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TECHNICAL NOTE

Isolation and characterisation of 19 microsatellite loci from the Amazonian frog *Adenomera andreae* (Amphibia: Anura: Leptodactylidae)

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Abstract Nineteen novel microsatellite loci were isolated from *Adenomera andreae*, a widespread Amazonian frog considered to be a species complex. Three multiplex kits were optimized. Genetic diversity was assessed in 66 individuals sampled in three populations along the West of the Approuague River catchment (French Guiana). We also tested the multiplex kits in four other *Adenomera* and nine *Leptodactylus* species with 43.4 and 17.5% success respectively.

Keywords Amphibians · Amazonia · French Guiana · Enriched library · Multiplex PCR · Cross-amplification

The genus *Adenomera* (*Leptodactylus marmoratus* group *sensu* Frost et al. 2006) includes 12 described species, all small terrestrial burrowers. Of these, *A. andreae* is a forest litter species considered to be a species complex (Angulo et al. 2003) virtually continuously distributed throughout Amazonia, from sea level to 800 m above sea level. It is found in most type of forest and occurs in very large populations. Despite these characteristics, many non overlapping mtDNA lineages have been found in relatively restricted area within French Guiana (Fouquet et al. 2007; Fouquet 2008). This strong genetic structure can have several causes ranging from reproductive mode (e.g. no larval dispersion) to landscape barriers such as rivers, which could limit gene flow, and a complex evolutionary history with past range fragmentation linked to climatic fluctuations (Noonan and Gaucher 2005, 2006). For all of these reasons *A. andreae* represents an interesting species to study species boundaries and the evolutionary history of Amazonian forest biota. In order to investigate microevolutionary processes and fine scale genetic structure, and also to obtain data on the species' evolutionary history, highly variable, unlinked markers, such as microsatellites, are needed. To date, little is known of the true diversity of Amazonian amphibians, their geographic boundaries, levels of intraspecific diversity, and origins (Köhler et al. 2005); microsatellites will help fill these gaps and establish effective conservation strategies.

Microsatellites were developed following the biotin enrichment protocol of Glenn and Schable (2005). Total genomic DNA was extracted from liver tissue using Puregen Gentra DNA Tissue Kit (QIAGEN). Genomic DNA was digested with *Rsa*I and *Xmn*I. Double-stranded linkers (SuperSNX Forward; SuperSNX24+4P Reverse) were ligated to the blunt ends of these fragments using T4 DNA Ligase (NEB). Biotin-labelled oligonucleotides corresponding to microsatellites motif $(AG)_{12}$ was hybridized with the linker-ligated DNA and captured using Streptavidin magnetic beads and a magnetic separation rack (NEB). Unbound genomic fragments (lacking complimentary repeat motifs) were removed with successive washes (Glenn and Schable 2005). Recovered fragments were amplified in a 25 µl PCR reaction using SuperSNX Forward primer for 25 cycles of 95°C for 20 s, 60°C for

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Table 1 Characterization and level of variability at 19 microsatellite loci in three populations of *Adenomera andreae*

Locus	GenBank Accession	Fluorescent dye	Labelled primer (5′–3′)	Non labelled primer (5′–3′)	Core motif	PCR multiplex		Total size range (bp)	Total N_a	H_O/H_E		
						Kit	Primer concentration			Cisame West ($n = 24$)	Inselberg-Parare trail ($n = 20$)	Regina West ($n = 22$)
Adan-40	GQ339727	PET	F: CAATCCTTCAAATGGCTCCT	R: ATGGAAGGATTTTATGATCAGTG	$(AG)_8$	1	50 nM	103–116	7	0.660/0.417	0.524/0.556[a]	0.649/0.524
Adan-02	GQ339737	6-FAM	F: ACAATTTCATGGCAGAAAGC	R: ACTCTCCTTCACCACAATCTCC	$(GA)_6$		50 nM	80–105	6	0.642/0.625[a]	0.831/0.824	0.549/0.429
Adan-29	GQ339734	VIC	F: TTTCCGGTATCTTCCTGGTG	R: GCTGGTGGGTGAAGACTAGC	$(GA)_5AA(GA)_4$		50 nM	107–153	6	0.451/0.333	0.702/0.500	0.647/0.429[a]
Adan-22	GQ339741	PET	F: CGGAACGATGGGATATGAAC	R: TGATCCCTCCCTTTCATCTG	$(AG)_5AC(AG)_5$		100 nM	163–202	9	0.639/0.583	0.787/0.722	0.573/0.524
Adan-38	GQ339739	NED	F: ACTGCTCTTCCAGCACATCC	R: TTCTTTCCTGAAACACGAGGA	$(CTC)_4N_{16}(CTC)_5$		100 nM	117–153	5	0.595/0.292[a]	0.756/0.722	0.627/0.286[a]
Adan-30	GQ339742	PET	F: TAAGGTTCCAGGGCATTCAG	R: TTCAAAACGGTGTGTGCAAT	$(GT)_5$		100 nM	400–411	8	0.453/0.542	0.731/0.529	0.553/0.714
Adan-42	GQ339735	NED	F: AGATGGTCAGCAGCTTCACA	R: CACTAAACAAGCGGCTTAACG	$(AG)_{11}$		100 nM	232–270	21	0.911/0.750	0.841/0.778	0.902/0.857
Adan-26	GQ339730	6-FAM	F: GGCCCAAAAGTTTCAAGCTA	R: AGCAGGAGCAAACATGGACT	$(AG)_6$	2	100 nM	234–238	3	0.940/0.875[a]	0.949/0.550	0.928/0.773[a]
Adan-18	GQ339724	PET	F: TCAATACCAGTTCCCTGTTGC	R: GCAAAGTGGTCTTTTGCTCA	$(TC)_8N_{14}(TC)_5$		50 nM	94–131	16	0.923/0.875	0.928/0.950	0.849/0.818
Adan-15	GQ339726	6-FAM	F: GCTGACAATGTGGCTGAGAA	R: GTTACAGCAGCAAGCATGGA	$(GA)_7$		50 nM	117–123	7	0.676/0.542	0.677/0.750	0.654/0.773
Adan-05	GQ339738	VIC	F: TCGGACACGCATACTCACTC	R: TGAGACCTTTGTCAGTGTTTTTG	$(TCTA)_{16}$		100 nM	160–407	30	0.765/0.667	0.737/0.800[a]	0.362/0.273[a]
Adan-12	GQ339733	PET	R: GCCTGCCTTGTGTAGCTTTC	F: TGGTCAAATTCTCAGAGACACC	$(AG)_8ACTGCG(AG)_4$		100 nM	194–235	22	0.403/0.125	0.582/0.368	0.431/0.200
Adan-08	GQ339729	PET	F: CTGTGCGCAGTTCAGGTATC	R: CATTGCAGACATGATTTGTGG	$(GT)_8$	3	100 nM	172–189	9	0.619/0.542[a]	0.703/0.600[a]	0.424/0.364
Adan-34	GQ339732	PET	R: GCCGGCAAGTACGGTAAGTA	F: TCCATGTAGGTGGCAAAAGA	$(TA)_4N_9(GA)_{10}$		100 nM	303–326	19	0.861/0.625[a]	0.822/0.500	0.757/0.773
Adan-01	GQ339740	VIC	F: GGGGCATACAACAGCTAGGA	R: TGGCTGGAAATACGACCTCT	$(GA)_4N_{10}(GA)_9$		50 nM	143–185	10	0.623/0.188	0.723/0.474	0.686/0.111
Adan-20	GQ339725	6-FAM	F: GGCAATGCTCCTTCTGTTTC	R: TGCCCCCACACCTATTGTAT	$(CT)_5TT(CT)_{11}$		100 nM	280–300	10	0.965/0.917	0.944/0.600[a]	0.942/0.955[a]
Adan-27	GQ339731	VIC	F: CAGAGTCTGGGTTTGCAGATT	R: AGTGCAAAAGTGGTGCAATG	$(TATC)_{15}$		100 nM	342–433	34	0.856/0.200	0.861/0.737[a]	0.647/0.526
Adan-37	GQ339728	6-FAM	F: TTTTGTCACGGCAGTTCTGA	R: AAACATAGGGGGTTGGCTTT	$(CT)_{12}$		50 nM	124–184	22	0.902/0.478	0.930/0.294[a]	0.762/0.455[a]
Adan-43	GQ339736	NED	F: TGAGATTTGGGGTTTCCATC	R: GTGGTCAATAAGGCCGAGAG	$(CT)_9$		100 nM	193–246	11	0.681/0.625	0.792/0.750	0.772/0.636

[a] Significant departure from Hardy–Weinberg equilibrium

Table 2 Cross-species amplification results (size range and, between parentheses, number of alleles per locus) in four *Adenomera* species and nine *Leptodactylus* species

Species	Adan-01	Adan-02	Adan-05	Adan-08	Adan-12	Adan-15	Adan-18	Adan-20	Adan-22	Adan-26
A. heyeri ($n = 4$)	141(1)	78(1)	–	–	–	113(1)	–	–	175–217(2)	251(1)
A. hylaedactyla ($n = 6$)	–	–	–	–	–	111–114(3)	92(1)	–	173(1)	–
A. lutzi (Guyana) ($n = 1$)	–	–	–	–	–	112(1)	–	–	–	211(1)
A. sp. (Peru) ($n = 1$)	141(1)	–	–	–	203–207(2)	112(1)	117–121(2)	–	201–207(2)	232–234(2)
L. knudseni ($n = 4$)	–	–	–	–	–	–	–	–	–	–
L. longirostris ($n = 3$)	–	–	–	–	–	–	–	–	–	–
L. myersi ($n = 2$)	–	–	–	–	–	–	–	–	–	–
L. mystaceus ($n = 4$)	–	–	–	–	–	–	–	–	–	–
L. pentadactylus ($n = 1$)	–	–	–	–	–	116–120(2)	–	–	–	–
L. rhodomystax ($n = 3$)	–	–	–	–	–	–	–	–	–	–
L. wagneri A ($n = 2$)	–	–	–	–	–	–	–	–	–	–
L. wagneri B ($n = 4$)	–	–	–	–	–	–	–	–	–	–
L. wagneri E ($n = 2$)	–	–	–	–	–	–	–	–	–	–

Species	Adan-27	Adan-29	Adan-30	Adan-34	Adan-37	Adan-38	Adan-40	Adan-42	Adan-43
A. heyeri ($n = 4$)	375–570(7)	114(1)	–	–	–	79(1)	–	–	216(1)
A. hylaedactyla ($n = 6$)	213–297(6)	–	–	–	137(1)	138–170(4)	–	–	203–214(3)
A. lutzi (Guyana) ($n = 1$)	388(1)	–	402(1)	–	131(1)	–	–	–	201(1)
A. sp. (Peru) ($n = 1$)	376–402(2)	112(1)	–	–	129–149(2)	164(1)	–	–	201(1)
L. knudseni ($n = 4$)	–	–	–	–	–	117(1)	118(1)	266–301(2)	–
L. longirostris ($n = 3$)	–	–	–	–	154(1)	117–143(2)	118(1)	–	220(1)
L. myersi ($n = 2$)	–	–	–	–	78(1)	117(1)	118(1)	–	–
L. mystaceus ($n = 4$)	–	–	–	–	155(1)	117(1)	118(1)	–	–
L. pentadactylus ($n = 1$)	–	106–151(2)	–	–	–	117(1)	118(1)	266(1)	–
L. rhodomystax ($n = 3$)	–	140(1)	–	–	–	117(1)	118(1)	264–266(3)	–
L. wagneri A ($n = 2$)	–	–	–	–	–	117(1)	118(1)	–	–
L. wagneri B ($n = 4$)	–	–	435(1)	–	–	117(1)	118(1)	–	–
L. wagneri E ($n = 2$)	357(1)	–	–	–	–	117–169(3)	118(1)	–	–

–, no amplification

20 s, and 72°C for 90 s and a final extension step of 30 min. Amplified fragments were then cloned using the TOPO TA Cloning Kit (Invitrogen). Recombinant clones were identified via blue-white selection and replated to allow for additional growth. Colony scrapes of these clones were placed directly into 25 µl PCR reactions (cycle as above, except 47°C for annealing temperature) containing M13F (−20) and M13R primers. One hundred and nineteen inserts of appropriate size (300–1000 bp) were obtained and bidirectionally sequenced on an ABI 3130 Genetic Analyser (Applied Biosystems). Vector sequence was trimmed and fragments were aligned and edited using Geneious 4.0.4 (Gene Codes Corp.). Among them, 42 sequences for which primers could be designed (mainly containing a perfect motif with more than 5 repeats) were isolated. Sixty-seven primer pairs were designed for these 42 loci (for a total of 109 primers, two pairs designed for 25 loci). Primers pairs were optimized for both size range and homogeneity in annealing temperatures (57–63°C) using Geneious 4.0.4. Amplifications were performed for each of the 57 primers pairs in a total volume of 10 µl containing 0.5 µl of total DNA extract using QIAGEN Multiplex PCR Kit following the manufacturer's protocol, with 30 cycles and an annealing temperature at 57°C. Thermocycling was performed on a Mastercycler® gradient (Eppendorf) with the following protocol: 95°C for 10 min, followed by 30 cycles (94°C for 1 min, 57°C for 1 min, 72°C for 1 min), and 60°C for 45 min. Only 28 primer pairs were retained, which displayed a clear amplification pattern. All the 28 loci were then amplified separately as described above; one primer of each pair was labeled with either 6-FAM (Eurogentec), PET, NED or VIC (Applied Biosystems) fluorescent dye. Visualization of the amplicons was conducted on an ABI 3130xl Genetic Analyser (Applied Biosystems). Alleles sizes were scored against an internal GeneScan-500 LIZ® Size Standard and genotypes

were obtained using GeneMapper 3.7. Among the 28 loci, a total of 19 loci that were associated with clear genotype profile (low noise, high single peak and weak stutter) were selected and combined into three PCR multiplex kits (Table 1). Among these 19 loci, 11 were displaying perfect motifs in the clone sequences and eight interrupted motifs (Table 2). PCRs were conducted as described above.

Polymorphism was screened on 66 individuals of *A. andreae* from three populations sampled along the West part of the Approuague River catchment (French Guiana). The number of alleles per locus ranged from 3 to 34 and expected heterozygosity values ranged from 0.362 to 0.965 (Table 1). After Bonferroni correction, there was no indication of linkage disequilibrium among loci. 70% of loci were at Hardy-Weinberg equilibrium (HWE), except for Adan-02, Adan-38, Adan-26, Adan-08, Adan-34, Adan-20 and Adan-37 in the Cisame West sample, Adan-40, Adan-05, Adan-08, Adan-20, Adan-27 and Adan-37 in the Inselberg-Parare trail sample, and Adan-29, Adan-38, Adan-26, Adan-05, Adan-20 and Adan-37 in the Régina West sample, which show significant excess of homozygotes. Departure from HWE could be caused by: (i) null alleles; (ii) consanguineous mating or biased sampling of related individuals; or (iii) a Wahlund effect. Departure from HWE was observed in most loci in a previous study using microsatellites in a Neotropical frog with comparable life history traits and sampled at a fine geographical scale (Elmer et al. 2007). They suggested that in this type of species such pattern could be caused by a lack of panmixia of inter-individual isolation by distance.

The multiplex kits were also tested on four other *Adenomera* species (successful in 43.4% cases) and nine *Leptodactylus* species (successful in 17.5% cases) (Table 2). Polymorphism seems low in most cases, particularly for *Leptodactylus* ssp. Consequently, the applicability of these markers seems taxonomically circumscribed to the *Adenomera* genus and may be even limited to *Adenomera andreae*.

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References

Angulo A, Cocroft RB, Reichle S (2003) Species identity in the genus *Adenomera* (Anura: Leptodactylidae) in southeastern Peru. Herpetologica 59:490–504

Elmer KR, Davila JA, Lougheed SC (2007) Applying new inter-individual approaches to assess fine-scale population genetic diversity in a neotropical frog, *Eleutherodactylus ockendeni*. Heredity 99:506–515

Fouquet A (2008) Diversity and phylogeography of Eastern Guiana shield frogs. PhD Dissertation. University of Canterbury

Fouquet A, Gilles A, Vences M, Marty C, Blanc M, Gemmell NJ (2007) Underestimation of species richness in neotropical frogs revealed by mtDNA analyses. PLoS ONE 2:e1109. doi: 10.1371/journal.pone.0001109

Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CFB, de Sà RO, Channing A, Wilkinson M, Donnellan SC, Raxworthy CJ, Campbell JA, Blotto BL, Moler P, Drewes RC, Nussbaum RA, Lynch JD, Green DM, Wheeler WC (2006) The amphibian tree of life. Bull Am Mus Nat Hist 297:1–370

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. In: Zimmer EA, Roalson EH (eds) Methods in enzymology 395, molecular evolution: producing the biochemical data, Part B. Academic Press, San Diego, CA, pp 202–222

Köhler J, Vieites DR, Bonett RM, Garcia FH, Glaw F, Steinke D, Vences M (2005) New amphibians and global conservation: a boost in species discoveries in a highly endangered vertebrate group. Bioscience 55:693–696

Noonan BP, Gaucher P (2005) Phylogeography and demography of Guianan harlequin toads (*Atelopus*): diversification within a refuge. Mol Ecol 14:3017–3031

Noonan BP, Gaucher P (2006) Refugial isolation and secondary contact in the dyeing poison frog *Dendrobates tinctorius*. Mol Ecol 15:4425–4435