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PERMANENT GENETIC RESOURCES NOTE

Eight novel tetranucleotide and five cross-species dinucleotide microsatellite loci for the ornate chorus frog (*Pseudacris ornata*)

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Abstract

We describe the cloning and characterization of eight novel tetranucleotide microsatellite loci in the ornate chorus frog (*Pseudacris ornata*). We also screened 26 loci from GenBank that were isolated from other *Pseudacris* species and obtained consistent product from five of these dinucleotide loci. All loci are polymorphic. In our sample of 26 frogs from a natural population, polymorphism ranged from 1 to 22 alleles per locus with expected heterozygosities ranging from 0 to 0.958. These loci enable high-resolution studies of *P. ornata*. Moreover, cross-species amplification success suggests they will also be useful for other chorus frog species.

Keywords: biotin enrichment, chorus frog, microsatellite, *Pseudacris ornata*

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The ornate chorus frog (*Pseudacris ornata*) is a small terrestrial frog occurring in the coastal plains of the southeastern USA. Moriarty & Cannatella (2004) suggest some degree of phylogeographical structure in this species although gene flow and high-resolution population genetic structure has yet to be investigated. Moreover, this frog exhibits a striking green/brown colour polymorphism, offering a unique opportunity to study the natural selection of this trait in wild populations (Hoffman & Blouin 2000). The microsatellite markers we develop here will provide important tools to test for evidence of natural selection as well as to investigate the evolutionary history and population structure in *P. ornata*.

Our microsatellite enrichment protocol followed that of Hoffman *et al*. (2003) and is described here in brief. Genomic DNA was extracted using a DNeasy Tissue Kit (QIAGEN). Random DNA fragments (~200–2500 bp) were generated using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), using the K6-MW primer and protocol (Macas *et al*. 1996). Microsatellite enrichment of the fragments employed a 3′-biotinylated $(GATA)_8$ repeat motif bound to streptavidin-coated particles (Promega) enriched via magnetic separation. Enriched product was cloned using TOPO TA cloning kits (Invitrogen). PCR screening followed the procedure from Cabe & Marshal (2001). Briefly, two PCRs were carried out per sample. One PCR included T3 and T7 primers while the second included an additional $(GATA)_8$ primer. The PCR products of these two reactions were visualized side-by-side on a 2.0% agarose gel. Positive clones give a distinctive smear in contrast to that produced in the T3–T7 reaction. We screened 1056 clones of which 183 (17%) indicated the presence of a $(GATA)_n$ repeat. Sequences were aligned and edited with Sequencher version 4.1 (Gene Codes Corporation) to correct for errors and to identify microsatellite loci. Primers flanking the repeat motif were developed for 45 unique loci using Primer 3 (Rozen & Skaletsky 2000). Of the 45 loci, eight (18%) were polymorphic and amplified consistently. Additionally, 26 loci developed in other *Pseudacris* species (J. Van Buskirk, unpublished data; GenBank Accession nos EF190892–EF190917) were screened for amplification in *P. ornata* and five (19%) of these were polymorphic and amplified consistently.

For genotyping, six of these 13 loci (Por 002, Por 010, Por 026, Por 105, Por 106, and Por 111) were directly labelled (DL) on the 5′-end of the forward primer with a fluorescently labelled dye. The seven remaining loci were labelled with

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Table 1 Basic information for 12 polymorphic microsatellite loci used in this study (see text for information on Ptri29). Locus name, GenBank Accession number, species cloned, repeat motif, primer sequence, and annealing temperature (T_a) are shown. Population data (number of diploid genotypes obtained, n; observed and expected heterozygosities; allele size range; and number of alleles, N_A) for *Pseudacris ornata* individuals collected from Colleton County, South Carolina, are given. Allele sizes do not include 21 bp M13 primer. Loci cloned from *P. crucifer* were developed in J. Van Buskirk *et al*. (unpublished data)

Locus	GenBank Accession no.	Species cloned	Repeat motif in clone	Primer sequence (5′–3′)	T_a	Allele size range	n	N_A	H_O	H_E
Por002	EU910888	*P. ornata*	$(GATA)_{11}(GATA)_4$	F-AGAAACAGACAGACAGGAAGAGA R-TTCAAATCTCAAAGCATTTTTGT	52	300–352	20	7	0.632	0.791
Por010	EU910889	*P. ornata*	$(GATA)_2(GATA)_2$ $(GATA)_2(GATA)_7$	F-ATGTGGCAAAAGGTTGAAAA R-ACACAAGGTTTCCTGATCCA	55	366–406	22	9	0.818	0.405
Por026	EU910890	*P. ornata*	$(GATA)_2$ $(GATA)_{13}$	F-GATATACACACTTTGTGGCAGAAT R-AGGACTTGACCCCTGGATAG	53	98–144	20	9	0.950	0.872
Por105	EU910891	*P. ornata*	(GATA) imperfect	F-ACGACCATAGTGGGCTATCA R-TGGTTAGGCCATTGTCAAGC	52	162–280	25	14	0.920	0.878
Por106	EU910892	*P. ornata*	(GATA) imperfect	F-ATGTCGGTAAGAAACTCTTAATGT R-TTGCTGTCCTTGGATTAGGC	62	286–686	23	22	0.826	0.958
Por111	EU910893	*P. ornata*	(GATA) imperfect	F-AAAGGAATTATTACATTCTGGCATTC R-TGTGTGAACTAGTTTAAAAAACACA	53	260–390	20	10	0.700	0.858
Por151	EU910894	*P. ornata*	$(GATA)_2(GATA)_8$	F-ACCAGGACAGTCAAACAGAG R-CCGATGTGGATAGATGGA	54	110–198	25	13	0.760	0.910
Por165	EU910895	*P. ornata*	$(CA)_7(TA)_4(GATA)_6$ $(GATA)_4$	F-CTCTGTCCTGTGCATTGAA R-TAGGTAGGAAAAACCAAGCA	54	236–290	22	10	0.682	0.883
Pcru09	EF190895	*P. crucifer*	$(CA)_{15}$	F-ACAATTAACACAATTGGCTCTCC R-AGTAAGGGAGCGTGCAAGAG	54	143–153	25	3	0.600	0.635
Pcru10	EF190896	*P. crucifer*	$(CA)_{13}$	F-CATTTGGAACAAAGCAGCAAG R-GTGGAGTTCCAGCCTAATGC	54	392–499	21	5	0.190	0.838
Pcru14	EF190899	*P. crucifer*	$(GT)_{13}$	F-GATCAGACAGTCTACAGTAATGAGGAG R-CATAACACAGGGCAACCAAG	54	179–183	26	3	0.231	0.278
Pcru24	EF190902	*P. crucifer*	$(CA)_{17}$	F-TGCCATGGGGATGTTATATG R-CGAGCTATAGGAAAAGGCAGAG	54	244–278	25	6	0.360	0.322

an M13 (–21) tail (5′-TGTAAAACGACGGCCAGT-3′) attached to the forward primer for fluorescent labelling (M13) of PCR product (Schuelke 2000). PCRs were performed in 20 μL volumes and contained approximately 10 ng DNA template, 1× PCR buffer, 0.2 μM each dNTP, and 1 U *Taq* polymerase. Other PCR components were 2.5 mM $MgCl_2$ (DL) or 2 mM $MgCl_2$ (M13), 0.3 μM each primer (DL) or 0.5 μM (M13), 0.13 μM M13 tag and 0.5 μM reverse primer (M13). Thermocycling conditions for loci Por105 and Por106 are as follows: initial denaturation at 95 °C for 4 min; 40 cycles of 95 °C for 30 s, optimized annealing temperature (T_a; Table 1) for 30 s, 72 °C for 30 s, followed by an extension of 7 min at 72 °C. Following initial denaturation (95 °C for 4 m), locus Por111 was amplified with the following conditions: 48 °C for 45 s; 69 °C for 60 s; 35 cycles of 95 °C for 30 s, 53 °C for 30 s (decreasing 0.1 °C/cycle), 72 °C for 30 s, followed by an extension of 7 min at 72 °C. All other loci had the following thermocycling conditions: initial denaturation of 5 min at 95 °C; 36 cycles of 95 °C for 45 s, T_a for 45 s, 72 °C for 45 s; eight cycles of 95 °C for 45 s, 52 °C for 45 s, 72 °C for 45 s, followed by an extension t of 20 min at 72 °C. Amplified PCR products were sized using the CEQ 8000 genetic analysis system and software (Beckman-Coulter).

To test genetic variability, we amplified 26 individuals from a natural population of *P. ornata* from Colleton County, South Carolina. Genetic analyses were carried out using GenePop version 3.3 (Raymond & Rousset 1995). One cross-species amplified locus (Ptri29; forward primer CACCATCTGGGAGGTGCTAC; reverse primer ATGTCAAAGCCGACCAAATC; T_a = 54 °C; GenBank Accession no. EF190917) was fixed in this population. However, preliminary data from other populations indicate that it is polymorphic. Among other loci, number of alleles per locus ranged from 3 to 22 averaging 9.25 per locus. Expected heterozygosities ranged from 0.278 to 0.958 (Table 1). Tests for departures from Hardy–Weinberg equilibrium (Fisher's exact tests) were corrected for multiple comparisons by applying a sequential Bonferroni correction (Rice 1989) and revealed no strong deviation at the 5% level. Fisher's exact tests for genotypic disequilibrium revealed one comparison that displayed a significant association at the 5% level after applying a sequential Bonferroni correction. However, we feel that this association is trivial because preliminary

data from other populations indicate no evidence of linkage between these loci. To our knowledge, these are the first microsatellite loci developed specifically for *P. ornata*. Moreover, our ability to cross-amplify among congeneric species indicates that these 13 loci will be useful in a plethora of other chorus frogs.

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