Published in final edited form as:

. 2014 ; 35(2): 243–246. doi:10.1163/15685381-00002940.

Characterisation of nine new polymorphic microsatellite loci in the reticulated glass frog *Hyalinobatrachium valerioi* (Centrolenidae)

Eva Ringler^{1,2,*}, Alexandra Mangold¹, Katharina Trenkwalder¹, Max Ringler^{1,3}, and Walter Hödl¹

¹Department of Integrative Zoology, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria

²Department of Cognitive Biology, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria

³Department of Tropical Ecology and Animal Biodiversity, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

Abstract

Here we document the development of thirteen novel microsatellite markers for the reticulated glass frog *Hyalinobatrachium valerioi* (Centrolenidae). Nine of those markers were polymorphic and contained between 4 and 34 alleles per locus (mean = 20.3) in 138 individuals (91 males, 47 females) from the field site ‘La Gamba’, Costa Rica. Average observed heterozygosity was 0.76. Two loci (*Hyval19* and *Hyval21*) significantly deviated from Hardy-Weinberg equilibrium. We did not find evidence for linkage disequilibrium among any of the loci. These markers will serve to identify the genetic mating system in *H. valerioi*, investigate gene flow between local populations, and reconstruct parent-offspring relationships for studies on individual mating and reproductive success. Therefore, these markers will serve to answer a wide range of scientific questions in conservation, behavioural ecology, and also evolutionary biology.

Keywords

Costa Rica; *Hyalinobatrachium valerioi*; microsatellite markers; population genetics

The reticulated glass frog *Hyalinobatrachium valerioi* (Centrolenidae) is distributed from central Costa Rica to the Pacific coast of Ecuador and inhabits lowland forests, premontane wet forests and rainforests below elevations of 400 m (Savage, 2002). *Hyalinobatrachium valerioi* is nocturnal and occurs along small lowland streams (Guyer and Donnelly, 2005). Throughout the rainy season males are highly territorial and call from elevated positions in the vegetation (Savage, 2002; Vockenhuber et al., 2008). Clutches are laid on leaves up to 6 m above the water. The female leaves the breeding site soon after the eggs are fertilized,

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*Corresponding author; eva.ringler@univie.ac.at.

while the male stays and guards the clutch (approx. 30 eggs per clutch) and continues advertising for females (Vockenhuber et al., 2009). So far nothing is known about any form of clutch piracy in this species. *Hyalinobatrachium valerioi* is listed in the IUCN list as a species of “Least Concern”, as a result of its wide distribution, the tolerance for a certain degree of habitat modification, and its presumed large populations (Solís et al., 2004). However, population sizes are generally decreasing, as it is the case for most amphibians, which are considered the most threatened vertebrate class on earth (Alford, 2011). Still, for most *H. valerioi* populations long-term monitoring studies are lacking. Furthermore, little is known about population connectivity and persistence, as well as about the genetic mating systems, mostly due to the lack of suitable molecular markers.

Herein we describe the characteristics of new microsatellite loci for *H. valerioi*. DNA samples were collected from a study population near the field station ‘La Gamba’, Costa Rica (8°42′61″N, 83°12′97″W). Toe clips were immediately transferred to absolute ethanol and adult frogs were promptly released at their initial sampling location. Genomic DNA was extracted using a standard phenol-chloroform protocol (Sambrook et al., 1989) and sent to Geno-Screen, Lille, France (www.genoscreen.fr). Genomic DNA from seven individuals was used for the development of the microsatellite library through 454 GsFLX Titanium pyrosequencing of enriched DNA libraries as described in (Malausa et al., 2011). Total DNA was enriched for microsatellite loci using the eight probes TG, TC, AAC, AAG, AGG, ACG, ACAT and ACTC and subsequently amplified. PCR products were purified and quantified, and GsFLX libraries were established following the manufacturer’s protocols (Roche Diagnostics) and sequenced on a GsFLX-PTP. All bioinformatical analyses were conducted with the program QDD (Megléc et al., 2010). In that way 12 969 sequences containing microsatellite motifs and 348 sets of primers were identified. Twenty-four out of 348 sets of primers were tested for amplification on agarose gels, giving priority to loci that contain only one tetra-nucleotide microsatellite sequence. Primer sets were discarded if they failed to amplify or led to multiple fragments. Thirteen out of the tested 24 microsatellite loci produced visible products in the expected size range on agarose gels.

For the characterisation of these 13 microsatellite loci we genotyped 138 individuals of *H. valerioi* (91 males, 47 females). PCR amplifications were performed using reaction volumes of 10 µl containing about 10 ng of genomic DNA, 0.2 mM of each dNTP, 1 µM of each forward and reverse primer, 0.5 U of Taq DNA polymerase (Axon) and 1 µl of 10 × NH₄ reaction buffer (Axon), at a final concentration of 1.5 mM MgCl₂. We used the following PCR programme: 10 min at 95°C, 40 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by a final extension step for 10 min at 72°C. Differences in the sizes of the amplified alleles and in the fluorescent dye labels of the primers allowed for pooling of multiple loci for the subsequent genotyping process (see table 1). The products were mixed at equal volume and then diluted with water at 1:25, mixed with HiDiFormamid and the internal size standard ROX350 (Applied Biosystems), and run on an ABI 3130xl Genetic Analyser. Alleles were manually inspected with Peakscanner Software (Applied Biosystems), and final allele sizes were calculated using TANDEM v1.08 (Matschiner and Salzburger, 2009). Four loci appeared to be either monomorphic (*Hyval08* and *Hyval12*) or produced stutter bands (*Hyval02* and *Hyval14*) in our population of *H. valerioi* and were

thus discarded from further analyses. Number of alleles, observed and expected heterozygosities, and PIC (mean polymorphic information content) were calculated using CERVUS 3.0.3 (Kalinowski et al., 2007). Tests for departures from Hardy-Weinberg equilibrium and linkage disequilibrium between all sets of loci were carried out using FSTAT v.2.9.3.2 (Goudet, 2001). MICROCHECKER v.2.2.3 (van Oosterhout et al., 2004) was used to test for the possibility of scoring errors, allelic dropout, and null alleles. The locus-specific primers, their optimized PCR conditions, and the characteristics of 138 *H. valerioi* genotypes are presented in table 1. The program IDENTITY v4.0 (Wagner and Sefc, 1999) was used to calculate the probability of identity (Paetkau et al., 1995).

We detected between 4 and 34 alleles per locus (mean = 20.3), with observed and expected heterozygosities ranging from 0.45 to 0.93 (mean = 0.76), and 0.41 to 0.95 (mean 0.82), respectively. Two out of the nine analysed = microsatellite loci (*Hyval19*, *Hyval21*) showed significant deviations from Hardy-Weinberg equilibrium (p -value for F_{is} within samples = 0.0011, adjusted p -value for 5% nominal level = 0.0056) likely due to the presence of null alleles. We did not find any evidence for linkage disequilibrium among the nine loci (all p -values above the adjusted p -value for sequential Bonferroni correction). With the exception of loci *Hyval19* and *Hyval21*, MICROCHECKER did not detect evidence for scoring errors due to stuttering, neither for large allele dropout, nor for a high frequency of null alleles in any of the tested loci (van Oosterhout values are given in table 1). We did not find any sex-specific differences in allele frequency, observed or expected heterozygosity (Wilcoxon signed rank test, all p -values > 0.05), thus we assume all loci to be autosomal. The probability of identity was $P_{ID}[9] = 1.36 \times 10^{-14}$ for our dataset.

The herein described nine novel microsatellite loci will serve to reveal the mating system and patterns of mate choice and reproductive success, as well as the assessment of gene flow between local populations in *H. valerioi*. We intentionally do not provide any recommendation which loci should be used in further studies and also provide information on all of the tested loci, as in other *H. valerioi* populations or in other centrolenid species these loci might amplify at different frequencies.

Acknowledgments

Fieldwork of K. Trenkwalder and A. Mangold was supported by the University of Vienna (Förderungsstipendium). A. Mangold was further supported by the grant 'Vorarlberg Stipendium'. Work in the lab was supported by the Department of Tropical Ecology and Animal Biodiversity (University of Vienna) and by the Austrian Science Fund (FWF): P24788-B22 (PI: Eva Ringler, <http://www.fwf.ac.at>). Permissions for collection and exportation of all *H. valerioi* samples were provided by Costa Rican authorities (permissions n°100.620 from 05/12/2012, R-021-2012-OT-CONAGEBIO, R-022-2012-OT-CONAGEBIO). We are very grateful to Werner Huber for logistic support in Costa Rica. Thanks to Susanne Hauswaldt and one anonymous reviewer for valuable comments on the manuscript.

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Table 1
Characterisation of 13 microsatellite loci genotyped in 138 *H. valerioi* from a single population in Costa Rica

Locus	Repeat motif	Dye and primer sequence (5'-3')	k	Size range of amplified product (bp)	AT (°C)	mix	H _O	H _E	PIC	P _{HWE}	P _{Null}	GenBank accession no.
<i>Hyal04</i>	TTC	F: ATTO550-CAAAAACCTCATAAAGCTCACTTCC R: TACTCTTGGCCCAACCAAGTT	4	122-137	55	A	0.453	0.409	0.338	NS	-0.0624	KJ126994
<i>Hyal10</i>	TCTA	F: HEX-GACGTACCCAGGAGAACCCAC R: GCCATTTCATAGTAGAACCTTGC	14	96-156	55	B	0.789	0.808	0.779	NS	0.0121	KJ126995
<i>Hyal16</i>	AGAT	F: ATTO550-CAATCTGCGACTTGTCTCTCA R: TGAGATGTGCTGAATGTCTC	14	161-213	55	B	0.884	0.869	0.852	NS	-0.0106	KJ126996
<i>Hyal17</i>	TATC	F: HEX-CATACACGGTTGCATGTAAAA R: CGCCATATGTACAATATACCCCTG	26	215-343	55	A	0.898	0.933	0.925	NS	0.0172	KJ126997
<i>Hyal19</i>	TCTA	F: FAM-ACAGTCTTGGAGACGGGAAG R: GCCAGCTACAGTAGATAGATAGGAA	28	207-359	55	B	0.661	0.904	0.891	**	0.1334	KJ126998
<i>Hyal20</i>	ATCT	F: FAM-ACCTCTGTGGCTCTCTCAG R: TTTTGCCTTGTAAATGCATAGTG	16	275-343	55	A	0.881	0.901	0.889	NS	0.0093	KJ126999
<i>Hyal21</i>	ATCT	F: HEX-ATTGCGTGTGCTTACTTTAGG R: TCCCAAAACAAACAGCAGAA	31	147-279	55	B	0.69	0.952	0.946	**	0.1361	KJ127000
<i>Hyal22</i>	AGAT	F: FAM-CTGGCACCTTTCCTCAACTC R: GCTGTAGAGTTGTTCTCTCGAT	16	70-158	55	A	0.688	0.632	0.618	NS	-0.0775	KJ127001
<i>Hyal24</i>	ATCT	F: FAM-GCAGAAAGTGGAGAAAGGTGCT R: TGGTGACAGTGGATACAGTAGATT	34	144-368	55	B	0.93	0.94	0.933	NS	0.0031	KJ127002
<i>Hyal02</i>	GGA	F: HEX-GACAGATGGGAGGGTGAAGA R: GAAAATTGGATAGTTACAGCAC	s	-	55	-	-	-	-	-	-	KJ127003
<i>Hyal08</i>	ATAG	F: FAM-ACCTCATATCTGCTGCATGGC R: CACAGGTAAAGCAGCATGGA	1	154	55	-	-	-	-	-	-	KJ127004
<i>Hyal12</i>	TAGA	F: TET-TGGCTGAAACATTGCCATTGA R: TGGAAACACAAAATGAGCCA	1	150	55	-	-	-	-	-	-	KJ127005
<i>Hyal14</i>	CTAT	F: TET-ATCGAGTCACCTAAATACCCCT R: CAGCAACAACACATTGGAGG	s	-	55	-	-	-	-	-	-	KJ127006

k: number of alleles observed; AT: annealing temperature; mix: pooled PCR products in the genotyping analysis; H_O: observed heterozygosity; H_E: expected heterozygosity; PIC: mean polymorphic information content; P_{HWE}: probability of deviation from Hardy-Weinberg equilibrium following Bonferroni correction; P_{Null}: van Oosterhout estimate for the frequency of null alleles at each locus; s: stutter bands.