Characterization of nine polymorphic microsatellite loci in the dyeing poison frog *Dendrobates tinctorius* (Dendrobatidae), and their cross-species utility in two other dendrobatoid species

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While field and laboratory based studies have provided significant insights into the parental care and courtship behaviour of dendrobatoid frogs, a comprehensive assessment of their genetic mating systems and population genetic parameters has been precluded because of the lack of highly variable DNA markers. Here we document the development of nine novel polymorphic microsatellite markers for the dyeing poison frog *Dendrobates tinctorius* (Dendrobatidae). We found between three and 16 alleles per locus in 60 individuals (30 males, 30 females) from the field site Saut Pararé, French Guiana, with an average observed heterozygosity of 0.75. None of the loci deviated significantly from Hardy–Weinberg equilibrium or showed linkage disequilibrium. We also report successful cross-species amplification of the nine markers in two other dendrobatoid species (*Allobates femoralis* and *Oophaga pumilio*). These markers have the potential to aid in determining the genetic structure of local populations, identifying small-scale phylogenies such as parent-offspring relationships and will allow for cross-species comparisons within dendrobatoid species. Therefore, these markers can be applied to a wide range of scientific fields, such as conservation, behavioural ecology and evolutionary biology.

Key words: cross-species amplification, *Dendrobates tinctorius*, Dendrobatidae, microsatellite marker, polymorphic

Dendrobates tinctorius (Dendrobatidae) occurs throughout the eastern part of the Guiana Shield and is typically found in canopy gaps of primary forests from the sea level up to 600 m (Noonan & Gaucher, 2006; Wollenberg et al., 2006; Born et al., 2010). *Dendrobates tinctorius* is one of the largest dendrobatoids (37–53 mm in the population studied, B. Rojas unpubl. data; Lötters et al., 2007), features toxic skin alkaloids (Summers & Clough, 2001), and its bright colouration acts as an effective warning signal against potential predators (Noonan & Comeault, 2009, Comeault & Noonan, 2011). *Dendrobates tinctorius* also exhibits a few behavioural traits that are exceptional in the family: neither males nor females have been documented to display strong territoriality and males do not produce a prominent advertisement call (Born et al., 2010). The inter- (Wollenberg et al., 2008) and intra-populational (Rojas, unpubl. data) variation in colour patterns is extremely high and has even led to the description of some populations as distinct species (e.g. *Dendrobates azureus*, synonymized by Wollenberg et al., 2006).

Here we describe the characterization of nine *D. tinctorius* microsatellite loci and assess their cross-species amplification in two other dendrobatoid species: *Oophaga pumilio* as another member of the Dendrobatidae and *Allobates femoralis*, a less closely related species but still within the family Aromobatidae (Grant et al., 2006). The markers were developed to investigate the genetic mating system of this species, determine the relationship between colour patterns and individual reproductive success, and should also reveal insights into population genetic structure within and between different phenotypic type localities.

Samples (toe tips of adult individuals) were collected from a study population near the field station Saut Pararé situated in the nature reserve Les Nouragues, French Guiana (3°59'N, 52°35'W), from a fairly large population of *Dendrobates tinctorius* (approx. 4.3 individuals/100m^2, BR pers. obs., Devillechabrolle, 2011). Toe clips were stored in absolute ethanol and individuals were immediately released at their initial sampling location. Genomic DNA was extracted using a phenol–chloroform protocol (Sambrook et al., 1989) and sent to GenoScreen, France (www.genoscreen.fr). Genomic DNA from seven individuals was used for the development of microsatellites libraries through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in (Malausa et al., 2011). Total DNA was enriched in 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 then carried out following manufacturer's protocols (Roche Diagnostics) and sequenced on a GsFLX-PTP. In that way, 3349 potential markers were identified. All bioinformatical analyses were conducted with the program QDD (Meglécz et al., 2010). Twenty-four out of the 76 validated sets of compound primers were tested for amplification. Primer sets were discarded if they failed to amplify or led to multiple fragments. Twelve out of the 24 microsatellite loci were tested for polymorphism, nine of which appeared to be polymorphic and produced consistent products across all seven individuals. For the characterization of the microsatellite loci we genotyped

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Table 1. Details of nine polymorphic microsatellite loci characterized in 60 *D. tinctorius* individuals from French Guiana. *k* (number of alleles observed), H_o (observed heterozygosity), H_e (expected heterozygosity), P_{HWE} (probability for deviation from Hardy–Weinberg equilibrium), P_{Null} (Oosterhout estimate for the presence of null alleles at each locus).

Locus	Repeat motif	Primer sequence (5'–3')	*k*	Size range of amplified product (bp)	H_o	H_e	P_{HWE}	P_{Null}	GenBank accession no
Dtinc09	$(TAGA)_7$	F: FAM-GGACAGAAACGTCGCCATA	9	131–163	0.6731	0.7511	0.4559	0.0495	JX120777
		R: TTGCCGAATGATTAATGACA							
Dtinc10	$(CA)_8$	F: VIC-TGAACATGAGGTTCTTTGATTAACAT	3	147–155	0.5	0.4738	0.8671	-0.0412	JX120778
		R: TGTATGGTATATGTATGAACGGTGT							
Dtinc14	$(AC)_8$	F: NED-GCGGTGGAATTTTCTGGAG	5	174–192	0.6	0.5546	0.4839	-0.0616	JX120779
		R: CCCAAGAAATTAGGACCATCC							
Dtinc17	$(TC)_{10}$	F: FAM-GGAATCGTGGAAGTTCGTGT	7	136–156	0.661	0.732	0.4052	0.0485	JX120780
		R: TGGACAGGAGAGGGAAAGAG							
Dtinc19	$(CTAT)_{11}$	F: NED-ATCATCCTGTCTTCAATGTGTCTC	11	108–156	0.8667	0.8478	0.8825	-0.0149	JX120781
		R: CAGTGCAAGCTCAACAAAGC							
Dtinc21	$(TATC)_{12}$	F: VIC-CTTGTGCCTGGAAACACTCA	10	120–156	0.8305	0.8469	0.557	0.0022	JX120782
		R: GGGAAAACTTGGAAAATAAGCA							
Dtinc22	$(TTC)_{14}$	F: PET-ATGTCTGATTTCCCCAGCAG	14	141–183	0.8983	0.8731	0.7245	-0.022	JX120783
		R: CACATAATGTTAAAGTTGGAATGGA							
Dtinc23	$(ATCT)_{14}$	F: PET-CCCTATTCTCATGTCTGCAATG	15	195–251	0.9333	0.9078	0.9897	-0.0174	JX120784
		R: CCTGCAAGGAAAACAGATCA							
Dtinc24	$(CTAT)_{17}$	F: PET-GTCATGAAATGTATTCTTCCAACC	16	100–172	0.8246	0.8907	0.2106	0.0347	JX120785
		R: GGTGTAGTCTGTCTCTGGCGA							

60 individuals of *D. tinctorius* (30 males, 30 females). Additionally, we checked for cross-species utility of the markers in two other dendrobatoid species, *A. femoralis* (*n*=8) and *O. pumilio* (*n*=8). Genetic material of *A. femoralis* was available from a recent population genetic study (Ursprung et al., 2011). DNA samples of *O. pumilio* from Panama and Costa Rica were provided by H. Pröhl. PCR amplifications were performed using reaction volumes of 10 µL containing 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: 5 min at 95 °C, 39 cycles at 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, followed by a final extension step for 5 min at 72 °C. Although the annealing temperature was 55 °C for all loci, we decided against multiplexing several loci in PCRs. The amplified products were diluted with water (1:80), mixed with internal size standard LIZ600, and run on an ABI 3130xl Genetic Analyzer. Alleles were manually inspected with Peakscanner Software (Applied Biosystems), and binned to their final sizes using TANDEM v1.08 (Matschiner & Salzburger, 2009). Observed and expected heterozygosities were calculated using CERVUS (Kalinowski et al., 2007), and GENEPOP v4.0 (Rousset, 2008) was used for calculations of Hardy–Weinberg 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. One out of the 12 tested microsatellite loci was found to be monomorphic (*Dtinc05*) and two others failed to amplify. Locus-specific primers, their PCR conditions and fragment size data which were obtained from 60 *D. tinctorius* genotypes are presented in Table 1. We detected three to 16 alleles per locus, with observed heterozygosities ranging from 0.50 to 0.93 (mean=0.75). Loci showed neither significant deviations from Hardy–Weinberg equilibrium nor significant linkage disequilibria (*p*-values>0.21). Microchecker did not detect evidence for scoring errors due to stuttering, for large allele dropout or for the presence of null alleles in any of the tested loci (Oosterhout values are given in Table 1). We did not find any sex-specific differences in the allele frequencies between males and females (data not shown), thus assuming all loci to be autosomal.

Cross-species tests revealed that several loci are potentially useful in two other dendrobatoid species, *O. pumilio* and *A. femoralis*, albeit showing lower variability and amplification success (Table 2).

Here we demonstrate the potential of the nine loci for revealing the mating system and patterns of mate choice and reproductive success, as well as the assessment of population genetic structure in *D. tinctorius*. This

Table 2. Cross-species utility of the nine microsatellite loci in *Oophaga pumilio* and *Allobates femoralis* (*n*, number of tested individuals). When polymorphic, the numbers of alleles are given; numbers in brackets indicate weak PCR amplification success; m, multiple bands within one PCR product.

Species	*n*	*Dtinc09*	*Dtinc10*	*Dtinc14*	*Dtinc17*	*Dtinc19*	*Dtinc21*	*Dtinc22*	*Dtinc23*	*Dtinc24*
O. pumilio	8	6	3	3	m	(3)	m	6	5	2
A. femoralis	8	(5)	6	3	(2)	4	1	m	m	2

information will contribute to the knowledge of mating strategies in this species and offer new insights into the evolution of mating systems in dendrobatoid frogs. Furthermore, these markers can be used for investigating population genetic differences across different geographical scales.

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