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Identification of Microsatellite DNA Markers for use in Parentage Analysis of *Ambystoma opacum* (Marbled Salamander)

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Abstract

Polyandry is well documented as a female driven mating phenomena among several species in the animal kingdom. Further, this extends to a subset of species in the genus *Ambystoma*, though it does not include the marbled salamander (*Ambystoma opacum*). In an attempt to help understand mating systems across this genus, this study aimed to characterize microsatellite DNA markers, originally developed for the spotted salamander (*Ambystoma maculatum*), for cross-species use and eventual parentage analysis in *A. opacum*. DNA was extracted from wild female adults who were found hovering egg masses. Moreover, the egg masses were harvested and DNA was also extracted from all embryos. 6 microsatellite markers were tested, and yielded limited success in amplification. A review and optimization of amplification methods and more markers would be paramount to the continuation of this preliminary research.

1. Introduction

Mating system research over the last 70 years has centered on male driven approaches[1,2]. Further, females were originally thought to gain very little from mating with more than one male[1]. More recently, several studies recognize the idea that there are many potentially selective factors for females, suggesting a reevaluation of mating systems[1,3]. Moreover, many of these mating systems, including polyandry, are now being looked at as a product of female driven mechanisms[2,4].

Polyandry, or the postulate that females will mate with multiple males, was first noted in insects[5]. Some hypotheses for this observation include the intrinsic male-quality hypothesis, sperm limitation, and genetic bet hedging. The first of these hypotheses suggests that females will mate with the most viable male present, which can be expressed both socially and physiologically[6]. The second hypothesis, that of sperm limitation, suggests one male's sperm may not be enough to fertilize all of a female's eggs, thus multiple mating would dispel this limitation[7,8]. Finally, genetic bet hedging is the hypothesis that females may mate with as many males as possible in order to spread genetic diversity among offspring[9]. These hypotheses are simply a few of many, but give keen insight into how expansive polyandry can be.

In *Ambystoma*, a genus of salamanders localized to eastern North America and the Appalachian Mountains, polyandry has been observed heavily[3,4,10]. Further, it has previously been reported that as many as 8 fathers have been found in one clutch of the spotted salamander (*Ambystoma maculatum*)[4]. This phenomenon is not particularly surprising, as all members of *Ambystoma* are known to have internal sperm transfer[4]. Moreover, this leads to the potential for aggregate breeding and larger amounts of genetic mixing, making polyandry an unsurprising reproductive strategy[4]. The issue to date is that limited research has been done to prove polyandry in other species of this genus, including the commonly found marbled salamander (*Ambystoma opacum*).

Several methods for studying polyandry in insects have since been adapted and applied to other animal species, justifying polyandry as a very common mating system[1,4]. Genetic parentage analysis is one of the most common, and it involves the use of microsatellite DNA markers[11]. However, currently there are minimally available microsatellite markers developed specifically for *A. opacum*[12]. In this study, we aim to characterize viable cross-species microsatellite markers for *A. opacum* using previously developed markers for *A. maculatum*[12,13,14]. We hope that these

markers will be used to address the many hypotheses related to multiple paternity in the *Ambystoma* genus, and invariably aid in the preservation of these species.

2. Methods and Materials

2.1 Specimens

Egg masses and associated mothers from one population of *A. opacum* were collected from the Savannah River Site in Aiken, GA in November of 2019. Tail tissue was collected from females at the field site and stored in 70% ethanol on ice. Embryos were placed in plastic cups with moist soil. Specimens were then transported to UNC Asheville where tissue samples were stored at -20°C. Embryos were reared to hatching in aerated, aged tap water in the Aquatic Vivarium. Hatched embryos were euthanized with an overdose of Tricaine-S nerve toxin. Hatchlings were kept in 70% ethanol and stored at -20°C until DNA could be extracted.

2.2 DNA Extraction and Amplification

DNA was extracted from specimens using Qiagen DNEasy DNA extraction kits™ (Qiagen, Hilden, Germany) before the ND-2,000 was used to perform nanodrop spectrophotometry to confirm extraction (ThermoFisher Scientific Inc., Waltham, MA). For this experiment, six microsatellite loci were amplified using a modified PCR recipe (Table 1, Table 2)[13,14]. We then used a touchdown protocol, which calls for the following: denaturation at 95˚C for 5 min followed by annealing of 10 cycles at 95˚C for 20 s, 60-50˚C for 60 s, and 72˚C for 40 s stepping down 1˚C each cycle from 60 to 50˚C; 20 cycles at 95˚C for 20 s, 48˚C for 20 s, and 72˚C for 40 s; and a final extension at 72˚C for 10 min[15]. All PCR was done using Applied Biosystems SimpliAmp™ PCR Thermal Cyclers (Applied Biosystems, Foster City, CA). Untagged primers were initially used and these PCR products were run on 115 mL 1.5 % agarose gels, using 5 µl of amplified DNA, 1 µl of 6x loading dye, and an Invitrogen 100bp DNA ladder (ThermoFisher Scientific Inc., Waltham, MA).

Table 1. *A. maculatum* microsatellites used for amplification in *A. opacum*.

Locus (M13 Dye)	Primer Sequences (5'-3')	Repeats	Size Range (base pairs)	Number of Alleles Reported in Source	Source
Ama34 (PET)	F: GAACCGCTTGTTCAGTATAG	Dinucleotide	90-118	12	Wieczorek et al. 2002
	R: TCAGGTAAGCACGATTTAAC				
Ama61 (VIC)	F: CCAATCTAGTGCTCTCTCCC	Dinucleotide	229-255	5	Wieczorek et al. 2002
	R: ACATACTCCCCTCTGCTCAC				
AmaD42 (NED)	F: GATGGAAAATCAATCAAGTGTG	Tetranucleotide	125-160	7	Julian et al. 2003
	R: TAACTAGCTGTCAATCGCTCTC				
AmaD95 (NED)	F: AGCGCTTAGATACCTCTCGG	Tetranucleotide	125-210	15	Julian et al. 2003
	R: TATTGCATGTGAATATCGATGG				
AmaD321 (6-FAM)	F: GATGCCTTGAAACTTGTTCTTC	Tetranucleotide	120-175	13	Julian et al. 2003
	R: TGGTGCATCTATATTCCTCAAG				
AmaD328 (6-FAM)	F: CCCCAGTTTGTTTGTTTTGTAG	Tetranucleotide	260-305	10	Julian et al. 2003
	R: ATGACCCTTCCAGCTAATACAG				

Table 2. PCR recipe modified from Julian et al. 2003.

Ingredient	Working Concentration	16 µl reaction
PCR Buffer (Phenix)	1x 15mM MgCl	1.6 µl
Taq polymerase (GoTaq Promega)	1.0 unit	0.2 µl
dNTPs 10mM	0.25 mM	0.4 µl
Forward primer 10mM (M13 Tagged)	0.25 µM	0.4 µl
Reverse primer 10mM	0.5 µM	0.8 µl
M13 Dye	0.25 µM	0.4 µl
Water	NA	10.2 µl
DNA	2.0 µl	2.0 µl

2.3 Genotyping and Analysis

PCR was epeated using forward primers that were previously 5' tagged with an M13 dye before being sent for fragment analysis to North Carolina State University's Genomic Sciences Laboratory (GSL)[16]. These samples were sent in several cohorts, ranging from the early spring of 2020 to late fall of 2020. Samples contained either pooled PCR product or individual PCR product, for a total DNA volume of 4 µl. Samples also contained 0.5 µl GeneScan™ 500 LIZ® size standard (Applied Biosystems, Foster City, CA), and 5.5 µl Hi-Di™ Formamide (Applied Biosystems, Foster City, CA).

Peak files from fragment analysis were all screened using the microsatellite plugin in Geneious Prime® 2020.1.2 (Biomatters Ltd.). LIZ® size standard peaks were manually adjusted to fit as a ladder before the binning function was used to call genotypes. Peaks were then manually adjusted to incorporate all successful amplification within microsatellite ranges.

3. Results

From gel electrophoresis, we can see that Ama61 and AmaD95 might have been amplified (Figure 1). Additionally, both of these microsatellites appear to have multiple oligonucleotide dimers along their respective lanes (Figure 1).

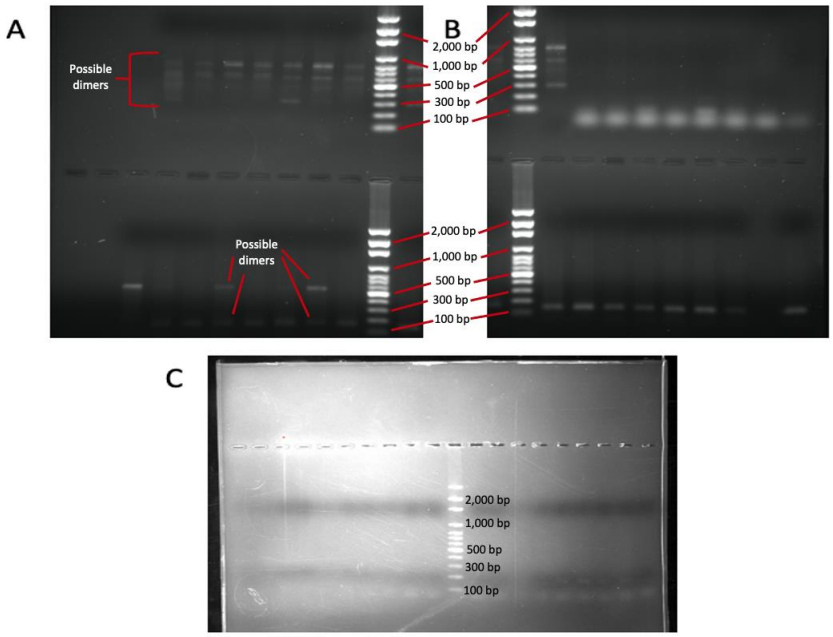

Figure 1. 1.5% agarose gel images under UV light for (A) Ama61 on top and AmaD95 on bottom, (B) Ama34 on top and AmaD42 on bottom, and (C) AmaD321.

We found limited numbers of alleles at the six amplified microsatellites, though 6 were amplified at Ama34 (Table 3). Additionally, Ama34 proved to have the most success with 9.5% of samples amplified; most other loci showed no amplification at all. Unfortunately, 21 samples that used AmaD95 primers were corrupted in transit, and these data may be negligible. Though there was limited amplification, several peaks were also dismissed due to size.

Table 3. Microsatellites amplified in *A. opacum*.

Locus	Number of Alleles Found at Locus	*n*	Percent Amplified
Ama34	6	63	9.5%
Ama61	0	63	0
AmaD42	1	63	1.6%
AmaD95	0	63*	0
AmaD321	0	164	0
AmaD328	0	82	0

*Some samples corrupted in transit

4. Discussion

Overall, the data from these experiments suggest that more testing should be done using the amplified regions of Ama34 and AmaD95, though other microsatellites should also be considered for parentage analysis of *A. opacum.* This is largely due to the contradictory information from fragment analysis and gel electrophoresis, which disputes previous documentation of amplification in *A. opacum*[12].

Amplification of both the Ama61 and AmaD95 loci exhibited by the gels suggests potentially positive results, but the clear dimers suggest inefficient primer bonding. However, because there is some amplification for the locus AmaD95 on the gel, and a few samples were corrupted before fragment analysis, it may be advantageous to reevaluate this microsatellite. Additionally, fragment analysis was mostly unsuccessful, but we did see minor peaks for the

Ama34 locus. For the purposes of using these loci in parentage analysis, it would be most suitable to identify reliable and consistent microsatellites. So, in the case of both Ama34 and AmaD95, fine tuning of PCR methods may prove useful to increase amplification and to benefit future research.

Most notably, it may be beneficial to adjust PCR mixes and thermocycler conditions. DNA extraction was generally successful as indicated by 260 nm/280 nm absorbances, though concentrations of DNA were inconsistent. Moreover, concentrations ranged from 20 μg/mL to 650 μg/mL, therefore these methods may benefit from the dilution of DNA to a standard working concentration for PCR mixes[17]. The thermocycler conditions were also originally set for Central American reptiles, and may not be ideal for this system[15]. Revisiting the thermocycler conditions, including denaturation temperatures and annealing temperatures, might not only reduce primer dimer occurrence, but yield feasible peaks from greater concentrations of target sequence amplification[18].

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