

## PERMANENT GENETIC RESOURCES

# Isolation and characterization of microsatellite markers in the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*), two declining frogs of the North American desert southwest

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## Abstract

We characterized 15 microsatellite loci for the lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*) for future studies of population genetic structure and relatedness. Analysis of 20 individuals from single populations of each species showed that all markers were polymorphic in at least one species. Observed and expected heterozygosities ranged from 0 to 0.94 and from 0.11 to 0.85, respectively, and there were three to 11 alleles per locus. No loci were in linkage disequilibrium, but six loci deviated significantly from Hardy–Weinberg equilibrium, and the presence of a null allele was detected in two of these loci.

*Keywords:* anuran, microsatellite, polymorphism, *Rana onca*, *Rana yavapaiensis*

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The lowland leopard frog (*Rana yavapaiensis*) and the relict leopard frog (*R. onca*) are sister species within the *R. pipiens* complex (*Pantherana*) (Hillis & Wilcox 2005). *R. onca* was once considered extinct (Jennings 1988), but recently, several extant populations were confirmed as *R. onca* based on mitochondrial haplotypes, assessment of randomly amplified polymorphic DNA (RAPD) markers, and morphology (Jaeger *et al.* 2001). Taxonomic designations may be uncertain, however, because these two species are very similar at mitochondrial DNA markers (approximately 98.8% similarity at the 12 S and 16 S genes), a level of divergence comparable to that among other ranid subspecies (Hillis & Wilcox 2005).

Both *R. yavapaiensis* and *R. onca* are species of conservation concern. *R. onca* was known from only 24 locations in Utah, Nevada and Arizona prior to the 1970s, but by 2001, its range dwindled to five localities in Nevada (Bradford *et al.* 2004). In contrast, *R. yavapaiensis* is still patchily distributed throughout much of Arizona and Sonora (Hillis 1988), although the species has also experienced considerable

declines and extirpations since the 1970s (Sredl *et al.* 1997), at least some of which were caused by the fungal disease chytridiomycosis (Bradley *et al.* 2002). Characterization of fine-scale levels of genetic variability within and among these species is therefore critical to establish conservation priorities and to inform questions of species designation.

To accomplish this objective, 15 microsatellite loci were isolated and characterized from two enriched partial genomic libraries (Hamilton *et al.* 1999), one prepared with tissue from an adult *R. yavapaiensis* individual from Maricopa County, Arizona, and one prepared with tissue from an adult *R. onca* individual from Clark County, Nevada. For the *R. yavapaiensis* library, genomic DNA was extracted using a QIAGEN DNeasy tissue kit, followed by digestion with *AluI* and *HaeIII* (New England Biolabs). Digested genomic fragments were then ligated to linkers and enriched for microsatellites with biotinylated dimer, trimer and tetramer probes bound to streptavidin-coated magnetic beads (Dynabeads, Dynal Biotech). Subsequently, the microsatellite-containing DNA fragments were magnetically captured. These fragments were amplified by polymerase chain reaction (PCR) using linker-specific primers, followed by digested with *NheI* (New England Biolabs). Resulting

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**Table 1** Primer sequences, repeat motif, annealing temperatures ( $T_a$ ), size range, and variability measures for 15 microsatellite loci from 20 individuals each of *Rana yavapaiensis* ( $R_y$ ) and *R. onca* ( $R_o$ ). Sequences of original clones have been accessioned in GenBank (Accession nos EU853423–EU853437).  $A$ , no. of alleles;  $N$ , no. of successfully genotyped individuals;  $H_E$ , expected heterozygosity;  $H_O$ , observed heterozygosity.  $P$  values represent significance values for exact tests of deviation from Hardy–Weinberg equilibrium (significant deviations are listed in bold); NA, not applicable

Locus	Repeat motif	Primer sequence (5'–3')	$T_a$ (°C)	Size range	$N$	$A_{R_y}$	$A_{R_o}$	$A_{tot}$	$A_{shared}$	$R_y$ $H_E/H_O$	$R_y$ $P$ value	$R_o$ $H_E/H_O$	$R_o$ $P$ value
RoC4	(ATAG) <sub>19</sub> (ATAC) <sub>12</sub>	F: (6-FAM)TTACCAAGGTAGCACTCTTTG R: ACTGCAAACCGAAATGT	55	195–243	37	5	3	7	1	0.84/0.65	<b>0.02</b>	0.55/0.60	1.00
RoC123	(TGTA) <sub>10</sub>	F: (PET)GGCTTACTTCTTGCCTTTAGC R: CATCCATTTTTCCCTTGTTTC	51	124–156	37	3	3	5	1	0.57/0.78	0.08	0.34/0.46	1.00
RoD102	(TATC) <sub>19</sub>	F: (NED)ATGGAGATTTAAGTGAAGAGT R: CTTCCAATATGGCATAGATAT	60	195–251	38	9	4	11	2	0.85/0.82	0.12	0.70/0.50	0.07
RoD125	(AGAT) <sub>15</sub>	F: (6-FAM)TCAATGGTGGTGTGTGCAC R: GCTCTGAAGTCAACTGGTC	60	282–298	37	5	3	5	3	0.79/0.71	<b>0.01</b>	0.44/0.58	0.46
RoC9	(AT) <sub>5</sub> (AGAT) <sub>10</sub> (ACAT) <sub>8</sub>	F: (NED)CCAGCTCTAAACACATTAGCTC R: CAGCATAGGTTGGAATACAAAT	59	184–239	37	8	3	9	2	0.80/0.82	0.58	0.23/0.20	0.24
RoD124	(TCTA) <sub>9</sub> (TCTCTA) <sub>6</sub> (TC) <sub>3</sub>	F: (VIC)ACCCTCCAAAGTCAAAATC R: AAAGGTGGGCAAACCTCAA	55	232–292	37	7	4	11	0	0.83/0.63	<b>0.03</b>	0.71/0.53	<b>0.00</b>
RoD120	(ATCT) <sub>11</sub>	F: (NED)TATCCGAGGCTTAAAAATCCTTC R: ATATCGGTGCAACCCTAATACA	60	100–124	38	6	2	6	2	0.75/0.47	<b>0.00</b>	0.11/0	<b>0.03</b>
RoD122	(TATC) <sub>19</sub> (TA) <sub>1</sub> (TATC) <sub>2</sub>	F: (NED)CTCTGAGTCTGTCTGTCTGTCT R: TAGTGGCTTAGTCCATTCTATG	51	190–260	39	5	1	6	0	0.75/0.33	<b>0.00</b>	NA	NA
RoC110	(ATAC) <sub>7</sub> (AC) <sub>6</sub>	F: (VIC)GGACCTGTGCATACACAATGTC R: AAATGCTCTCAAACCAAGTAAC	60	124–148	37	4	1	5	0	0.53/0.47	0.26	NA	NA
RyDI-7	(GT) <sub>14</sub>	F: (VIC)AAACTCTGTTCATCTTTGTTCATCTGTTCTG R: AAATCCTTAGCACTCCTTCTGGTCACT	65	232–280	40	4	3	7	0	0.50/0.40	0.41	0.59/0.65	0.05
RyTET-G	(ATCT) <sub>13</sub>	F: (PET)GGTGGTGTACAGAGCCAAAAGGATTAGAATTGTGTTGAT R: AACCCCTAGACGGCAGGAGTGAAGAAG	55	124–224	40	7	1	8	0	0.75/0.74	0.05	NA	NA
RyTET-f	(CTAT) <sub>16</sub>	F: (6-FAM)CTTTACCCTTAAAGTTGTTTAGTGGATAA R: ACTTACAAAACACAATAATATAACAGGACCGAGTA	61	219–289	39	2	5	7	0	0.41/0.25	0.11	0.51/0.61	0.06
RyTET-e	(CAAA) <sub>6</sub>	F: (PET)ATAGTTTCAGCAATTTTGTATATTTTGTGCAAGATGTATG R: ACTTCTCCGAGGGGTCAGCAGATGT	55	219–271	37	4	3	6	1	0.53/0.40	0.27	0.54/0.94	<b>0.01</b>
RyDI-2r	(AC) <sub>11</sub>	F: (NED)TTCAACGTCCCATTAAGAGGAAACT R: GACTATTTGGGCGATATCAGAAAA	50	182–194	40	3	1	3	1	0.57/0.45	0.08	NA	NA
Ry2	(CA) <sub>6</sub> CC(AC) <sub>4</sub> ACC(AC) <sub>6</sub>	F: (PET)GTGTGCGGCAGAGCCATGTGC R: GGCATATCCATTTTGTATGGG	62	166–186	40	4	2	5	1	0.69/0.60	0.05	0.48/0.55	0.65

fragments were cloned into pUC19 vector and transformed with DH5 $\alpha$  competent cells (Invitrogen). Colonies were grown on X-Gal/IPTG-coated agar containing 0.5 g/L of ampicillin and transferred to Magna Lift nylon membranes (Osmonics Inc.) that were probed with the same di-, tri- and tetramer radiolabelled repeats. Positive clones were transferred to 30  $\mu$ L of de-ionized water, boiled at 99 °C for 5 min, and 1  $\mu$ L of the resulting solution was used as template for PCR amplification with vector-specific primers (M13 F and R). Amplification products > 300 base pairs were sequenced in one direction with the M13 F primer using dGTP BigDye terminator cycle sequencing components on a 3100 Genetic Analyser (Applied Biosystems). For the *R. onca* library, a private company (Genetic Identification Services) was employed to isolate, clone and sequence microsatellite-containing DNA fragments.

PCR primers were designed in the flanking regions of 22 microsatellites from the *R. yavapaiensis* library using Seqman version 5.05 (DNASTar Inc.) and 46 microsatellites from the *R. onca* library using the program DesignerPCR version 1.03 (Research Genetics Inc.). Eight *R. yavapaiensis* from four sites and eight *R. onca* from four sites, representing a broad area of the current range for each species, were screened for amplification reliability and microsatellite polymorphism. Of the evaluated primers, 15 polymorphic loci were identified. These loci were used for a broader assessment of population and species differentiation.

Genotyping was conducted for 20 *R. yavapaiensis* individuals from a single location on the Santa Maria River (Yavapai County, Arizona) and 20 *R. onca* individuals from Bighorn Sheep Spring along the Colorado River (Clark County, Nevada). DNA was extracted from toe clips in 150  $\mu$ L of a 5% Chelex-100 solution (Bio-Rad) with 1  $\mu$ L Proteinase K by incubation at 55 °C for 180 min and 99 °C for 10 min; supernatants were used directly as PCR templates. Each PCR had a total volume of 10  $\mu$ L, consisting of 1  $\mu$ L of each template, 0.5 U *Taq* DNA polymerase (Roche), 1.5 $\times$  PCR buffer (15 mM Tris-HCl, 2.25 mM MgCl<sub>2</sub>, 75 mM KCl), 1.0 mM dNTPs, and 5  $\mu$ M of each primer. Forward primers were 5'-end labelled with a fluorescent dye (Applied Biosystems). Amplification was performed in a Hybaid PCR thermal cycler under the following conditions: 5 min initial denaturation at 94 °C; 35 cycles of 1 min denaturing at 94 °C, 1 min annealing at primer-specific annealing temperatures (Table 1), 1 min extension at 72 °C, and a final extension of 75 °C for 5 min. Amplified products with different labels or nonoverlapping size ranges were multiplexed and electrophoresed on a 5% polyacrylamide gel on a 3100 Genetic Analyser (Applied Biosystems). Fragment sizes were determined with the LIZ-500 standard using GeneMapper version 3.5 (Applied Biosystems). Tests of significant deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD), and calculations of the number of alleles, observed and expected

heterozygosities were performed using GenePop version 3.4 (Raymond & Rousset 1995).

The majority of the microsatellite loci identified were tetramers or complex repeats mostly containing tetramers, while only two loci were pure dimers (Table 1). All 15 loci were polymorphic among *R. yavapaiensis* individuals, while only 10 loci were polymorphic among the *R. onca* sampled. Lower genetic diversity was expected in *R. onca* because the current remnant populations comprise a very small geographical range (Bradford *et al.* 2004). The total number of alleles per locus across both species ranged from three to 11 (mean 6.7), with two to nine alleles per locus in *R. yavapaiensis* (mean 5.1) and one to five alleles per locus in *R. onca* (mean 2.6). For nine of the loci, one to three alleles were shared among *R. yavapaiensis* and *R. onca* individuals. Across all samples, observed and expected heterozygosities ranged from 0 to 0.94 and from 0.11 to 0.85, respectively.

No significant LD was detected among any pair of loci; however, six loci showed significant deviations from HWE. Loci *RoC4*, *RoD125*, and *RoD122* showed significant heterozygote deficiency for *R. yavapaiensis* only, loci *RoD120* and *RoD124* showed significant heterozygote deficiency in population samples from both species, and locus *RyTET-e* showed significant heterozygote excess for *R. onca* only. The program Micro-Checker (Van Oosterhaut *et al.* 2004) was used to further evaluate these loci, and the presence of a null allele was detected at loci *D120* and *D122* for the *R. yavapaiensis* population sample. The 15 loci characterized here will be used in range-wide studies of population structure within and among *R. yavapaiensis* and *R. onca* populations, providing a comprehensive understanding of relatedness and conservation priorities for these threatened species.

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