TECHNICAL NOTE

Ten microsatellite loci for the strawberry poison frog (*Oophaga pumilio*)

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Abstract We describe primers and PCR conditions to amplify nine new tetranucleotide loci and one new dinucleotide locus isolated from the strawberry poison frog (*Oophaga pumilio*). In 21 individuals from Costa Rica, the number of alleles ranged from 4 to 16, observed heterozygosities from 40 to 100%, and polymorphic information content ranged from 0.60 to 0.90 per locus. Evidence for linkage disequilibrium was found only between two loci, but this pattern was not found in other populations tested. All primer pairs cross-amplified in *Oophaga vicentei* from Panama.

The distribution of the strawberry poison frog (*Oophaga pumilio*) ranges along the Caribbean lowlands of Central America, from Nicaragua across Costa Rica to northern Panama. Like other species of poison frogs (Dendrobatidae) it has a bright aposematic colouration. Unusual, however, is the colour polymorphism this species exhibits in the Bocas del Toro archipelago in northwestern Panama. While in most part of the range, frogs are bright red on dorsal and ventral side with dark (black/blue) limbs, here its colour patterns are strikingly different and range from a yellow dorsal side with black spots and a white ventral side, to a dark green dorsal side with bright yellow venter. While colour patterns vary among sites, some locations even have striking variation within. Unfortunately, intensive development on some of these islands is threatening the habitat of several colour morphs.

Several phylogeographic studies have been carried out on *O. pumilio*, in particular to address populations from these islands (Hagemann and Pröhl 2007; Rudh et al. 2007; Wang and Shaffer 2008). However, the amount of colour polymorphism at Bocas del Toro still remains difficult to explain, although dorsal colouration appears to play a role in female preference (Maan and Cummings 2008). Fast-evolving loci such as microsatellites may hold the key to decipher in more detail the genetic variation of this species, but so far such markers are unavailable for *O. pumilio*. We here report on the development of specific microsatellite markers for this species, aiming at loci with tetranucleotide motifs which we have found easier to score. For the enrichment, genomic DNA was used from an individual strawberry poison frog. DNA was enriched with two oligo mixtures containing tetranucleotide probes following the protocol by Glenn and Schable (2005). The mix of biotinylated oligonucleotides contained $(AAGT)_8$, $(AGAT)_8$, $(AACT)_8$, $(ACAT)_8$, and $(AAAT)_8$ as probes. In brief, genomic DNA was digested with *Rsa* I, ligated to Super-SNX linkers, hybridized to the probes and captured with Dynabeads (Dynal Biotech Inc.). Unspecifically bound DNA was washed away according to the protocol and specifically bound DNA was recovered by PCR with the SuperSNX-f (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′) primer. PCR products were enriched twice. The resulting

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Table 1 Characterization of ten microsatellite loci for *Oophaga pumilio*. For *O. p.* number of alleles (NA), size ranges, observed (Ho), expected heterozygosity (He), and polymorphic information content (PIC) are given for each locus. Size range refers to the observed distribution of alleles at each locus for 21 *O. p.* and for two *O. vicentei* (*O. v.*) (except for Oop_C3) all alleles found are listed

Locus	Primer sequence 5′→3′	Genbank accession number	Repeat sequence	N	NA	Size range (bp) *O. p.*	Alleles (bp) *O. v.*	Ho	He	PIC
Oop_C3-F	GGTCCTAACAACATGCACCTTTC	FJ645912	(TATC)$_{13}$	21	11	93 – 153	129 (N = 1)	1.0	0.88	0.84
Oop_C3-R	TCCAGCCTCCTCTTTTACATTTG									
Oop_B8-F	TTTCCCATTAGAATGATTTTTGACTC	FJ645913	(TATC)$_{11}$	21	11	294 – 338	310, 314, 318, 338	0.76	0.87	0.83
Oop_B8-R	CTTGCTACACTTGATCCCAAACC									
Oop_H5-F	GCGGAGAAGAAGGATGAAATAGC	FJ645914	(TATC)$_{17}$	21	13	258 – 314	266, 282, 286	0.67	0.92	0.88
Oop_H5-R	CCGGACTCTCCTTTTCTCTTACTG									
Oop_B9-F	AAAATGTCCACCCATCATTTGTC	FJ645915	(TATC)$_{21}$	21	10	136 – 176	128, 132, 172	0.67	0.89	0.85
Oop_B9-R	GAACAATTTGAAAGGATGCCAAC									
Oop_F1-F	CGCAGTTTCCTATTGTCTTTTCC	FJ645916	(TAGA)$_{19}$	21	16	308 – 372	320, 324, 328	0.86	0.93	0.90
Oop_F1-R	GGGCCAAAATCCTAACAAATTC									
Oop_E3-F	AGACATGGCCATAACAAATGGAC	FJ645917	(TCTA)$_{20}$	20	8	390 – 422	386, 398	0.40	0.77	0.72
Oop_E3-R	CATAGGGATATGCCTTGTTTGC									
Oop_G5-F	CCATTGACTTACATTGGATTCG	FJ645918	(TATC)$_{26}$	21	14	286 – 358	306, 334	0.86	0.92	0.89
Oop_G5-R	TCGGAACTGTTTGCTTTGC									
Oop_01-F	ATACTGCGACCGCTGTGTATAG	FJ645921	(TG)$_{13}$	21	4	178 – 190	178, 186	0.62	0.68	0.60
Oop_01-R	GACAGACAGACACACTCCCTTG									
Oop_D4-F	GCAGGGGCGTCACAATAG	FJ645919	(TAGA)$_{14}$	21	8	186 – 254	202, 210, 234, 254	0.76	0.66	0.61
Oop_D4-R	CGCTAAAGGTCAGGTTTTGG									
Oop_C11-F	TTGCGGTGGTCTCTTAATTTTTG	FJ645920	(TAGA)$_{22}$	21	6	154 – 178	162, 178	0.52	0.68	0.62
Oop_C11-R	ATTCTTTACGAGTGGAGGGGTTG									

PCR products were cloned using the TOPO TA Cloning System (2.1) (Invitrogen). Colonies were amplified using M13 primers and for 50 colony-PCR products of 500–1,000 base pairs good sequences were obtained with T3 and T7 primers. Sequencing was done using BigDye version 3.1 chemistry (Applied Biosystems) on an ABI 3730XL sequencer.

Sequences of 39 clones contained microsatellite motifs but in nine of these the motives were repeated less than nine times and they were not considered further. Two other clones contained composite microsatellites and one a dinucleotide locus, therefore neither were used for primer design. In five clones the flanking sequence was too short for successful primer design. Primers were designed for 19 loci with PRIMER3 version 0.4.0. For ten loci primers did not render amplicons of the appropriate size or generated more than two bands per individual. Primers for nine polymorphic tetranucleotide loci were identified (Table 1). A further dinucleotide locus had previously been isolated by one of us (SH). This was done using a modified protocol by Fischer and Bachman (1998) and the detailed procedure is described in Hagemann (2005).

Two quadruplex and one duplex PCR were performed using the Type-it Microsatellite PCR Kit (Qiagen). All reactions were done in volumes of 5 µl and all primers were at 10 µM concentrations. Forward and reverse primers for each locus were mixed at equal proportion, and these mixes were used for the following recipes. For the first multiplex mix 1.25 µl Master Mix, 0.35 µl of Oop_B8, 0.3 µl of Oop_C3, 0.25 µl of Oop_H5, and 0.15 µl of Oop_01, 1 µl DNA (10–20 ng/µl), and 2.15 µl dH$_2$O were used. The second mix contained the same amount of master mix and water, 0.3 µl each of Oop_B9, Oop_F1, and Oop_E3 and 0.15 µl of Oop_D4. Oop_G5 and Oop_C11 were amplified in a duplex reaction using 0.3 µl of each primer mix. All reactions were performed using the same thermal cycling program: following an initial denaturation at 95°C for 5 min, 35 cycles were run with: 95°C for 30 s, 60°C for 90 s and 72°C for 45 s. This was followed by a 30 min extension at 60°C. Amplicons were diluted appropriately before fragment size determination was done on an ABI 3130XL automated sequencer using LIZ 600 (Applied Biosystems) as an internal size standard and analysed using GENEMAPPER software (Applied Biosystems).

We genotyped 21 *O. pumilio* individuals from Costa Rica. Because we did not have a large enough sample from one population, we combined samples from two sites, Guápiles (N10° 11.298′ W83° 49.274′) and Pueblo Nuevo (N10° 19.305′ W83° 34.440′). We calculated allele frequencies, observed and expected heterozygosity, and polymorphic

information content (PIC) values using CERVUS vs. 3.0 (Kalinowski et al. 2007). We further analysed the loci for deviation from Hardy–Weinberg Equilibrium and tested for linkage disequilibrium with GENEPOP web vs. 3.4 (Raymond and Rousset 1995). The results of testing the ten primer pairs on 21 *Oophaga pumilio* DNA samples are summarized in Table 1. We detected an average number of 10.1 alleles per locus (range 4–16), an average observed heterozygosity of 0.71 (range 0.40–1.0), and an average PIC of 0.77 (range 0.60–0.90). Linkage disequilibrium was detected between Oop_C3 and Oop_G5, but this pattern was not confirmed in other populations tested (data not shown). In the sample studied here, one locus (Oop_E3) was found to be deviating from Hardy–Weinberg equilibrium after Bonferroni correction. We also tested all primers on two individuals of *Oophaga vicentei* from Panama (Provincia de Veraguas and Provincia Coclé): all primers amplified and alleles were within the size range of *O. pumilio*.

We now have a set of codominant markers developed that may aid in resolving some of the questions regarding the population genetic structure of this species, for example, by comparing the level of genetic diversity among populations with different colour pattern and by assessing the amount of gene flow among populations.

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