Title

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Isolation and characterization of nine tetranucleoide microsatellite loci for the secretive limbless lizards of the genus *Anniella* (Anguidae)

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ABSTRACT

Limbless lizards of the genus *Anniella* are found in the western United States and Mexico. Until recently only two species were known, but four new species have since been described. Since these lizards are fossorial, not much is known about the nature of gene flow within species, or if gene flow occurs across species boundaries in regions of overlap. Since these lizards are of conservation interest, we isolated and developed nine tetranucleotide microsatellite loci for the recently described species *Anniella alexanderae*. We characterized the polymorphism of each locus in *A. alexanderae*, and then cross-amplified these loci in five other *Anniella* species. These nine loci have high observed levels of heterozygosity and polymorphism information content, and were in Hardy–Weinberg equilibrium within the *A. alexanderae* samples we tested, indicating that they will have high utility in assessing population genetic and demographic patterns within *Anniella*.

Abstract

Limbless lizards of the genus *Anniella* are found in the western United States and Mexico. Until recently only two species were known, but four new species have since been described. Since these lizards are fossorial, not much is known about the nature of gene flow within species, or if gene flow occurs across species boundaries in regions of overlap. Since these lizards are of conservation interest, we isolated and developed nine tetranucleotide microsatellite loci for the recently described species *Anniella alexanderae*. We characterized the polymorphism of each locus in *A. alexanderae*, and then cross-amplified these loci in five other *Anniella* species. These nine loci have high observed levels of heterozygosity and polymorphism information content, and were in Hardy-Weinberg equilibrium within the *A. alexanderae* samples we tested, indicating that they will have high utility in assessing population genetic and demographic patterns within *Anniella*.

Keywords: genetic resources, North America, microsatellites, lizards, herpetology, California, Mexico, Anguidae, Reptilia

1. Introduction

The genus *Anniella* is presently comprised of six species of legless fossorial lizards. Due to their fossorial nature, the distributions and natural history of these lizards are poorly characterized. Until recently only two species of *Anniella* were known, *A. geronimensis* from Baja Mexico, and *A. pulchra* with a range extending throughout much of California and Baja Mexico. The recent discovery of extensive genetic variation and substructure across the range, as well as diagnostic morphological features unique to each clade, has led to the description of four new species and the restriction of *Anniella pulchra* to encompass two disjunct Californian populations (Parham and Papenfuss 2009, Papenfuss and Parham 2013). Of the four newly described taxa, three (*A. alexanderae*, *A. grinnelli*, and *A. campi*) have highly restricted ranges in the San Joaquin Valley, the Carrizo Plain, and eastern Sierra Nevada, respectively, while the fourth *A. stebbensi* is more widely distributed in Southern California and Baja Mexico. Prior to the discovery of the four new species, *A. pulchra* was considered to be a Species of Special Concern by the California Department of Fish and Wildlife, now that its range is further reduced, assessment of its demographic and population structure is required to better evaluate its conservation status. Furthermore, little is known of the newly described species given their recent discovery, and their conservation status has not been assessed. It is likely that given their small ranges and the ongoing habitat degradation across the region that these species might also warrant conservation protection. Towards that end, we developed and characterized nine tetranucleotide microsatellite loci in *Anniella alexanderae* and demonstrate that these same markers can be cross-amplified across additional species in the genus. These microsatellite markers will allow us to characterize

contact zones as well as more fine-scale genetic structure within and across species of *Anniella* to better understand population and landscape genetics dynamics among these fossorial lizards.

2. Materials and Methods

2.1 Isolation of microsatellite markers and primer design

We used the same approach as in Wogan *et al*. (2015a, b) to develop and analyze microsatellites for *Anniella alexandrae*. Microsatellites were developed following the protocol of Glenn and Schable (2005). First genomic DNA was extracted using the DNeasy kit (Quigen, USA). DNA from one individual was digested with the restriction enzymes RsaI and XmnI before SuperSNX24 linkers were ligated onto the fragments. We next hybridized the fragments with four biotin-labeled tetranucleotide probes [$(ACAG)_8$; $(AAGT)_8$; $(AGAT)_8$; $(ACAT)_8$]. This complex was then attached to streptavidin-coated magnetic beads (Dynabeads M-270, Invitrogen) and washed twice with 2X SSC, 0.1% SDS and four times with 1X SSC, 0.1% SDS at 52 °C before ethanol precipitation. We sequenced a total of 118 colonies and then preferentially selected colonies that contained repetitive elements with eight repeats for primer design. Primers were designed using Websat (Martins et al. 2009) which integrates Primer3 (Rozen and Skaletsky 2000). The forward primer was 5'tagged with either a HEX or FAM flourophore.

2.2 PCR-amplification and genotyping

68 We then selected a test panel of 16 samples of *A. alexanderae*. To test each microsatellite
69 we first ran a series of gradient PCRs with annealing temperatures ranging from 54-64
70 °C. All PCR reactions were carried out in a 10 µl volume consisting of 1 µl diluted DNA
71 (1:10 dilution), 0.12U of Taq polymerase (Invitrogen), 1 µl 10X buffer, 0.3 µl 50mM
72 MgCl2, 0.6 µl 10 µg/ µl BSA, 0.25 µl 10mM dNTPs, 0.6 µl 10 mM of each primer, and
73 dH_20. The thermocycling profile was 94 °C for 3 min followed by 30 cycles of 94 °C for
74 45 s, annealing temperature (Table 1) for 30 s, and 72 °C extension for 45 s, followed by
75 a final extension at 72 °C for 30 min. Genotyping was performed using LIZ500 size
76 standard on an ABI 3730. All samples were PCR-amplified and genotyped three times
77 and then compared to ensure consistency. Alleles were binned using Genemapper v. 4.0
78 (Applied Biosystems, USA). We then tested cross-species PCR-amplification of each
79 locus for all five species of *Anniella* using the same PCR-amplification conditions as in
80 *A. alexanderae*. Our primary objective for doing so was to assess if the microsatellite loci
81 would amplify in other members of the genus, and so our samples sizes for this test panel
82 are small (four individuals of each species).
83
84 2.3 Data analyses
85 To evaluate the presence of null alleles and the probability of large allele dropout, we
86 used Microchecker (van Oosterhout et al. 2004). We next calculated the number of
87 alleles, the polymorphism information content (PIC)(Botstein et al. 1980), and the
88 expected and observed heterozygosity (with 1000 bootstrap replicates), and then used the
89 exact test (with 1000 replicates) to check for deviations from Hardy-Weinberg
90 Equilibrium for each marker using the R packages PopGenKit (Paquette 2013) and pegas

(Paradis 2010). We also tested for linkage disequilibrium among the microsatellite loci using the log likelihood ratio statistic in Genepop v. 4.2 3 (Raymond and Rousset 1995, Rousset 2008). For the other five species for which we tested cross-amplification, we report the number of alleles and the size range of the alleles. The small sample sizes of these species preclude the meaningful application of population genetics statistics such as used above.

3. Results and discussion

Of the 118 colonies sequenced 32 did not contain repeat motifs, and several colonies contained non-unique fragments, leaving us with 57 potential loci. Of these, several did not meet our criterion for further development (i.e. fewer than 8 repeats), whereas others did not contain suitable surrounding sequence for primer design. We designed and tested seventeen microsatellite primer sets, of these, nine amplified consistently for *Anniella alexanderae*. We found no evidence of null alleles or large allele drop out among the nine loci. All nine microsatellite loci were polymorphic and contained between 3-11 alleles (Table 1). Observed heterozygosity values ranged from 0.438 - 0.938, and PIC values ranged from 0.3666-0.8461, indicating that the majority of the loci have high information content that should prove useful for population genetic analyses (Table 1). All nine loci were found to be in Hardy-Weinberg equilibrium within the sample we tested. There was no statistically significant linkage disequilibrium detected among the loci.

We were able to cross-amplify each of the nine microsatellite loci in five additional *Anniella* species (Table 2) under the same PCR-amplification conditions as used for *A.*

alexanderae (Table 1). The loci were polymorphic across species, although in two instances, a single allele was recovered for one locus (Table 2), which suggests that the microsatellite locus may be fixed in those instances. Larger sample sizes are required to adequately address this finding. Overall, these microsatellites will have high utility in addressing much needed research into population genetics and conservation genetics questions relating to these secretive fossorial species.

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Locus	Primer sequence 5'-3'	repeat motif	Ta (°C)	Size range (bp)	N_A	Ho	He	HWE (*p* value)	PIC	Genbank accession
Ann1	F: FAM-AGATAGAAACCAGCAGCCAAAG R: GTAGCCTGCAAACTGGGAATTA	$(TAGA)_{14}$	58	289-317	7	0.812	0.777	0.470	0.7494	KR872018
Ann3	F: HEX-GTTGCTTTCATGCTCTCCTTTT R: TTCACAAAATCCAGACTTCCCT	$(TAGA)_{9}$	58	382-438	11	0.750	0.821	0.590	0.8006	KR994684
Ann7	F: FAM-TTTATCTGGCATCCCTATTTGC R: AAGCTCTCTGGGTGGTTTACAA	$(ATAG)_{8}$	62	230-250	6	0.625	0.756	0.175	0.7209	KR872019
Ann22	F: HEX-AAAGAACATGGAGTAGTGCGGT R: GTATTCCCCGTAAACATCATCC	$(GATA)_{10}$	60	329-373	11	0.938	0.875	0.946	0.8632	KR872020
Ann34	F: FAM-TTTCTTGGTGACGTGTAAATGG R: CATGGTGTATCTGTCATGCCTT	$(ACAG)_{8}$	58	395-403	3	0.438	0.404	0.687	0.3666	KR872021
Ann38	F: HEX-TTGAATGGGTGGTATAGGTGC R: TGGTTTCTCTGGAGTTAGACAGG	$(TAGA)_{8}$	60	352-384	8	0.688	0.760	0.079	0.7314	KR872022
Ann79	F: FAM-TAGTGAGTGTGTGCATGTTTGC R: CATCCAGGTGATGTGTCTCAAT	$(TCTA)_{13}$	60	261-285	7	0.750	0.805	0.284	0.7767	KR872023
Ann86	F: FAM-AACTGGTTGACACATCTCCAAA R: GACACCATTCCTCTCAAGGTCT	$(ATAG)_{10}$	62	224-314	11	0.750	0.861	0.173	0.8461	KR872024
Ann117	F: HEX-ACCATTGAAAAGAGAGGTCCAG R: GATACATCGAGAGATTCCCAGC	$(TCTA)_{12}$	62	192-212	6	0.875	0.789	0.925	0.7583	KR872025

Table 1. Characterization of microsatellite loci isolated from *Anniella alexanderae*. For each microsatellite locus we have included the forward and reverse sequences, the specific repeat motif of the locus, the annealing temperature, and the size range of the repeat found within the test panel. N_A is the number of alleles recovered for the test panel, Ho and He are respectively the observed and expected heterozygosities, HWE is the p-value obtained for Hardy-Weinberg Equilibrium, with a non-significant value indicating that there are no departures from HWE. PIC, the polymorphism information content is a measure that ranges from zero to one with values closer to one having high information content, and finally the Genbank accession number for the original sequence containing the microsatellite locus.

	A. campi n=4	*A. geronimensis* n= 4	*A. grinnelli* n= 4	*A. pulchra* n= 4	*A. stebbinsi* n= 4
Ann1	2 [273 - 277]	4 [289 – 305]	3 [289 - 297]	5 [277 – 301]	5 [289 – 321]
Ann3	3 [410 - 426]	3 [386 – 394]	4 [378 - 394]	7 [374 – 414]	5 [386 – 410]
Ann7	3 [242 - 250]	4 [242 – 266]	2 [230 - 238]	6 [242 – 286]	4 [234 – 250]
Ann22	3 [329 - 361]	2 [341 – 349]	4 [341 - 349]	5 [325 – 377]	3 [349 – 369]
Ann34	3 [375 - 383]	5 [375 – 427]	2 [379 - 387]	4 [375 – 391]	1 [375 - 375]
Ann38	3 [368 - 380]	4 [352 – 380]	4 [360 - 374]	6 [356 – 400]	4 [364 – 380]
Ann79	3 [249 - 269]	4 [253 – 293]	3 [245 - 253]	5 [249 – 273]	5 [253 – 281]
Ann86	1 [188 - 188]	3 [188 – 258]	4 [200 – 304]	5 [148 – 242]	5 [228 – 250]
Ann117	6 [168 – 192]	3 [172 – 196]	3 [184 – 200]	5 [176 – 196]	5 [148 – 184]

Table 2. Results from PCR-cross-amplification for the remaining five described species of *Anniella*. The number of individuals for each species and the number of unique alleles recovered from genotyping are provided for each of the microsatellite loci. The number in brackets is the size range of the alleles.