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PRIMER NOTE

Development and characterization of polymorphic microsatellite DNA loci for the endangered dusky gopher frog, *Rana sevosa*, and two closely related species, *Rana capito* and *Rana areolata*

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Abstract

A microsatellite library was developed using genomic DNA of the endangered dusky gopher frog, *Rana sevosa*. Polymerase chain reaction (PCR) primers and conditions are presented for *R. sevosa* (eight loci) and two sister taxa — other gopher frogs, *Rana capito* (seven loci) and crawfish frogs, *Rana areolata* (three loci). Polymorphism of each microsatellite locus was evaluated for each species. All loci have moderate to high genetic variation in terms of allelic richness (four to 10 alleles per locus), observed heterozygosity (0.595–0.946), and expected heterozygosity (0.531–0.856).

Keywords: amphibian, anuran, enrichment method, microsatellite, PCR, population genetics, primer, Ranidae

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Dusky gopher frogs, *Rana sevosa*, are listed as endangered in the US Endangered Species Act and Critically Endangered in the Red List of the International Union for Conservation of Nature and Natural Resources (IUCN). The only two known populations are geographically separated by *c*. 32 km. Extensive ecological and demographic data exist for one of the populations (Glen's pond, Harrison County, Mississippi) (e.g. Richter & Seigel 2002; Richter *et al*. 2003), whereas the status of the other is unknown because it was only recently (March 2004) discovered (Mike's pond, Jackson County, Mississippi).

Following the listing of *R. sevosa* in the US Endangered Species, a recovery team was assembled to develop conservation priorities and recovery plans. Conservation efforts implemented through a recovery team include assessing current and historical population genetic variation and monitoring genetic variation in natural and captive populations. The primary goal of the microsatellite library was to develop genetic markers that could be used to address the genetic consequences of reduction in population size and complete isolation of *R. sevosa*. Nonisolated populations of *R. sevosa* are not known to exist. Therefore for comparison purposes, loci were developed that were also informative for two closely related species, other gopher frogs (*Rana capito*) and crawfish frogs (*Rana areolata*). Additionally, one management strategy being considered by the recovery team is the use of the two extant populations as a stock to reintroduce the species to historic localities where it has gone locally extinct. If this management strategy is implemented, genetic variation will be monitored following successful establishment of populations.

In this study, the development of eight microsatellite DNA loci is described. DNA was extracted from a single *R. sevosa* individual using a QIAGEN DNeasy tissue kit. A microsatellite library was developed following a modified protocol of T. Glenn and M. Schable (unpublished data; see Hauswaldt & Glenn 2003), which was modified from Hamilton *et al*. (1999).

Genomic DNA (*c*. 3.3 μg) was digested using the restriction enzyme, *Rsa*I. Resulting DNA fragments were then ligated to linkers, which provided a primer binding site for polymerase chain reaction (PCR) (*SuperSNX24* forward: GTTTAAGGCCTAGCTAGCAGAATC; *SuperSNX24 + 4p* reverse: GATTCTGCTAGCTAGGCCTTAAACAAAA). Ligated genomic fragments were then allowed to hybridize

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to a mixture of the following biotinylated probes: $(TG)_{12}$, $(AG)_{12}$, $(AAG)_8$, $(ATC)_8$, $(AAC)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACCT)_6$, $(ACAG)_6$, $(ACTC)_6$, $(ACTG)_6$, $(AAAT)_8$, $(AACT)_8$, $(ACAT)_8$, $(AGAT)_8$, $(AACC)_5$, $(AACG)_5$, $(AAGC)_5$, $(AAGG)_5$, and $(ATCC)_5$. Following hybridization, the biotin on each probe was attached to streptavidin-coated magnetic beads (Dynabeads; Dynal Biotech). Magnetism was used to selectively retain microsatellite-containing fragments, and unbound fragments were discarded. PCR employing the linker priming site was used to increase the number of copies of each microsatellite fragment. These were ligated into pGEM-T Easy vectors (Promega), and transformed into *Escherichia coli* competent cells. Plasmid DNAs from individual clones were isolated and sequenced using BigDye version 3 and an ABI Prism 310 genetic analyser (Applied Biosystems).

Of the 192 fragments screened, 27 microsatellite loci were identified with flanking regions sufficiently large to allow primer development. Of these, 17 appeared appropriate for population genetic work (i.e. the microsatellite repeat was the only repetitive sequence in the fragment). Primers were designed for these 17 loci and evaluated using three-temperature touchdown PCRs with the highest temperatures ranging from 60 to 46 °C (Don *et al*. 1991). Reaction mixtures (20 μL total) contained 1 × reaction buffer (10 mM Tris-HCl [pH 8.5] and 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1.0 U *Taq* polymerase, and 50–100 ng template DNA. Fourteen loci amplified for *R. sevosa* only, 12 loci amplified for *R. sevosa* and *R. capito*, and three amplified for all three species. These loci were screened for polymorphism by first amplifying them with PCR under the same conditions as previously described and then visualizing the PCR products on a 3%-agarose gel ($n = 15$ for each species). For loci found to be polymorphic, another PCR was performed using a fluorescently labelled forward primer for each locus for *R. sevosa* ($n = 46$), *R. capito* ($n = 37$), and *R. areolata* ($n = 32$). An aliquot of each PCR product was loaded into an ABI Prism 310 genetic analyser, and allele lengths were scored using GENESCAN analysis software version 3.1.2 (Applied Biosystems). For all species and loci, microsatellite data were analysed using FSTAT version 2.9.3.2 to determine allelic diversity, observed (H_O) and expected heterozygosities (H_E) within Hardy–Weinberg equilibrium (HWE), and deviations from HWE and from genotypic equilibrium among loci (Goudet 1995, 2002). Deviations from HWE were evaluated for each locus and species using global tests with Bonferroni corrections (Weir 1996). Deviation from genotypic equilibrium was assessed across loci using *G*-statistics with Bonferroni corrections (Goudet 1995).

Of the loci that amplified, eight of 14 were polymorphic in *R. sevosa* (Table 1). Of these eight loci, seven amplified and were polymorphic in *R. capito*, and three amplified and were polymorphic in *R. areolata* (Table 2). Thus, differences in the number of suitable loci among species were caused by lack of amplification and not the lack of polymorphism. All loci had moderate to high genetic variation in terms of allelic richness (four to 10 alleles per locus), H_O (0.595–0.946), and H_E (0.531–0.856) (Tables 1 and 2). One locus (*RsevMs3*) in one species (*R. sevosa*) deviated significantly from HWE. All loci were found to be in linkage equilibrium.

Genetic variability observed for these loci revealed their potential as suitable population genetic markers in studies of conservation and management for all three species, and

Table 1 Description of eight microsatellite loci developed for *Rana sevosa*. Size range of alleles is shown as total size range (bp) and as range in number of repeat motifs. Allelic data and genetic variation were derived from 46 individuals. Annealing temperatures (T_a) are presented as the highest temperature in a three-temperature touchdown PCR with two degree decrements

Locus	Primer sequence	GenBank Accession no.	Repeat motif	T_a (°C)	Size range (bp)	Size range (motifs)	No. of alleles	H_E	H_O
RsevMs3	F: ATGTAAGCAATGCTTGTCC R: AAGGACATTGCCACTCAGGC	AY787861	CA	55	274–306	14–30	6	0.787	0.761
RsevA05	F: CCATGTCACTAATGCACCTG R: AGCTTTGCTGTATATTGAGC	AY787862	CT	48	263–269	15–15	3	0.423	0.435
RsevB12	F: TATAAGTGTGCCAACGCAGAC R: CAATCATTTCCAAAAACAC	AY787863	ATAG	55	208–236	10–17	7	0.779	0.826
RsevC02	F: TGCATGACTGAGTAATTGTC R: GATGTATAGTAAGCCATCCG	AY787864	AC	55	165–169	5–7	2	0.502	0.435
RsevC05	F: TACATTAGTGTGATGGTCAG R: GATTGTAAGCTCTTCTGAGC	AY787865	TAT	55	172–178	2–4	2	0.196	0.174
RsevE03	F: ATCTCGGCTTCACTGATTGC R: GCCTACTATGTAACTACTAT	AY787866	GA	55	276–304	9–23	6	0.727	0.391
RsevF01	F: GTGGCGTAACATGCCAGTC R: CTGTGGATTGAAAGTGTACGC	AY787867	ATAG	55	163–195	9–17	6	0.729	0.609
RsevG11	F: GTCTTCCATTACAAGGCTGC R: ACTTCTGACAGTCTAGTTAA	AY787868	TCTA	55	226–276	8–21	8	0.856	0.869

Table 2 Microsatellite loci data for *Rana capito* and *Rana areolata*. Allelic data and genetic variation were derived from 37 individuals for *R. capito* and 32 for *R. areolata*. Annealing temperatures are the same as for *R. sevosa* (Table 1)

Species	Locus	Repeat motif	Size range (bp)	Size range (motifs)	No. of alleles	H_E	H_O
R. capito	*RsevB12*	`ATAG`	212–240	11–18	7	0.791	0.838
	RsevC02	`AC`	163–171	4–8	5	0.777	0.703
	RsevA05	`CT`	255–283	8–22	9	0.681	0.487
	RsevC05	`TAT`	172–184	2–6	4	0.531	0.649
	RsevE03	`GA`	278–308	10–25	10	0.622	0.595
	RsevF01	`ATAG`	163–195	9–17	9	0.847	0.946
	RsevMs3	`CA`	266–292	10–23	9	0.801	0.703
R. areolata	*RsevA05*	`CT`	255–265	8–13	6	0.650	0.688
	RsevE03	`GA`	286–308	14–25	7	0.732	0.625
	RsevF01	`ATAG`	163–195	9–17	9	0.841	0.875

for monitoring genetic health in captive and reintroduced populations of *R. sevosa*. Especially important is their suitability for determining the genetic health of the isolated population of endangered *R. sevosa* via comparison to non-isolated populations of *R. capito* and *R. areolata*.

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