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Identification of Polymorphic Loci in *Ambystoma annulatum* and Review of Cross-species Microsatellite Use in the Genus *Ambystoma*

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We screened 74 published microsatellite primers in *Ambystoma annulatum*, a species for which no microsatellite markers have been developed. Overall, we had a moderate success rate, identifying 11 polymorphic microsatellites previously developed in four different species of *Ambystoma*. We also conducted a review of the literature, collecting all published cross-species applications of microsatellite markers within the genus *Ambystoma*. From this, we identified 20 loci that have amplified in three or more species. Our synthesis of microsatellite use within the genus *Ambystoma* should prove valuable to future molecular research, especially in species without developed markers and for studies in species that may already have microsatellites, but are being conducted far from the region where individuals were collected for original development of species-specific loci.

MICROSATELLITES have become very prevalent in molecular ecological studies of amphibians over the last ten years, and have been used to differentiate species (Bogart et al., 2007), study reproductive ecology (Tennessen and Zamudio, 2003; Gopurenko et al., 2006), assess population history (Spear et al., 2006), test hypotheses concerning the evolution of phenotypes (Wang and Summers, 2010), measure mutation rates and inbreeding (Williams et al., 2008; Bulut et al., 2009), describe dispersal (Stevens et al., 2006; Zamudio and Wieczorek, 2007; Bartoszek and Greenwald, 2009), and relate population genetic patterns with landscape features (Spear et al., 2006; Giordano et al., 2007; Murphy et al., 2010). Microsatellite markers have been extensively used for such studies because they are highly polymorphic, bi-parentally inherited markers that are generally believed to be selectively neutral (Jarne and Lagoda, 1996). Although powerful tools for a variety of questions, microsatellites are costly and time consuming to develop, and are generally developed for use with a single target species. As such, studies using microsatellites have largely been limited to species in which primers have been previously developed. Additionally, because microsatellite primers are developed and optimized from a single population, the transferability of these primers to novel geographic regions or populations can be problematic. When confronted with these obstacles, researchers may have a limited number of informative loci, and are left with three options. First, they can develop new primers, requiring substantial time and resources. Second, they can screen primers developed in related species. There are no guarantees that the same priming region and polymorphic microsatellite will exist, and often cross-amplified (heterologous) markers have fewer alleles than the species in which they were originally developed (Ellegren et al., 1995; Hutter et al., 1998; Keever et al., 2008). If neither of the previous avenues are pursued or are successful, the study will have to proceed with a reduced number of microsatellites, possibly hindering the resolution of analysis and limiting conclusions that can be drawn.

With increasing computational abilities and advanced algorithms (Beerli, 2006; Faubet and Gaggiotti, 2008), researchers can gain increased resolution in their analyses by increasing the number of polymorphic loci sampled (Manel et al., 2005). In order to utilize microsatellite primers in species that they were not developed for (i.e., non-target species) or increase the number of loci beyond those specifically designed for a species, it is necessary to screen potential loci for amplification and polymorphism. In this study, we screened 74 primers in *Ambystoma annulatum*, the Ringed Salamander. This ambystomatid species is endemic to the Interior Highlands of the Ozark and Ouachita mountains of central Missouri, north central and western Arkansas, and eastern Oklahoma. Across this range, *A. annulatum* is characterized as patchily distributed, but locally abundant (Petranka, 1998). In this study we describe a set of cross-amplified polymorphic loci that would be suitable for population genetic studies on *A. annulatum*. We also review the literature for all published cross-species applications of microsatellite primers in species of *Ambystoma*, and include unpublished findings from other primer screenings.

MATERIALS AND METHODS

Primers selected for screening in this study consisted of all the primers published in Wieczorek et al. (2002), Julian et al. (2003a, 2003b), Williams and DeWoody (2004), and Savage (2009). Samples from *A. annulatum* were collected from a single pond located in Warren County (38.764, –91.268) by taking approximately 0.5 cm of tail tissue from larval animals. Samples were stored in 95% EtOH at −20°C prior to DNA extraction. DNA was extracted from tissue using chelex-based resin (InstaGene, BioRad). Approximately 2 mm × 2 mm of tissue was finely chopped with a sterile razor and was incubated at 60°C for 2 hrs in 250 μL of InstaGene, vortexed, incubated for 20 min at 100°C, then vortexed again. A 100 μL aliquot was removed and used as template DNA and the remainder was kept at −20°C. Initial screening of primers was carried out on a single individual of *A. annulatum*. For this screening, the polymerase chain reaction (PCR) conditions were standardized to a final volume of 25 μL containing 1X GoTaqPCR buffer (Promega, pH 8.5), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 U *Taq* polymerase (GoTaq, Promega), 2 μg bovine serum albumin (BSA), 0.25 μM of each primer, and 30 ng genomic DNA. The PCR was run using a gradient of eight annealing

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Table 1. Summary for Polymorphic Microsatellites Screened in 25 *A. annulatum* from a Single Population. T_A is optimized annealing temperature, pool represents the group into which primers were pooled together prior to fragment analysis, size is the amplicon fragment length, N_A is the number of alleles present, and *n* is the number of samples in which the primer amplified.

				This study					Original publication				
Primer	T_A	Label	Pool	Size (bp)	N_A	Ho	He	*n*	Size (bp)	N_A	Ho	He	*n*
AcroD037	60	PET	1	153–165	4	0.560	0.695	25	124–164	6	0.720	0.791	24
AcroD300	60	VIC	1	134–158	7	0.800	0.812	25	192–228	10	0.720	0.847	24
AcroD330[a]	56	VIC	2	108–132	5	0.286	0.717	22	136–176	7	0.840	0.827	24
AjeD162	60	PET	2	150–170	7	0.520	0.745	25	115–170	13	0.833	0.822	95
AjeD23[b]	57	FAM	1	123	1	0.000	NA	25	195–235	10	0.700	0.785	95
AjeD346	60	FAM	2	130–142	4	0.667	0.723	24	160–195	9	0.600	0.845	91
AjeD422[b]	60	FAM	1	176	1	0.000	NA	25	230–265	9	0.867	0.798	95
AmaD184[a]	56	PET	1	94–102	3	0.042	0.386	21	115–175	10	0.690	0.819	89
AmaD321	56	VIC	2	136–156	6	0.400	0.664	25	120–175	13	0.624	0.724	88
AmaD42	60	NED	2	158–194	5	0.680	0.735	25	125–160	7	0.615	0.604	83
Atex 65	56	FAM	1	158–194	5	0.680	0.699	25	272–384	18	0.910	NA	23
Mean[c]					5.32	0.515	0.686						

[a] Significantly different from HWE expectation; null alleles detected.
[b] Locus was polymorphic when screened in seven samples from across the range of *A. annulatum.*
[c] Monomorphic loci excluded.

temperatures from 50–60°C using the following cycling conditions: initial denaturing at 95°C for 2 min; followed by 35 cycles of 95°C denaturing for 45 s, gradient temperature (°C) annealing for 45 s, and 72°C extension for 90 s; and a final 5 min extension at 72°C. PCR products were separated in a 2% agarose gel with 100 bp ladder (GeneRuler, Fermentas), and nucleic acids were visualized with Gel Star (Lonza) under a UV light. All visible bands were noted, and the annealing temperature yielding the most intense bands was designated as the optimum temperature for each primer. This PCR profile most closely follows that of Julian et al. (2003a, 2003b).

All primer pairs yielding clear bands were screened for polymorphism. PCR conditions for polymorphism screens were identical to the gradient screens with the exception that the gradient temperature was replaced with the optimum temperature for each primer. Polymorphism screens were initially done on seven individuals from three different populations in Missouri (mean distance between sample locations = 74.5 ± 36.2 km SD). PCR products were separated in a 4% agarose gel. Fragment sizes were estimated using the 100 bp ladder, and alleles were enumerated.

Eleven primers with clear diploid polymorphic genotypes were 5′-modified with either 6-FAM, VIC, NED, or PET fluorophores for precise scoring and analysis. These primers were screened in 25 samples of *A. annulatum* collected from larvae at a single pond in Warren Co., Missouri. Each primer was run singly in a PCR reaction standardized to a final volume of 15 μL containing 1X PCR Gold Buffer (Applied Biosystems), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U *Taq*Gold DNA polymerase (Applied Biosystems), 1.2 μg BSA, 0.25 μM of each unlabeled primer, and 30 ng genomic DNA. The PCR conditions followed as previously described, using the optimal annealing temperature (Table 1). PCR products were sized on an ABI 3730 DNA Analyzer using Liz 600 as a size standard, and were scored using GeneMarker version 1.95 (Softgenetics). We tested for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium using Fisher's exact test approximations and Markov chain algorithms (100 batches, 10,000 iterations) using Genepop (v. 4.0.10; Rousset, 2008). Bonferroni corrections were applied to these computations, and all other multiple comparisons, to avoid chance occurrence of significance (Rice, 1989). Values for mean expected and observed heterozygosity were calculated using MS toolkit in Microsoft Excel (Park, 2001). We tested for linkage among the loci using Genepop (Raymond and Rousset, 1995; Rousset, 2008). Lastly, we tested for the presence of null alleles using Micro-Checker (Van Oosterhout et al., 2004).

To conduct our review of the literature for cross-species applications of microsatellite primers in species of *Ambystoma*, we used the Scopus online database, accessed through the University of Missouri library on 15 September 2011. We searched titles, abstracts, and keywords for all years available using the search term ''Ambystoma* AND Microsatellite*''. The asterisk represents a wildcard operator to make for a more general search.

RESULTS

In total, we screened 74 primers pairs from the 146 published microsatellite sequences for cross-species amplification in *A. annulatum*. Twenty-four primer pairs were found to amplify in *A. annulatum*, and 16 appeared to be polymorphic on 4% agarose when screened in seven samples from across the range (Table 2). Of these 16 loci, 11 gave clear, consistent diploid genotypes, and these primer pairs were then screened in 25 individuals from a single population. These primer pairs produced scorable amplicons in 21–25 samples, and had 1–7 alleles per locus (mean = 5.32; Table 1). Two loci, AjeD23 and AjeD422, previously determined to be polymorphic in *A. annulatum* following screening of samples from geographically disparate populations were monomorphic when screened in our single population. The mean fragment length from our samples generally did not differ substantially (max = 95 bp smaller; mean = 32 bp smaller) from the fragment lengths in the original primer publications (Table 1). None of the linkage comparisons among the loci were significant. Following Bonferroni correction for multiple comparisons, loci AcroD330 and AmaD184 were found to be significantly out of Hardy-Weinberg equilibrium. Further, Micro-Checker anal-

Table 2. Summary of Cross-species Amplification of Microsatellite Primers in *Ambystoma*. Assessment of cross amplification comes from published literature and unpublished data of the authors. Source represents the source publication of primer sequences. Primers listed here were not necessarily screened in all species; only positive amplifications (+) are reported, which we define as working in at least five of seven screened samples when visualized on agarose gel. Numbers refer to the corresponding numbered citation in Table 3, which are the cross-species applications of each primer.

		Species[a]												
Locus	Original source	AMAN	AMBA	AMCA	AMJE	AMLA	Auni	AMMA	AMMacro	AMOP	AMTA	AMTE	AMTI	Total
AcalB142	Savage, 2008												5	1
AcalB148	Savage, 2008								18[b]					1
AcalD001	Savage, 2008												5	1
AcalD019	Savage, 2008								18					1
AcalD021	Savage, 2008												5	1
AcalD031	Savage, 2008												5	1
AcalD032	Savage, 2008												5	1
AcalD071	Savage, 2008								18					1
AcalD088	Savage, 2008												5	1
AcalD098	Savage, 2008								18				5	2
AcalD108	Savage, 2008												5	1
AcroB192	Savage, 2009	+[e]		18	+				11,12,18			+[f]		5
AcroD037	Savage, 2009	+							11,12,18					2
AcroD114	Savage, 2009	+[f]			+[f]				18			+[e]		4
AcroD137	Savage, 2009								18[b]					1
AcroD166	Savage, 2009								11,12,18					1
AcroD167	Savage, 2009	+[e]			+				18			+[e]		4
AcroD176	Savage, 2009	+[e]			+				18			+[e]		4
AcroD190	Savage, 2009								18					1
AcroD206	Savage, 2009								18[b]					1
AcroD231	Savage, 2009								18					1
AcroD300	Savage, 2009	+							11,12,18					2
AcroD315	Savage, 2009					18		+[f]	18[b]			+		4
AcroD327	Savage, 2009								18					1
AcroD330	Savage, 2009	+							18			+[f]		3
Aje294	Julian et al., 2003b							+[f]						1
AjeD03	Julian et al., 2003b						16		10,11,12					2
AjeD108	Julian et al., 2003b	+[e]				15								2
AjeD162	Julian et al., 2003b	+						+	10,18	6,7, 14		+[f]		5
AjeD212	Julian et al., 2003b							+[f]				+		2
AjeD23	Julian et al., 2003b	+				15	16			7, 14	7	+[f]	5	7
AjeD283	Julian et al., 2003b		1			8,9,15	1,2,16	+[f]						4
AjeD346	Julian et al., 2003b	+	1			8,9,15,17	1,2,16	+				+[f]		6
AjeD37	Julian et al., 2003b					17	16							2
AjeD378	Julian et al., 2003b		1				1,2,16							2
AjeD422	Julian et al., 2003b	+	1			8,9,15	1,2,16		10	7		+	5	8
AjeD448	Julian et al., 2003b								18					1
AjeD46	Julian et al., 2003b						16					+		2
AjeD75	Julian et al., 2003b					8,9,15								1
AjeD94	Julian et al., 2003b		1			8,15,17	1,2,16	+[f]	10					5

Table 2. Continued.

Locus	Original source	Species[a]												Total
		AMAN	AMBA	AMCA	AMJE	AMLA	Auni	AMMA	AMMacro	AMOP	AMTA	AMTE	AMTI	
AmaC151	Julian et al., 2003a								18					1
AmaC40	Julian et al., 2003a	+[e]							10			+[e]		3
AmaD184	Julian et al., 2003a	+[g]							10			+		3
AmaD226	Julian et al., 2003a				+							+		2
AmaD321	Julian et al., 2003a	+			+					6,7		+[f]	5	5
AmaD328	Julian et al., 2003a									6,7,14				1
AmaD367	Julian et al., 2003a	+[e]				8,9								2
AmaD42	Julian et al., 2003a	+			+					7,14	7	+[e]		5
AmaD95	Julian et al., 2003a	+[e]			+				10,18	7,14		+[e]		5
AmmF142	Savage, 2009[c]	+[f]			+[f]				11,12,18[b]			+[f]		4
AmmH123	Savage, 2009[c]	+[f]				18		+[f]	11,12,18					4
AmmH136	Savage, 2009[c]					18			11,12,18					2
Aop2	Croshaw et al., 2005										7			1
At52.1	Parra-Olea et al., 2007												3,4,19	1
At52.34	Parra-Olea et al., 2007												3,4,13,19,20	1
At52.6	Parra-Olea et al., 2007												3,4,5,19	1
At60.3	Parra-Olea et al., 2007												3,4,19	1
At60.9	Parra-Olea et al., 2007[d]												13,20	1
Ata17	Croshaw et al., 2005									7				1
Ata18	Croshaw et al., 2005									7				1
Atex102	Williams and DeWoody, 2004	+[g]												1
Atex143	Williams and DeWoody, 2004	+[g]						+[f]						2
Atex65	Williams and DeWoody, 2004	+											3,4,13,19,20	2
Atex74	Williams and DeWoody, 2004	+[e]	1				1							3
ATS5-7	Mech et al., 2003												3,5,13,20	1
	Total	24	6	1	9	12	10	9	30	9	3	19	18	

[a] AMAN = *A. annulatum*, AMBA = *A. barbouri*, AMCA = *A. californiense*, AMJE = *A. jeffersonianum*, AMLA = *A. laterale*, Auni = Unisexual and/or polyploid species of *Ambystoma*, AMMA = *A. maculatum*, AMMacro = *A. macrodactylum macrodactylum*, AMOP = *A. opacum*, AMTA = *A. talpoideum*, AMTE = *A. texanum*, AMTI = *A. tigrinum*

[b] Subspecies of *A. macrodactylum*

[c] Sequence data published on GenBank, primers developed by Savage (2009)

[d] Primers for this locus have been published by Gopurenko et al. (2006)

[e] Inconsistent amplification; not screened for polymorphism, but may be useable

[f] Monomorphic in all screened samples

[g] Polysomic genotype

Table 3. Review of Literature with Successful Cross-species Amplification of Microsatellite Primers in *Ambystoma*. The citation numbers in this table correspond with the numbers in Table 2.

Citation number	Source study
1	Bogart et al., 2009
2	Bogart et al., 2007
3	Bos et al., 2008
4	Bulut et al., 2009
5	Cosentino et al., 2011
6	Croshaw, 2010
7	Croshaw et al., 2005
8	Demastes et al., 2007
9	Eastman et al., 2007
10	Giordano et al., 2007
11	Goldberg and Waits, 2010a
12	Goldberg and Waits, 2010b
13	Gopurenko et al., 2006
14	Greenwald et al., 2009
15	Julian et al., 2003b
16	Ramsden, 2008
17	Ramsden et al., 2006
18	Savage, 2009
19	Williams and DeWoody, 2004
20	Williams et al., 2009

ysis suggested there was a high probability of null alleles in both of these loci. All other loci conformed to HWE expectation and were free of null alleles.

Our literature search for ''Ambystoma* AND Microsatellite*'' resulted in 39 citations. Of these, 17 specifically reported cross-species use of microsatellites of *Ambystoma* (Table 3). Three studies that use cross-species primers of *Ambystoma* (Greenwald et al., 2009; Williams et al., 2009; Cosentino et al., 2011) were not identified through this search, but were included in the summary tables. Including unpublished data from our lab, as well those of colleagues (Cosentino, pers. comm.), we identified 98 microsatellites developed in a target species that cross-amplify in another species (Table 2). Twenty of these microsatellites amplified in three or more congeneric species, and ten amplified in five or more congeneric species.

DISCUSSION

With 146 species-specific microsatellite sequences identified for ten different species of *Ambystoma*, there is a wealth of information available for researchers seeking to conduct microsatellite-based genetic research on *Ambystoma* salamanders. As we have shown in *A. annulatum* through our lab screenings, and across the genus through our literature review, several primers are in fact suitable for cross-species use. There are several potential drawbacks or caveats to using microsatellites across species, including homology, ascertainment bias, null alleles, and a reduction of allelic richness (Ellegren et al., 1995; Hutter et al., 1998; Kim et al., 2004; Keever et al., 2008). These factors can be of considerable concern when implementing a population genetics study using microsatellites. Issues of ascertainment bias and homology are predominantly of concern in comparative studies using the same microsatellites in two or more different species, but presence of null alleles and reduced polymorphism are of serious concern in any study. Null alleles occur when mutations arise in the target flanking region of the microsatellite, resulting in no or inconsistent amplification, and ultimately will lead to genotyping errors (Jarne and Lagoda, 1996). To correct for null alleles, one either has to estimate the frequency of the missing alleles and correct for them in the data set (Van Oosterhout et al., 2004), or one can attempt to redesign the primer to target a different region of the flanking sequence. Finally, the high polymorphism of microsatellites is one their most appealing attributes for many applications. Reducing this polymorphism is not ideal, but limited levels of polymorphism can be ameliorated by increasing the number of loci without loss of overall power for detecting population genetic structure (Kalinowski, 2002).

Overall, 32% of primers screened in this study successfully cross-amplified in *A. annulatum*. Of the 24 primer pairs that amplified in *A. annulatum*, 16 appeared to be polymorphic, 11 of which gave clear diploid genotypes, and nine of these were found to be polymorphic in a large sample from a single population. Even though we ran each primer pair individually, we were able to pool-plex them for fragment analysis (Table 1). With the right selection of fluorescent labels and pre-screening for compatibility, these primers could be run together in 2–3 multiplex reactions, reducing both time and resources in the lab. Although difficult to directly compare or extrapolate to the original published primer results, the allelic diversity present in our small sample is encouraging, and adequate for use in more extensive studies of *A. annulatum*.

Although we ultimately developed a set of effective primers for *A. annulatum*, our reported success rate for cross-species screening is rather low, and it is difficult to compare to other systems or taxa in which cross-amplification studies have been conducted. Perhaps of greater relevance, though, is that this entire study was completed for <$1000 (U.S.) and modest amounts of time in the lab. In comparison, starting microsatellite development from scratch using traditional methods (e.g., Hamilton et al., 1999) is very time intensive, costly, and generally would provide only slightly greater yield in terms of total microsatellites as was found through our cross-species screenings. In contrast to both of these methods, next generation sequencing (NGS) technologies have drastically increased identification of potential microsatellite loci (average >2000) for non-model organisms (Gardner et al., 2011). This sequence data comes at a modest cost ($2000–$3000) using today's technology, but as NGS technology continues to improve, these costs will go down, making the relative cost per microsatellite locus extremely affordable. Even with thousands of putative loci available, traditional optimization and screening of primers as conducted in our study is still required, but software and algorithms to increase likelihood of success are further reducing lab-time requirements (Faircloth, 2008; Castoe et al., 2010). Screening of previously developed microsatellites is the most affordable approach, but it suffers from limited success and potentially lower quality of the identified microsatellites. It is one of the goals of this study to consolidate this cross-species information so that future researchers can prioritize cross-species microsatellite screening based upon past success in other ambystomatid species.

The majority of species diversity of *Ambystoma* occurs in the central and eastern United States (Petranka, 1998), and nearly all primers for species in this region (*A. talpoideum, A. jeffersonianum*, *A. maculatum*, *A. opacum*, *A. texanum*) have been developed in populations in the eastern portions of the species' range (Julian et al., 2003a, 2003b; Zamudio and Savage, 2003; Williams and DeWoody, 2004; Croshaw et al., 2005). This may lead to limited transferability of microsatellites across the range of the species. Phylogenetic studies including *A. maculatum* and *A. texanum* (Shaffer et al., 1991; Zamudio and Savage, 2003) have discovered the presence of at least two strongly supported clades in each species, suggesting isolation between populations in different regions. Isolation can lead to divergence and population- or region-specific mutations, some of which may occur in microsatellite priming regions. We experienced this problem when attempting to optimize primers for *A. maculatum* and *A. texanum* collected from Missouri and Illinois populations, respectively. For both of these species, species-specific primer pairs generally performed poorly, and/or were not polymorphic (WEP, unpubl. data). Additionally, many cross-species primer pairs were also not polymorphic (Table 2). Detection and description of phylogenetic patterns of cross-species microsatellite amplification was not a specific goal of this study. Although there is no clear phylogenetic pattern to successful cross-species amplification, future studies using more species may uncover phylogenetic patterns analogous to those in other taxa, although microsatellite homology must first be determined (Eggert et al., 2009).

Our screening of microsatellite primers developed for different species of *Ambystoma* in *A. annulatum* was successful, yielding 11 polymorphic loci, and at a fraction of the cost that would have been incurred to develop this many markers. Additionally, we have compiled a comprehensive table of all cross-species applications of microsatellite primers for species of *Ambystoma* (Table 2). The information in our tables can be used to help narrow the search for useful, polymorphic loci in species without developed microsatellites, or to increase the number or polymorphism of primers in a study. One limitation of our study, and indeed all of the studies we reviewed, is that microsatellite homology has not been determined. As such, we cannot state with certainty that the polymorphic loci presented in Tables 1 and 2 represent the same microsatellites as identified in the species from which they were originally sequenced. This may preclude use of such loci for comparative studies until homology can be verified, but single-species population genetics studies will be unaffected by this issue as long the previously discussed issues of null alleles and polymorphism are adequately addressed. We recognize that there may still be a need for future researchers to develop highly polymorphic species- and/or site-specific microsatellites to address specific research questions. Further, this endeavor is continually being made easier and more affordable with the continued advancement of next generation sequencing (Castoe et al., 2010; Gardner et al., 2011) and software to efficiently screen and develop primers from thousands of candidate microsatellite sequences (Faircloth, 2008; Castoe et al., 2010) for less than or equal to the cost of traditional microsatellite development. Future researchers in population and conservation genetics will have many resources at their disposal, and should not overlook the potential power and generality of previously developed microsatellite primers.

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