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Newly developed polymorphic microsatellite markers for frogs of the genus *Ascaphus*

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Thirteen polymorphic microsatellite loci were identified and developed for the coastal tailed frog, *Ascaphus truei*, from sites within the Olympic Peninsula of Washington, USA. These tetranucleotide repeat loci were highly variable, averaging 19 alleles per locus and expected heterozygosity of 0.91. In addition, these loci cross-amplify in the sister species, *Ascaphus montanus*. These markers will prove useful in identifying fine-scale genetic structure, as well as provide insight into the evolution and conservation of this group across fragmented landscapes.

Keywords: amphibian, *Ascaphus*, microsatellite DNA, Olympic Peninsula, tailed frogs, tetranucleotide

Received 18 November 2007; revision accepted 19 December 2007

The coastal tailed frog (*Ascaphus truei*) is an example of an amphibian species that is highly susceptible to habitat alteration. Tailed frogs are the most basal of all living amphibian species and are highly specialized for life in fast-flowing streams. Among these specializations are internal fertilization and the presence of large oral discs on larval individuals that maintain suction on rocks in currents. Tailed frogs also have among the lowest thermal and desiccation tolerances known in amphibians and are thus limited to cool forests (Claussen 1973; Brown 1975). The combination of the life-history requirements and physiological limitations make this a species of special concern, and timber harvest has been suggested to reduce populations (Corn *et al.* 2003). Previous research has used frog larval abundance to draw conclusions on the impact of logging on populations (Welsh & Ollivier 1998; Diller & Wallace 1999; Wilkins & Peterson 2000). However, these methods are influenced by yearly fluctuations in number of individuals (particularly for amphibians; Pechmann *et al.* 1991) and do not give information on key variables such as reproductive success or population isolation. One approach is to use variable genetic markers to investigate levels of genetic diversity and genetic connectivity across a fine scale. Thirteen polymorphic microsatellite DNA markers were developed to determine the influence of forested

stream buffers and harvested patches on genetic diversity and gene flow of tailed frog populations.

We extracted DNA from tail clips obtained from larval individuals collected from two separate streams within Olympic National Forest of northwest Washington State (USA). Using manufacturer's protocols for the QIAGEN DNeasy 96 tissue kit (QIAGEN Inc.), we obtained pure DNA at a concentration ranging between 20 and 200 ng/ μ L.

We sent 10 individuals to ecogenics GmbH to generate an enriched microsatellite library. Size-selected genomic DNA was ligated into SAULA/SAULB-linker (Armour *et al.* 1994) and enriched by magnetic bead selection with biotin-labelled (ACAG)₇ and (GATA)₇ oligonucleotide repeats (Gautschi *et al.* 2000a, b). Of 380 recombinant colonies screened, 94 gave a positive signal after hybridization. Plasmids from 94 positive clones were sequenced and primers were designed for 31 microsatellite inserts. Of these, 26 were tested for polymorphism, with the final 13 loci showing the greatest variation. Primer design was carried out using PRIMER 3 software (Rozen & Skaletsky 2000).

For primer optimization, the polymerase chain reaction (PCR) was used to amplify DNA for each individual at each of the 13 loci. PCRs consisted of 0.1 U of *Taq* polymerase (Applied Biosystems, Inc.), 1 \times PCR buffer with 1.5 mM MgCl₂, 1 μ L of 1.5 mM dNTPs, and 2 μ L of DNA in a 10- μ L reaction volume. All PCRs were run on a PTC-100 Thermal Cycler (MJ Research, Inc.) or iCycler (Bio-Rad Inc.), or ABI 2720 (Applied Biosystems Inc). An initial hold of 95 °C

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Table 1 Forward and reverse primer sequence, repeat motif, annealing temperature (T_A), number of alleles, allelic size range, observed heterozygosity (H_O), expected heterozygosity (H_E) and GenBank Accession numbers for 13 microsatellite loci developed for *Ascaphus truei* and tested in 64 individuals. The fluorescent dye label for each forward primer is indicated as a prefix before the sequence

Locus	Primer sequence	Repeat	T_A	No. of alleles	Range	H_O	H_E	GenBank Accession no.
A1	F: VIC-GGAAACGAAGACGGAGAACAG R: TTCAGAGCCAGTGAAAGTGC	(GATA) ₁₂	53	12	103–151	0.828	0.887	EU271857
A2	F: PET-GCGCTGTCTTCTTTTCGTTTC R: ATCCCCTGCTTCAATCCTG	(GATA) ₁₀	53	13	170–218	0.859	0.858	EU271858
A3	F: PET-GGAACGAGTTGAACCTATCTGG R: CGGATAGGTAGCAGAGATGG	(CTAT) ₂ (CTAC)(CTAT) ₁₄	60	20	147–223	0.891	0.936	EU271859
A4	F: NED-CGCGCTACATTTATGCAGGA R: TGCTGTTTCTTTTGCTGTTGC	(GATA) ₄ (GATT)(GATA) ₂₂	53	34	112–268	0.875	0.968	EU271860
A12	F: 6-FAM-TGTGAAAGGCGGTCTGATAG R: CCCAACCAGTTCCCAAGAATC	(GT) ₂ (GA) ₂ (CAGA) ₇	53	8	179–211	0.812	0.786	EU271861
A13	F: NED-GCCAGCAGGTACATACAGCAC R: CCTAGAGAGCAGGCAGCAAG	(CTAT) ₈ (CAAT) ₃	60	10	159–199	0.859	0.857	EU271862
A14	F: VIC-TCAGAAGGGAAATGCATAACG R: CAGCTTTGACCGGAGAGAAG	(GT) ₄ (CTAT)(CTGT)(CTAT) ₂₀	60	28	167–249	0.938	0.951	EU271863
A15	F: 6-FAM-ATCACTGTGGCAGGTTTCATC R: AATGCATTCAGAGCATAGAT	(CTAT) ₁₄	53	16	107–213	0.906	0.921	EU271864
A17	F: 6-FAM-GATCCAGCATTATTAGTGAGG R: TGGACAGTTTGACCCCTAATATC	(CTAT) ₄ (CTGT) ₁₁	60	22	163–259	0.922	0.936	EU271865
A24	F: 6-FAM-TATCCCTTGTCCACCCCTTC R: CAATAAATACACAGATGTAGCATACCC	(TCTA) ₁₈	53	17	221–285	0.891	0.921	EU271866
A26	F: NED-GCTTTGCTGGTTTCTACAAGTG R: TGTGGAGAGAAACAGGTACAGAT	(GATA) ₁₈	53	24	136–240	0.828	0.937	EU271867
A29	F: PET-GCATTTTGTAGCCACAGAGC R: GATCCTTCCCCTATACGTCTATC	(GATA) ₂₈	56	30	129–267	0.938	0.954	EU271868
A31	F: VIC-TCTGTCAAATTAGAGAAGGACAG R: GCAATATGTGGACCCCAATC	(CTAT)(TTAT)(CTAT) ₉	53	16	125–191	0.859	0.910	EU271869

for 2 min was performed before cycling started to ensure denaturation of DNA strands. The individual amplification cycle consisted of the annealing temperature for 30 s, 72 °C for 30 s, and 95 °C for 30 s (Table 1). Cycling consisted of 25 cycles (with the exception of locus A15, which required 30 cycles) and upon completion was followed with the annealing temperature for 1 min and a concluding hold of 72 °C for 10 min to ensure that all DNA strands had annealed. PCR products from the 13 loci were pooled into three multiplex sets and analysed with an ABI PRISM 3730 Genetic Analyser (Applied Biosystems, Inc.). Electropherograms for all loci were visualized using GENEMAPPER version 3.7 software (Applied Biosystems Inc.). We successfully amplified all loci in 64 individuals of *A. truei* (Table 1).

We tested for evidence for deviations from Hardy–Weinberg equilibrium at each locus, as well as for linkage disequilibrium between locus pairs using GENEPOP 3.4 (Raymond & Rousset 1995). After Bonferonni correction for multiple comparisons, there were no significant deviations from Hardy–Weinberg expectations at any loci or significant linkage disequilibrium between locus pairs. Overall, loci were highly variable, with an average of 19 alleles per locus and an average expected heterozygosity of 0.91 (Table 1). In addition, the repeat motif for most of the loci is a

Table 2 Cross-amplification results of all loci with 22 individuals of *Ascaphus montanus*. PCR conditions are the same as with *Ascaphus truei*

Locus	No. of alleles	Range	H_E
A1	17	99–189	0.933
A2	11	166–222	0.795
A3	9	135–203	0.570
A4	14	116–228	0.895
A12	4	175–187	0.720
A13	6	159–195	0.598
A14	25	209–335	0.967
A15	17	107–185	0.930
A17	21	207–335	0.957
A24	23	229–369	0.968
A26	14	158–226	0.924
A29	18	129–213	0.931
A31	21	137–253	0.944

tetranucleotide repeat, which reduces the potential for genotyping errors. Finally, we also tested 22 individuals of the sister species *Ascaphus montanus* for cross-amplification. All 13 loci successfully cross-amplified in *A. montanus* and had overlapping allelic ranges with *A. truei* (Table 2). In

addition, expected heterozygosity was similar between the two species, with the exception of locus A3 and A13.

These preliminary results indicate that these loci will be very useful for investigating the genetic response of tailed frogs to timber harvest, as well as other questions of population structure and genetic diversity.

Acknowledgements

This work was prepared for and funded by the Cooperative Monitoring, Evaluation and Research Committee under the Washington Department of Natural Resources Forest Practices Adaptive Management Program (IAA-06-047). We thank A. Giordano for assistance with GENEMAPPER software.

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