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Determination of microsatellite loci to be used for parentage analysis in *Ambystoma annulatum*.

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Abstract

Ambystomatidae is a family of salamanders commonly known as the mole salamanders. Most of the species within this family are characterised by polyandrous mating, in which females mate with and can lay clutches fathered by multiple males. The extent of polyandrous mating is thought to vary based on environment and with the occurrence of parental care, which varies across the genus *Ambystoma*. The extent of polyandrous mating can be explored using parentage analysis to determine how many males father a female's clutch. This study screens four microsatellite markers that have been identified and used for other species in the genus *Ambystoma* to determine their usefulness in the species *Ambystoma annulatum,* so that it may eventually be compared to other species in the genus.

1. Introduction

Polyandrous mating occurs when females mate with multiple males. While polyandry was originally considered an outlier, it is now considered a ubiquitous global phenomenon.[1] Polyandry has both direct and indirect benefits for males and females. In species where males provide nuptial gifts such as some insects, females benefit by receiving extra nutrition when they mate multiply.[9] In species where parents provide care to their young such as some birds, less energy is spent on this care when more individuals are involved. Indirect benefits of polyandrous mating include higher genetic diversity among offspring, fertilization of more eggs, and higher chance of encountering a mate with favorable genetics.[2]

In *Ambystomatidae*, males and females abandon eggs after laying, and males do not give nuptial gifts, so it can be assumed that the benefits of polyandry in this family are indirect. Multiple mating has also been demonstrated to confer a 4% increase in survival for offspring in *A. opacum* clutches.[3] The amount of polyandry varies across species as well as environmental conditions. [1,2] Studies have found that *A. maculatum* have clutches sired by multiple males approximately 70% of the time, however polyandrous mating rates for other species are unknown. [4]

Parentage analysis is an important tool that can allow us to quantify the amount of polyandry with which a female engages. Since it is extremely difficult to observe and track all of an individual's potential mates, parentage analysis of offspring serves as a useful technique for counting how many matings may have occurred. Parentage analysis commonly uses microsatellite markers, which are small regions of DNA repeats. Microsatellite markers are especially useful for parentage analysis since they are easily assayable and follow Mendelian rules of segregation.[5] Microsatellite markers have been developed for several species within the genus *Ambystoma*, including *A. annulatum*.[6] The goal of this study is to identify microsatellite markers that will work in *A. annulatum* for eventual use in parentage analysis by screening primers that have been shown to work by others.

2. Methods

2.1 Specimens

Gravid females were collected from Ozark National Forest in September 2019 by RE Hale. Females were then paired and placed in plastic tubs overnight to deposit eggs. Embryos were reared in the lab until hatching then euthanized in MS-222, a drug that is commonly used to sedate or euthanize fish. Tail tissue (~0.5cm) was collected from adult females and stored at -20° C until DNA was extracted.

2.2 DNA Extraction

DNA was extracted from females F001 through F012 (n=12) from thawed tail cuttings using Qiagen DNEasy DNA extraction kits™ (Qiagen, Hilden, Germany) and quantified using spectrophotometry. Four microsatellite loci were amplified using PCR: AmaD42, AmaD321, AjeD23, and AjeD162, all described by Peterman et al. and chosen based on primers that were availiable.[6] The PCR recipe (Table 1) was modified from one used by Julian et al.[7] One female (F006) was removed from the study due to low DNA extraction yield.

Table 1: PCR Recipe based on Julian et al. (2003)

Ingredients	Final [ ] in reaction	16ul reaction	With Dyes
PCR Buffer	1x	1.6 ul	1.6ul
Taq	1.0 units	0.2 ul	0.2ul
dNTPs 10mM	0.25 uM	0.4 ul	0.4ul
M13 Forward Primer 10uM	0.25uM	0	0.4ul
M13 Tagged Dye	0.25uM	0	0.4ul
Forward primer 10uM	0.5 uM	0.8 ul	0
Reverse primer 10uM	0.5 uM	0.8 ul	0.8ul
Water		10.2 ul	10.2ul
DNA		2 ul	2ul
		16ul	16ul

2.3 Thermocycler Protocol

A touchdown protocol originally developed by RG Reynolds was applied using SimpliAmp™ PCR Thermal Cyclers (Applied Biosystems, Foster City, CA).[8] The PCR protocol was run as follows: denaturation at 95˚C for 5 min; 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 [8].

2.4 Genotyping

PCR products of all four loci were sent to the North Carolina State University Genomic Sciences Laboratory for fragment analysis. Each sample contained 4ul of PCR product, 0.5ul GeneScan™ 500 LIZ (Applied BioSystems, Foster City, CA) and 5.5ul Hi-Di™ Formamide (Applied BioSystems, Foster City, CA). Primers were amplified on eleven *A. annulatum* females. Information on primers can be seen below in Table 2. Data files were processed in Geneious Prime version 2020.1.2 (Biomatters Ltd., Auckland, New Zealand) using the microsatellite plug-in.

Table 2: Microsatellite Information for Primer Sets Screened in the Study.

Locus	Size of Repeats (bp)	Size Range (bp)	Dye	Reference
AmaD42	4	125-160	NED	Julian et al. 2003
AmaD321	4	120-175	VIC	Julian et al. 2003
AjeD162	4	150-170	PET	Peterman et al. 2012
AjeD23	4	123	6-FAM	Peterman et al. 2012

3. Results

3.1 Gel Electrophoresis

Results from gel electrophoresis can be seen in Figure 1.

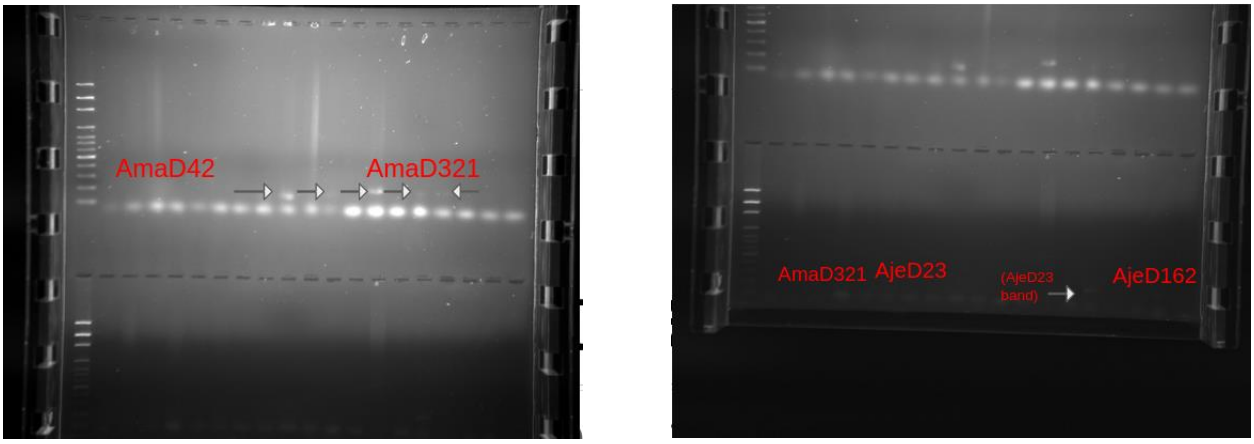

Figure 1. Left, upper lanes: Results of gel electrophoresis on primers AmaD42 (lanes 2-12) and AmaD321 (lanes 13-20 and lower lanes 2-4) from left to right. Right, lower lanes: Results of gel electrophoresis on primers AjeD23 (lanes 5-16) and AjeD162 (lanes 17-20) from left to right. Lane 1 contains ladder (100bp - 3Kbp). All fragments appear to be around 200bp in size. The bands staining consistently across all samples at < 100 bp represent primer. Bands > 100 bp represent amplified microsatellite product.

For each AmaD321 and AmaD42, 2-3 bands can be seen, while AjeD23 only appears to have 1 band. Not all samples for AjeD162 were run due to limited space on the gel.

3.2 Genotyping

Peak data for AmaD321 and AmaD42 from analysis in Geneious Prime 2020.1.2 can be seen below in Figures 2 and 3. The peaks for AmaD321 showed inconsistent amplification and low magnitude (Figure 2) and may not indicate the presence of DNA sequences in the sample. In contrast, the peaks for AmaD42 (Figure 3) are of similar magnitude as those of the ladder and show greater variability. Other loci did not show any peaks.

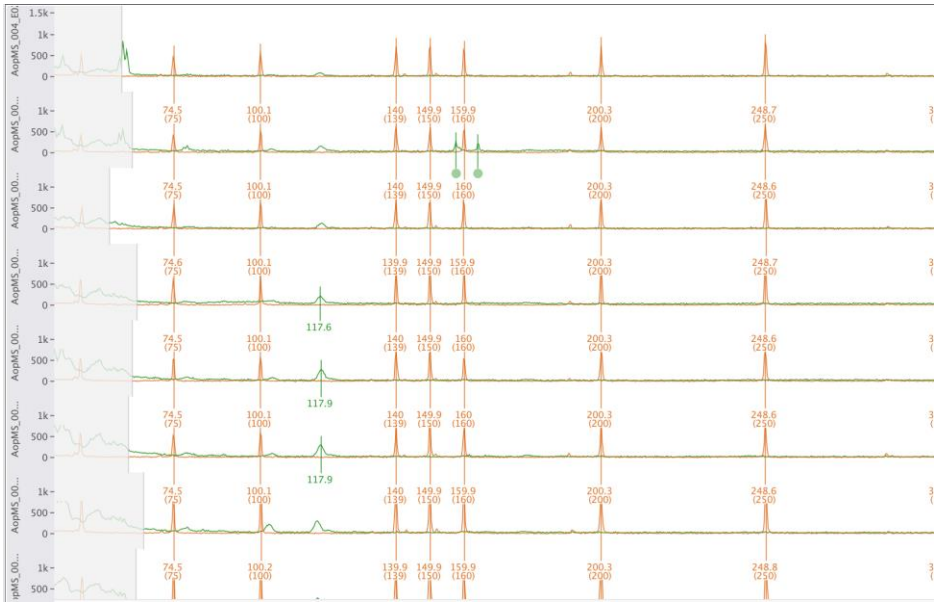

Figure 2. Peak data for AmaD321 with peaks shown in green. The numbers associated with each peak represent fragment length. The orange peaks are representative of a standardized ladder used. .

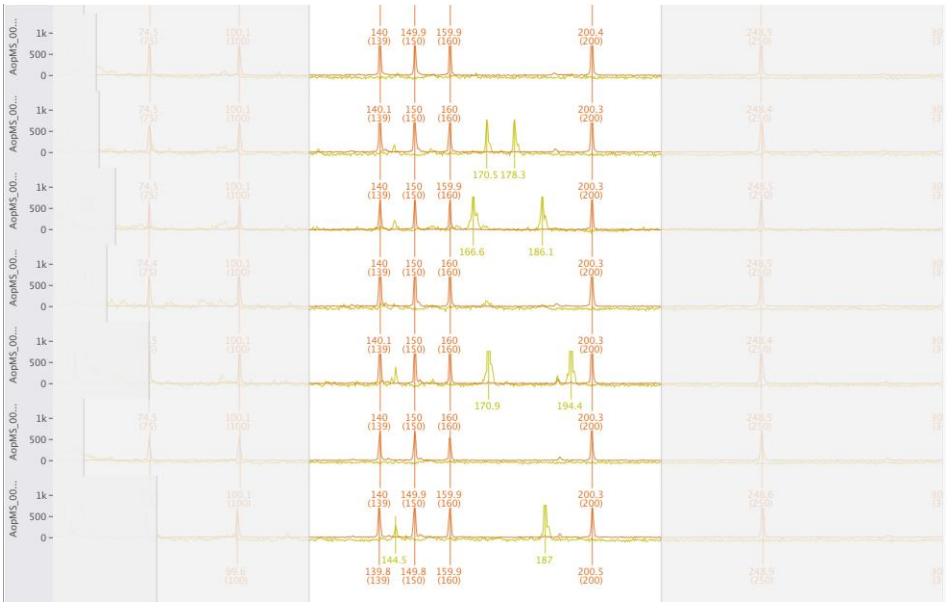

Figure 3. Peak data for AmaD42 with peaks shown in yellow.

4. Discussion

Of four microsatellite loci tested, only two were shown to be amplified in *A. annulatum* females. All primers used were selected from a list of 25 primers shown to be amplified in *A. annulatum* by Peterman et al.[6] The loci AmaD42 was shown to be amplified in 2-3 females based on gel electrophoresis and 4 based on fragment analysis. AmaD321

was shown to be amplified in roughly 3 females based on gel electrophoresis and, while peaks were seen in fragment analysis, these peaks are abnormally small and may represent an anomaly. AjeD162 shows faint amplification based on gel electrophoresis only, and AjeD23 did not show amplification via either method.

One source of discrepancy between the results found in this study and the results found by Peterman et al. could be due to differences in PCR recipe used. The PCR recipe used by Peterman et al. included 2mM $MgCl_2$ as well as bovine serum albumin.[6] In our recipe, the $MgCl_2$ was combined in the PCR buffer and no bovine serum was added. The addition of either of these to the PCR recipe may result in amplification of loci across more samples.

Future work should be carried further analyzing AmaD321 and AmaD42 on the same females as well as their offspring. AjaD162 should also be analyzed further as it showed slight amplification on gels. Other primers referenced in Peterman et al. should also be analyzed including AcroD300 and Atex65 since they were shown to amplify in numerous samples.[6] The results of this study as well as future studies can then be used to determine which primers to use for parentage analysis, however the results of this study are not enough to determine the usefulness of any primers tested. Had the primers tested shown strong peaks across samples tested, such as those shown in AmaD42, then these primers could then be used on samples from both mothers and embryos to carry out parentage analysis.

5. Acknowledgements

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6. References

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