     | 0.879   | 0.827   |
|         |              | R: CTTGATCTTCCAGTGGCC    |          |                 |         |         |
| Paur003 | (AATTTG)     | F: TGTACAGAGAAAAACTCTGTGTC | 56       | 6 (253–283)     | 0.667   | 0.712   |
|         |              | R: GCTCAATGCTGCTTGGG     |          |                 |         |         |
| Paur005 | (ATCT)       | F: GAAGATGTTCTCAGTCTG    | 56       | 17 (322–402)    | 0.880   | 0.903   |
|         |              | R: GATATGCTGCTCAACAGCG   |          |                 |         |         |
| Paur006 | (ACTG)       | F: GTGGACCGGCGATGATGCT   | 56       | 6 (246–266)     | 0.855   | 0.735   |
|         |              | R: CTATGGGCTGCTGATGGG    |          |                 |         |         |
| Paur011 | (ATCT)       | F: GAGATGTCATTGGTGGGTC   | 56       | 15 (368–424)    | 0.853   | 0.905   |
|         |              | R: CATGACTATCCTGCTGGG    |          |                 |         |         |
| Paur013 | (ATTT)       | F: CATACACCTGCAGTCTG     | 58       | 9 (181–213)     | 0.774   | 0.868   |
|         |              | R: AACACTATCTGGTCTAGC    |          |                 |         |         |
| Paur014 | (ATCT)       | F: GACATATGCTCAGTCTG     | 56       | 10 (252–292)    | 0.333   | 0.844   |
|         |              | R: GATCAATAAGTGTTAAGAGG  |          |                 |         |         |
| Paur016 | (ATCT)       | F: TGGTACACCTGCTGCTG     | 56       | 13 (319–379)    | 0.794   | 0.862   |
|         |              | R: TGTGCTATGGCTGCTGGG    |          |                 |         |         |
| Paur017 | (ATCT)       | F: TTGTGATGCTGCTACAGTC   | 54       | 15 (320–376)    | 0.875   | 0.898   |
|         |              | R: AACACATGCTGCTGCTG     |          |                 |         |         |
| Paur018 | (ATCC)       | F: AAACACCTAGCAGTATG     | 56       | 17 (132–216)    | 0.939   | 0.917   |
|         |              | R: TTGGTGGCTATATGTTG     |          |                 |         |         |
| Paur019 | (ATCT)       | F: TGGACATCTCATTATATAG   | 54       | 31 (299–505)    | 0.697   | 0.949   |
|         |              | R: GCAGAAGGTTTTGAAACAT   |          |                 |         |         |
| Paur020 | (AGGT)       | F: CATTTTACAAACTTTTCTG   | 54       | 14 (155–211)    | 0.882   | 0.885   |
|         |              | R: TCTAGTCTAGAATCTTCCCA  |          |                 |         |         |
| Paur022 | (TGCG)       | F: CTCAATTTTCTCTCATTCTG  | 54       | 8 (338–374)     | 0.724   | 0.782   |
|         |              | R: CCCACATTACACACCTTGGG  |          |                 |         |         |
| Paur023 | (ATGT)       | F: GCCCTAGCATGCTGCTCAC   | 56       | 7 (370–410)     | 0.594   | 0.554   |
|         |              | R: GAGATCCACATATCAAACAG  |          |                 |         |         |
| Paur026 | (ATTG)       | F: TTCACCTCTGGTACAAATGG  | NA       | NA              | NA      | NA      |
|         |              | R: AATGGTATTGCTTGAGGTTTC |          |                 |         |         |
| Paur027 | (TTTC)       | F: CTTGTAAGGGCTCTCTTTTA  | 56       | 12 (299–329)    | 0.765   | 0.777   |
|         |              | R: GATACGGGGGTACCTTGG    |          |                 |         |         |
| Paur028 | (TGGG)       | F: TACTGCAAGAGCTTCCAGG   | 56       | 10 (442–478)    | 0.938   | 0.851   |
|         |              | R: CACAGTTGGGCTTGGAGG    |          |                 |         |         |
| Paur030 | (TGGG)       | F: TCAGTTCTTGTGCTTCCG    | 58       | 11 (292–336)    | 0.735   | 0.859   |
|         |              | R: GACCCTCTGGTGCAAGTCC   |          |                 |         |         |
| Paur031 | (AAAG)       | F: AAGTCTCACCAGTGAATCG   | 56       | 14 (374–442)    | 0.931   | 0.902   |
|         |              | R: CAGGATGTGATTAGTAAATTCGTAATTAAGGG |          |                 |         |         |
| Paur032 | (ACAG)       | F: TCTCTCCGAGAAGCAGGAGGC | 56       | 25 (284–342)    | 0.706   | 0.926   |
|         |              | R: GCTGGCTATCCTACAGTGG   |          |                 |         |         |
| Paur033 | (ATCT)       | F: GGTTGATGCTGCTAGAGG    | 56       | 16 (208–288)    | 0.882   | 0.887   |
|         |              | R: GTGTAACATTACTAATAAAAATCTCGTGGG |          |                 |         |         |
| Paur034 | (ATCT)       | F: GTAATACCATGTAACTAAGGAGGC | 56       | 14 (292–348)    | 0.765   | 0.910   |
|         |              | R: TCGACTCTTCATCATCAGTGC |          |                 |         |         |
| Paur037 | (ACTG)       | F: TTTTCCAAAAGTGAGCAGTGC | 54       | 15 (326–390)    | 0.909   | 0.903   |
|         |              | R: GCGATCTTATTGAGCCTCTAGTC |          |                 |         |         |
| Paur038 | (ATAC)       | F: GCACATTTTCCATACTAATG  | 56       | 6 (341–377)     | 0.424   | 0.690   |
|         |              | R: CTCTCTCTGACACGAGGTTCC |          |                 |         |         |
| Paur040 | (ACTG)       | F: CATTCTCTGAGAACTCAAATGC | 56       | 7 (286–318)     | 0.636   | 0.776   |
|         |              | R: CAGACACTGAAAAGAATGCC  |          |                 |         |         |
| Paur042 | (AAAT)       | F: AATGGCCTTCTGATATCCATTC | 56       | 9 (198–234)     | 0.700   | 0.808   |
|         |              | R: CCAACTGTTGCTTTATCATGTGCTTCC |          |                 |         |         |
| Paur043 | (TTTC)       | F: GGGTAGACAAATCCTAACACC | 54       | 12 (412–460)    | 0.875   | 0.833   |
|         |              | R: CATATTAAAAGGTTAAGGTTGGG |          |                 |         |         |
### Table 1. Continued.

| Locus   | Repeat motif | Primer sequence (5’–3’) | \(T_A\) (°C) | \(N_A\) (size [bp]) | \(H_O\) | \(H_E\) |
|---------|--------------|--------------------------|-------------|----------------------|--------|--------|
| Paur045 | (ATT)\(_{12}\) | F: TTTGCTGCTACAGCTTTACATGG R: GACGAGATGTGGGATGAGGG | 58          | 8 (446–470)         | 0.656  | 0.805  |
| Paur046 | (ATG)\(_{13}\) | F: GACATGTCAGAAGCCGCTGC R: TCAATAAGGTGTTTGACGC | 56          | 10 (184–226)        | 0.750  | 0.832  |
| Paur047 | (TCC)\(_9\)  | F: TGGGAGTGAACTGAGGTGGCC R: ATGGCTTTTGTGAAAGGAGG | 56          | 6 (391–400)         | 0.333  | 0.451  |
| Paur048 | (AAC)\(_{13}\) | F: TAATTTCAAGTTAAGGTTTACGC R: AAATTCGGCGCCACAAGCC | 54          | 10 (328–361)        | 0.853  | 0.858  |
| Paur049 | (ATG)\(_8\)  | F: AGGTCAGGATGATGAGGAGG R: TGGGAGTGAACTGAGGTGGCC | 56          | 7 (259–286)         | 0.727  | 0.762  |
| Paur050 | (ATT)\(_{12}\) | F: GACATGTCAGAAGCCGCTGC R: TCAATAAGGTGTTTGACGC | 54          | 9 (230–263)         | 0.774  | 0.784  |
| Paur051 | (AT)\(_9\)    | F: GACATGTCAGAAGCCGCTGC R: TCAATAAGGTGTTTGACGC | 56          | 8 (345–366)         | 0.647  | 0.666  |
| Paur052 | (AAT)\(_{22}\) | F: GACATGTCAGAAGCCGCTGC R: TCAATAAGGTGTTTGACGC | 56          | 14 (231–279)        | 0.824  | 0.879  |
| Paur053 | (ATT)\(_9\)   | F: GACATGTCAGAAGCCGCTGC R: TCAATAAGGTGTTTGACGC | 56          | 6 (386–422)         | 0.594  | 0.597  |
| Paur054 | (AAC)\(_{19}\) | F: TCTAGCAAGGGCTTATGCTCC R: TCCAGTGCTGCTGCTGGGTGTC | 56          | 11 (471–504)        | 0.912  | 0.885  |
| Paur056 | (AAC)\(_8\)   | F: CAGTCTATACAGGGCTGCTGC R: CAATGCCTTACTACAGGGCTGCTGC | 56          | 4 (159–168)         | 0.235  | 0.287  |
| Paur057 | (AAT)\(_{13}\) | F: CTGCTTAATGAGGTTGTCGCAAG R: ACAATCAGGAGGAAATGCTCC | 56          | 15 (318–360)        | 0.594  | 0.888  |
| Paur058 | (ATT)\(_{16}\) | F: AAATTCGAGTTGTTTGGTGTGTCG | 56          | 14 (414–462)        | 0.581  | 0.889  |
| Paur059 | (AAT)\(_{18}\) | F: GAATTCCTCAATAAGGCTCC | 58          | 12 (242–281)        | 0.871  | 0.870  |

### Table S1. Information on Pearl Darter *Percina aurora*, Coal Darter *P. brevicauda*, and Channel Darter *P. copelandi* collected between 2004–2019 and used in this study to test the microsatellite loci characterized from the Pearl Darter by Illumina paired-end sequencing. Samples either came from the Mississippi Museum of Natural Science tissue collection (MMNS accession number provided) or from our own field collections.

### Table 2. Results of testing the sixty Pearl Darter *Percina aurora* microsatellite loci in the Coal Darter *P. brevicauda* and representatives of the Channel Darter *P. copelandi* from east of the Mississippi River and the Ouachita Highlands. Loci that were successfully amplified in 2020 are reported here. Sample sizes for these groups were 9, 10, and 10, respectively. For each locus, we report the number of alleles (\(N_A\)) and size range in base pairs (bp), including the 19 base pairs from the M13 tail added to the forward primer. Loci that failed to amplify are listed as NA.
(indicated by a field number). Sample sizes (N) are listed by site along with the geographic location (latitude/longitude) and a description of the locale (Site). Locations of Pearl Darter samples are in the first 11 rows in the table. Samples of the Coal Darter were from Collection MMNS 875. Channel Darter locales were Collections MMNS 167 and MMNS 667 for east of the Mississippi River and the Ouachita Highlands, respectively.

Found at DOI: https://doi.org/10.3996/JFWM-21-008.S1 (93 KB DOX).

**Table S2.** Genotype data for the microsatellite loci successfully amplified in Pearl Darter *Percina aurora* successfully amplified in Pearl Darter *P. brevicauda*, and Channel Darter *P. copelandi* in 2020. Individuals are identified with a sample name (ID) and species. Genotype data for each individual are listed by locus with zeros representing missing data or the fact that the locus did not amplify for that species.

Found at DOI: https://doi.org/10.3996/JFWM-21-008.S2 (76 KB XLSX).

**Reference S1.** Schaefer JF, Mickle P. 2011. Assessment of putative Pearl Darter populations in the upper Pearl River. Jackson, MS: U.S. Fish and Wildlife Service, Agreement No.401819G540, Final report.

Found at DOI: https://doi.org/10.3996/JFWM-21-008.S3 (729 KB PDF).

**Reference S2.** Wagner MD, Clark SR, Schaefer JF, Piller KR. 2017. Status survey for the Pearl Darter in the state of Mississippi. Jackson, MS: U.S. Fish and Wildlife Service, Agreement No. MS-E-F16AP00442, Final report.

Found at https://doi.org/10.3996/JFWM-21-008.S4 (5.51 MB PDF).

**Acknowledgments**

This work was supported by funding from the U.S. Fish and Wildlife Service Section 6 Cooperative Agreement with the Mississippi Department of Wildlife, Fisheries, and Parks. Collection permits were issued by the Mississippi Department of Wildlife, Fisheries, and Parks. Permission was obtained to collect individuals for DNA processing in two birds and a snake. PLoS ONE 7:e30953.

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