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Characterization of seven new polymorphic microsatellite loci in the brilliant-thighed poison frog *Allobates femoralis* (Dendrobatidae), and their cross-species utility in three other dendrobatid species

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Abstract

Here we document the development of seven novel polymorphic microsatellite markers for the brilliant-thighed poison frog *Allobates femoralis* (Dendrobatidae). We found between six and 27 alleles per locus in 100 individuals (50 males, 50 females) from the field site 'Saut Pararé', French Guiana, with an average observed heterozygosity of 0.79. One locus (*Afem*23) deviated significantly from Hardy–Weinberg equilibrium. We did not find any evidence for linkage disequilibrium among the new loci, or to seven of the already described markers for *A. femoralis*. We also report cross-species amplification of some of the markers in three other dendrobatid species (*A. talamancae*, *Dendrobates tinctorius* and *Oophaga pumilio*).

Keywords

Allobates femoralis; cross-species amplification; Dendrobatidae; microsatellite marker; polymorphic

The Neotropical poison frog *Allobates femoralis* (Dendrobatidae) has a pan-Amazonian distribution (Amézquita et al., 2009). Individuals of this species are rather small (approx. 27 mm snout-urostyle length, females slightly bigger than males, Ursprung et al., 2011a), do not possess highly toxic skin alkaloids (Lötters et al., 2007) and their appearance is rather cryptic. Males call from elevated structures on the forest floor to announce territory possession to male competitors and to attract females (Hödl et al., 2004). Pair formation, courtship and mating take place in the male's territory (Roithmair, 1992; Ringler et al., 2009; Montanarin et al., 2011). Both sexes are highly iteroparous and polygamous within prolonged but rather discrete reproductive periods that coincide with the local rainy seasons (Ursprung et al., 2011a).

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Previously developed microsatellite markers for *A. femoralis* served to investigate the genetic mating system of this species (Ursprung et al., 2011a), parental relatedness and individual reproductive success (Ringler et al., 2012a). Although ten microsatellite loci had been originally designed for *A. femoralis* (Jehle et al. 2008), only seven of those actually work for the population in French Guiana, and for two loci (*Afem*15 and *Afem*16) new primers had to be designed to enhance PCR amplification rates (see Ursprung et al., 2011a). This was likely due to the fact that markers were originally derived from individuals of a Brazilian population more than 800 km away (Amézquita et al., 2009), leading to sequence differences at primer binding sites. Microsatellite markers are a powerful tool to assess genetic differences on the individual and population level (Jehle & Arntzen, 2002; Selkoe & Toonen, 2006). However, their combined resolution capacity is strongly dependent on the number of markers used and their respective level of polymorphism (Kalinowski, 2002).

Here we describe the characteristics of seven new microsatellite loci for *A. femoralis* and assess their cross-species amplification in three other dendrobatid species: *A. talamancae*, another member of the subfamily Aromobatinae and *Dendrobates tinctorius* and *Oophaga pumilio*, two less closely related species from the sub-family Dendrobatinae (cf. Brown et al., 2011; Pyron & Wiens, 2011).

Tissue samples were collected via toe clipping of adult individuals (cf. Ursprung et al., 2011b) from the field station Saut Pararé situated in the nature reserve Les Nouragues, French Guiana (3°59′N, 52°35′W), which harbours a large population of *A. femoralis* (cf. Ursprung et al., 2011a). Toe clips were stored in absolute ethanol and individuals were immediately released where they were collected. Genomic DNA of 15 individuals was extracted using a phenol-chloroform protocol (Sambrook et al., 1989) and sent to Ecogenics GmbH (Zurich, Switzerland) to test twelve validated sets of compound primers for amplification from a previously established microsatellite library based on individuals from Brazil (Jehle et al., 2008). Five primer sets were discarded because they failed to amplify or led to multiple fragments. Seven of the tested microsatellite loci were polymorphic and produced consistent products across all 15 individuals.

For the characterization of these seven microsatellite loci we genotyped 100 individuals of *A. femoralis* (50 males, 50 females). We also checked for cross-species utility of the markers in the three other dendrobatid species *A. talamancae* (*n*=3), *D. tinctorius* (*n*=8) and *O. pumilio* (*n*=8). 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× NH_4 reaction buffer (Axon), at a final concentration of 1.5 mm$MgCl_2$. We used the following PCR programme: 5 min at 95 °C, 39 cycles at 95 °C for 45 s, the primer specific annealing temperature (Table 1) for 45 s, 72 °C for 45 s, followed by a final extension step for 5 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 sequencing process. The pooled products were diluted with water 1:20, mixed with HiDiformamid and the internal size standard ROX500 (Applied Biosystems), and run on an ABI 3130xl Genetic Analyzer. Alleles were manually inspected with Peakscanner software (Applied Biosystems), and final allele sizes were determined using TANDEM v.1.08 (Matschiner & Salzburger, 2009). Number of

alleles, observed and expected heterozygosities, and PIC values (mean polymorphic information content) were calculated using CERVUS v.3.0.3 (Kalinowski et al., 2007) and FSTAT v.2.9.3.2 (Goudet, 2001) was used for calculations of Hardy–Weinberg equilibrium and linkage disequilibria between all sets of loci. 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 100 *A. femoralis* genotypes are presented in Table 1. The program IDENTITY v.4.0 (Wagner & Sefc, 1999) was used to calculate the probability of identity (Paetkau et al., 1995) for different sets of loci.

We detected six to 27 alleles per locus, with observed and expected heterozygosities ranging from 0.27 to 0.95 (mean=0.79) and 0.66 to 0.947 (mean=0.864), respectively. Only one (*Afem*23) out of the seven tested loci showed significant deviations from Hardy–Weinberg equilibrium (*p*-value for F_{is} within samples=0.0071, adjusted 5% nominal level=0.00714), which was likely due to the presence of null alleles. We did not find any evidence for linkage disequilibrium among the new loci, or to any of the seven previously published markers for *A. femoralis*. With the exception of locus *Afem*23, MICROCHECKER did not detect evidence for scoring errors due to stuttering, for large allele dropout, or 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 or observed heterozygosity between males and females (Wilcoxon signed rank test, all *p*-values>0.05), thus assuming all loci to be autosomal.

The herein described microsatellite loci have similar properties than the previously published markers (old/new: mean alleles per locus=17.4/19.3; mean H_E=0.85/0.86; c.f. Ursprung et al., 2011a). By increasing the number of markers from 7 to 14, the probability of identity decreased from $P_{ID}[7]=4.93*10^{-11}$ to $P_{ID}[14]=6.46*10^{-23}$ in the present dataset, or $P_{ID}[13]=5.53*10^{-22}$ (omitting *Afem23*), respectively. Cross-species tests revealed that some loci are potentially useful in three other dendrobatid species, albeit showing much lower variability and amplification success (Table 2; cf. Ringler et al., 2012b).

The integration of the described new microsatellite loci will significantly enhance the explanatory power of future genotype analyses in studies on fine-scale population genetic structure in *A. femoralis*. Furthermore, these markers are designed to be used as a reliable tool for individual identification in mark-recapture studies across the life-history stages of *A. femoralis*; i.e. genetic tracking of individuals from the larval to the adult stage.

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Table 1

Details of seven polymorphic microsatellite loci characterized in 100 *A. femoralis* individuals from French Guiana; *k* (number of alleles observed), AT (annealing temperature), H_O (observed heterozygosity), H_e (expected heterozygosity), *PIC* (mean polymorphic information content), P_{HWE} (probability for deviation from Hardy–Weinberg equilibrium), P_{Null}(van Oosterhout estimate for the frequency of null alleles at each locus).

Locus	Repeat motif	Dye and primer sequence (5′–3′)	*k*	Size range of amplified product (bp)	AT (°C)	H_O	H_E	*PIC*	P_{HWE}	P_{Null}	GenBank accession no
*Afem*04	$(TAGA)_7$	F: HEX-GAGACGCCTGTTATAGATGGTG R: TTAAAATGCACCGACACTGG	21	248–336	56	0.95	0.919	0.908	NS	−0.0213	KF021569
*Afem*20	$(CA)_{15}$	F: TET-TCCTAAGCCAGAGGAAGCTG R: ACAATCACATGCACCGAGTC	6	139–151	56	0.73	0.72	0.669	NS	−0.0083	KF021570
*Afem*22	$(AGAC)_9(AGAT)_{14}$	F: NED-ACCGTGGAGTGGTTGATGAG R: CCTGCCAAGGATTGATAAGC	23	162–258	56	0.949	0.938	0.929	NS	−0.0094	KF021571
*Afem*23	$(TAGA)_8$	F: FAM-AGGCAGAGATTGCAGAGAATG R: GTGGCCATCTCCTGTTCATC	9	219–279	56	0.273	0.66	0.596	**	0.2689	KF021572
*Afem*24	$(ATAG)_{13}$	F: NED-AAAGTAGGGTCGCAGCACTC R: AGGTCAAGTCGGATGGTTTG	25	177–293	56	0.878	0.943	0.935	NS	0.0323	KF021573
*Afem*25	$(AC)_{15}$	F: HEX-GTAATCCCCCAATCCTGGTC R: GATCCCGGCATCGTTAAG	27	168–286	61	0.878	0.92	0.909	NS	0.0218	KF021574
*Afem*27	$(AC)_{12}$	F: FAM-AACGCAAACACACCCTTAGC R: ATCACTTGACATGGAATGACC	24	163–245	56	0,9	0,947	0.938	NS	0.0214	KF021575

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Table 2

Cross-species utility of 14 microsatellite loci in *A. femoralis* in *A. talamancae*, *D. tinctorius* and *O. pumilio* (*n*, number of tested individuals). When polymorphic, the numbers of alleles are given; m, multiple bands within one PCR product; x, no amplification.

Species	***n***	***Afem*05**	***Afem*12**	***Afem*09**	***Afem*03**	***Afem*15**	***Afem*13**	***Afem*16**
A. talamancae	3	1	2	x	2	2	x	x
D. tinctorius	8	1	3	x	2	2	x	x
O. pumilio	8	1	3	x	2	4	x	x
Species	n	*Afem*20	*Afem*22	*Afem*27	*Afem*25	*Afem*24	*Afem*23	*Afem*04
A. talamancae	3	2	m	m	2	3	x	x
D. tinctorius	8	4	m	m	2	m	1	x
O. pumilio	8	4	m	m	2	4	2	x