Isolation and characterization of microsatellite loci for the European tree frog (Hyla arborea)

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Isolation and characterization of microsatellite loci for the European tree frog (*Hyla arborea*)

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Abstract

We developed 11 new microsatellite markers for the European tree frog (*Hyla arborea*), and tested patterns of polymorphism in 54 adults (27 males and 27 females) from two ponds close to Lausanne (Western Switzerland). One marker was sex linked and two pairs displayed linkage disequilibrium. Comparisons of allele numbers with heterozygosity values support a stepwise-mutation model at neutral equilibrium, with mutation rates spanning nearly two orders of magnitude. These markers will prove useful for population genetic studies and fine-scale investigations requiring genetic assignment techniques.

Keywords: European tree frog, *Hyla arborea*, microsatellites, stepwise-mutation model

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The European tree frog (*Hyla arborea*) is widespread throughout Europe, from the coasts of the Atlantic Ocean to the shores of the Black Sea. Like many amphibians, this species has recently declined over its range, mostly due to anthropogenic landscape changes. A series of 15 microsatellite markers developed by Arens *et al*. (2000) have been used to document the effects of fragmentation on genetic variance, and estimate connectivity among residual populations from Denmark (Andersen *et al*. 2004) and the Netherlands (Arens *et al*. 2006). However, some of these markers later appeared to be sex linked (Ha5–22, Ha1–60 and Ha5–201; Berset-Brändli *et al*. 2006, 2007), difficult to amplify or score (Ha1–104, Ha1–140), or to display null alleles (Ha1–9) or insufficient polymorphism for investigations on Swiss populations (L. Berset-Brändli, J. Jaquiéry, T. Broquet, N. Perrin, unpublished results). We thus set out to characterize new markers for this species.

Specific markers were developed from genetic libraries enriched for microsatellite motifs constructed by Genetic Identification Services (GIS, www.genetic-id-services.com). Libraries were built using a pooled sample containing 100 μg of genomic DNA extracted from 10 tadpoles collected in Western Switzerland. Libraries were enriched for CA, GA, CAG, AAC, AAT, TAGA, CATC and TACA following Jones *et al*. (2002). Out of 63 different microsatellite-containing clones, 39 had flanking sequences of length sufficient for primer design using DESIGNERPCR version 1.03 (Research Genetics, Inc.). Of 39 pairs of primers tested, 11 pairs amplified microsatellites that were both easy to score and polymorphic enough to be potentially useful for population genetic analyses.

These microsatellites were amplified in 20-μL reaction volumes each containing 0.2 mM dNTP, 0.5 μM of each primer, 1× QIAGEN PCR buffer (with $MgCl_2$ 15 mM), 0.2 mM $MgCl_2$ (no $MgCl_2$ for Ha-H108, Ha-E2 and Ha-D115), 1 U QIAGEN *Taq* and 1 μL of extracted DNA. The polymerase chain reaction (PCR) programmes were performed on GeneAmp PCR Systems 2700 and 9700 (PerkinElmer), according to the following thermal profiles: initial denaturation at 94 °C for 5 min, followed by 40 cycles at 94 °C for 45 s, annealing at optimal primer temperature (Table 1) for 45 s, elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. Amplification products were run on an ABI PRISM 3100 (Applied Biosystems) automated DNA sequencer. Alleles were scored with GENEMAPPER 3.7 (Applied Biosystems).

The markers were tested on 54 adults (27 males and 27 females) from two ponds close to Lausanne (Switzerland). Individuals were sampled with two buccal swabs each (Broquet *et al*. 2007), then released at the place of capture. Buccal swabs were stored dry at –80 °C before analysis. DNA was extracted using a QIAGEN DNeasy Tissue Kit following the manufacturer's protocol, except that samples were incubated overnight at 56 °C in proteinase K. After

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Table 1 Characterization of 11 polymorphic microsatellite loci for the European tree frog *Hyla arborea*. {Reported are: locus name; repeat motif, sequences for forward (F) and reverse (R) primers; optimal annealing temperature (T_a); allele size range in base pairs; number of alleles (k); observed (H_O) and expected (H_E) heterozygosity calculated on 54 adults (27 males and 27 females); expected number of alleles [$E(k)_{SSM}$] and diversity value (θ_{SSM}) under simple stepwise-mutation model; and GenBank Accession no.}

Locus	Repeat motif	Primer sequence (5′ – > 3′)	T_a (°C)	Size range (bp)	k	H_O	H_E	$E(k)_{SSM}$	θ_{SSM}	Accession no.
Ha-A11	$(CA)_{14}$	F: NED-CCTCCCTCACTCTGCTGAC R: CAATCCCCGAAAAACATTG	58	133–145	3	0.34	0.38	2.91	0.80	EU029094
Ha-A119	$(CT)_5(CA)_6TA(CA)_{14}$	F: NED-CAACTTCCCCCTCTGTTC R: GCTGAGTGTGAGTGTGTTTG	58	238–250	3	0.488	0.503	3.59	1.52	EU029095
Ha-A127	$(TG)_{14}$	F: HEX-CTCTGGGTTGCACTACTTAGTC R: TTCAGGGCTAATTCTTTGTATG	56.8	277–284	3	0.375	0.392	2.97	0.85	EU029096
Ha-A130	$(CA)_{10}$ ... $(CA)_{13}$	F: FAM-ATTGCTCACACATACACACAGG R: GCAGTCACAACTCATTTTGATG	56.8	109–148	5	0.442	0.482	3.46	1.36	EU029097
Ha-B5R3	$(TC)_{13}$	F: FAM-CCCCTTTAGAGTCGCCATAC R: AGCCATCTTGTGGTCAGTCA	56.8	235–245	5	0.652	0.761	6.09	8.25	EU029098
Ha-B12	$(TC)_{21}$	F: HEX-AATGGTATCTCGGTGGTATCC R: TTGAAAAATCTCTCCCTACAGC	56.8	100–118	7	0.721	0.738	5.70	6.78	EU029099
Ha-D3R3	$(TATC)_{21}$	F: NED-ATCACCATCCCTGCATTAC R: CGACATGATAGATGTGAGATAA	56.8	157–169	3	0.242	0.283	2.43	0.47	EU029100
Ha-D115	$(TAGA)_{16}$	F: FAM-GTTTTTCGATTCCTGGATAAC R: TGGGAGTTTTCAAAAGTGAC	56.8	192–216	7	0.835	0.827	7.72	16.21	EU029104
Ha-E2	$(CAA)_7$	F: HEX-ACAACTTCCAACTGGAGTCAAC R: CCTTAGTGGGAGCTGTAATCAC	56.8	151–159	2	0.13	0.154	1.79	0.20	EU029103
Ha-H107	$(GT)_7$ ... $(TATG)_4$	F: FAM-CACCCTGGTAGGGAAATTC R: GGCAAATGGGGATGAGTA	56.8	267–269	2	0.405	0.368	2.85	0.75	EU029101
Ha-H108	$(TACA)_{10}$	F: FAM-GGGGGTGAGTAAGGGTTAAATC R: GCCACTGTATAGTCCCTCCCTA	56.8	250–252	2	0.5	0.377	2.90	0.79	EU029102

incubation, a QIA Shredder was used according to the manufacturer's conditions. DNA was eluted in a 200-μL volume (QIAGEN Buffer AE), and stored at –18 °C.

Allele numbers (k) ranged from two to seven, observed heterozygosities (H_O) from 0.13 to 0.84, and expected heterozygosities (H_E) from 0.15 to 0.83 (Table 1). We found no significant deviation from Hardy–Weinberg equilibrium after Bonferroni corrections (Goudet 1995) and no evidence for null alleles (MICRO-CHECKER 2.2.3; van Oosterhout *et al*. 2004). Two pairs of loci (Ha-D115/Ha-A127 and Ha-A119/Ha-A130) showed significant linkage disequilibrium after Bonferroni corrections (Goudet *et al*. 1996). One marker (Ha-H108) was sex linked: all females were homozygous for allele 250, and all males heterozygous for alleles 250 and 252, pointing to a location on the nonrecombining segment of the sex chromosomes.

Diversity values were calculated at each locus assuming either an infinite allele model ($\theta_{IAM} = H_E/1 - H_E$) or a one-step stepwise-mutation model [$\theta_{SSM} = 1/2[1/(1 - H_E)^2 - 1]$ Kimura & Ohta 1975]. These values ranged from 0.18 to 4.75 for θ_{IAM}, and from 0.20 to 16.21 for θ_{SSM}. The number of alleles expected at neutral equilibrium [$E(k)$] directly relates to this diversity parameter. Under infinite allele model (IAM), $E(k) = \sum_{i=0}^{N-1} \theta/(\theta + i)$ where N is the number of gene copies sampled, while under SSM, $E(k) = (\theta + \beta)/\beta[1 - \prod_{i=0}^{N-1}(\theta + i)/(\theta + \beta + i)]$ where $\beta = \theta(1 - H_E)/H_E - 1$ (Kimura & Ohta 1975). The regression coefficient of $E(k)_{SSM}$ vs. k (b = 0.98, intercept set to 0) was very close to unity and not significantly different from it [95% CI (0.85; 1.13)], with 76% of the variance explained. By contrast, $E(k)_{IAM}$, consistently overestimated the number of alleles [b = 1.71, 95% CI (1.28; 2.04), intercept set to 0]. We conclude that the mutation model does not differ significantly from SSM, and that the population investigated is at neutral equilibrium.

At this equilibrium, the diversity θ should equal the product of effective size N_e, mutation rate and gene copy number per mating pair (i.e. $\theta = 4N_e\mu$ for autosomal loci). Hence, the ratios of θ values among loci measure the ratios of their mutation rates. From the ratios of θ_{SSM}, the mutation rates span nearly two orders of magnitude, being 80 times larger for Ha-D115 than for Ha-E2.

We conclude that the range of diversity values and allele numbers documented for these 11 loci make them suitable tools for population genetic studies, and will also prove useful for investigations on fine-scale dispersal patterns as well as on the mating system of this lek-breeding species.

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