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Analysis of the mating system of *Podocnemis sextuberculata* in the lower Purus River of the Brazilian Amazon: another record of multiple paternity in chelonians

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When Geoffrey Albert Parker (1970) published a paper titled "Sperm competition and its evolutionary consequences in the insects", considerable discussion ensued on the mating systems of animals. Monogamous tendencies previously assumed to be common to most species turned out to be limited to only a few species and the concept of multiple mating became evident (Barash & Lipton 2007). The tendency to seek out several sexual partners used to be regarded as being exclusive to males, but that this behaviour has been shown also to be commonplace among females in the majority of species (Alcock 2010). While reptiles are good models of this polygamous mating system (Uller & Olsson 2008), little information exists on the reproductive behaviour of this group of animals due to difficulties associated with observing species in their natural environments (Simmons 2005, Wright et al. 2013).

Molecular tools have recently been used to facilitate inferences regarding the mating system of different chelonian species (e.g., Fitzsimmons 1998: *Chelonia mydas*; Valenzuela 2000: *Podocnemis expansa*; Hoekert et al. 2002: *Lepidochelys olivacea*; Moore & Ball 2002: *Caretta caretta*; Pearse et al. 2002: *Chrysemys picta*; Roques et al. 2006: *Emys orbicularis*; Fantin et al. 2008: *Podocnemis unifilis*; Refsnider 2009: *Emys blandingii*; Fantin et al. 2010: *Podocnemis erythrocephala*; Davy et al. 2011: *Gopherus agassizii*). These tools allow identifying the paternity of individuals in populations and examining important genetic connections regarding the evolution of organisms (Jones et al. 2010). Microsatellite DNA analyses, in particular, have allowed the recording of the promiscuous habits of chelonians, demonstrating that females regularly mate with more than one male in the same breeding season (Pearse & Avise 2001, Uller & Olsson 2008). It is possible that this plurality of sexual partners on the part of females enhances female fitness as well as the reproductive fitness of the species (Wright et al. 2013).

Despite this evidence, the benefits of promiscuous mating remain unknown (Jennions & Petrie 2000, Wright et al. 2013). Moreover, evidence of this behaviour is insufficiently documented for the majority of chelonians. In the present study, the mating system of the six-tubercled Amazon River turtle (*Podocnemis sextuberculata* Cornalia, 1849) was investigated to confirm the existence of multiple paternity in nests and contribute information to the reproductive biology of this species.

In September of 2012, two *P. sextuberculata* nests were found on the Uixi natural sand beach on the shores of Lake Ayapuá in the municipality of Anori, state of Amazonas, Brazil (04°26'030" S and 62°17'427" W). The site is a protected nesting area for chelonians managed by the Uixi community in the Piagaçu-Purus Sustainable Development Reserve (PP-SDR) along lower Purus River in central Amazonia. The nests were protected from natural predators and maintained in situ until hatching had been completed.

Table 1. Information on the microsatellite loci used in paternity analysis of *Podocnemis sextuberculata* from the Piagaçu-Purus Sustainable Development Reserve.

Locus	Primer sequence (5'–3')	Repeat motif	Size range (bp)	T (°C)
Puni_1B10	F-5'-CCAAACTAGGTTCATGTCCAAA-3' R-5'-GAAGCGTCAGGAAGGAAAGA-3'	$(GA)_8$	242–266	60
Puni_1B11	F-5'-CCAGACCTCTCCTGTTTTGG-3' R-5'-GGTTCTGGGCTCCTTACACA-3'	$(GA)_7gg(GA)_9$	265–273	60
Puni_1D12	F-5'-AGGAGCTGCAGGTGCAAAC-3' R-5'-GATCACCCAGATGCTGACCT-3'	$(GA)_{10}$	170–182	55
Puni_1D9	F-5'-GCTGGGGAACTGACTACCT-3' R-5'-CACGAGGTAGGAATGCCTGT-3'	$(GA)_{12}$	137	62
Puni_1E1	F-5'-GGCCTCTACTGTCTGAAAGTCC-3' R-5'-GAAGGAGAGCTCCAGGTGAA-3'	$(CT)_8$	213–219	60
Puni_2E7	F-5'-CTGGACCCATATGCAGTGAC-3' R-5'-CACTTGAGCTTCTGAGGGAGA-3'	$(GA)_5gc(GA)_8$	256–278	55
PE_344	F-5'-ATCCTGAGTTTAAAGGTGA-3' R-5'-AACTCTTCAAACTCCTCTAG-3'	$(AG)_{13}$	190–208	50

To understand and identify the mating system of this species, blood samples of offspring from the two nests (Nest 1: n = 10; Nest 2: n = 12) were obtained by puncturing the femoral vein with the aid of a 0.33-mm calibre needle on a 1.0-cm^3 syringe. The individuals were then released at the site of their hatching. Each sample was labelled, individually stored in microtubes containing 95% alcohol (Fantin et al. 2010) and deposited in the Animal Genetic Sample Collection (CTGA) of the Laboratório de Evolução e Genética Animal (LEGAL) of the Universidade Federal do Amazonas (UFAM), Brazil.

Genomic DNA was isolated from the blood samples according to the phenol-chloroform protocol of Sambrook et al. (2001) with modifications. Seven heterologous microsatellite loci [Puni_1B10, Puni_1B11, Puni_1D12, Puni_1D9, Puni_1E1, Puni_2E7) (Fantin et al. 2007) and PE344 (Valenzuela, 2000)] (Tab. 1) were amplified through polymerase chain reaction (PCR), applying the economic protocol described by Schuelke (2000). Genotype reactions were then carried out in a Veriti™ thermal cycler (Applied Biosystems, Foster City, CA, USA) at a final volume of 10 µL. Each reaction contained 2.7 µL of ultrapure water, 1.0 µL of 25 mM $MgCl_2$ (Fermentas), 1.5 µL of 10 mM dNTPs (Fermentas), 1.0 µL of PCR buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl) (Fermentas), 0.5 µL of 2 µM tailed forward primer, 0.5 µL of 2 µM fluorescence-labelled primer with FAM-6 or HEX fluorescent dyes, 1.0 µL of 2 µM reverse primer, 0.3 µL of 2.5 U Taq DNA polymerase (Fermentas), and 1.0 µL of DNA template (10 to 40 ng/µL). The amplifications were performed according to the following cycling programme: initial denaturation at 94°C for 1 min, followed by 25 denaturation cycles at 94°C for 30 s each, annealing of primers (at the specific temperature for each primer pair) for 30 s, extension at 68°C for 40 s; and the step of adding fluorescence consisted of 30 denaturation cycles at 94°C for 20 s, annealing at 53°C for 30 s, extension at 72°C for 1 min; and a final extension step at 72°C for 30 min.

Products from the PCR reactions were analysed using an ABI 3130 xl automatic sequencer (Applied Biosystems) with a ROX500 fluorescent size standard (DeWoody et al. 2004). Alleles were viewed with the aid of the GeneMapper software, version 4.0 (Applied Biosystems, Foster City, CA, USA). The number of alleles in all nests, expected heterozygosity (He), the probability of genetic identity among siblings for each locus (PISibs 1) and locus combination (PISibs 2), the probability of excluding multiple paternity for each individual locus (PX3) and the combined probability (P3Max) of the seven loci were calculated. All analyses were carried out with the software GenAIEx 6.3 (Peakall & Smouse 2006).

The number of fathers represented in each nest and reconstruction of the genotypes of the father and mother were analysed using a multilocus approach in the Gerud 2.0 software (Jones 2005). This software performs an exhaustive search, trying every possible combination of paternal genotypes until it will find a combination that can explain the composition of the progeny, and represents the true number of parents and the expected proportion of genotypes to be correctly reconstructed. In cases of multiple paternity, more than one combination of parental genotypes will be found amongst the progeny, in which case the program calculates a ranking of the solutions by likelihood for each paternal genotype combination. Thus, the paternal genotype solution with the highest likelihood score that is also consistent with genotypes for the candidate fathers within the examined group will be chosen.

Sixteen eggs were found in Nest 1, but only nine turtles hatched; six eggs were considered infertile and one embryo was dead. Fifteen eggs were found in Nest 2, 12 tur-

Table 2. Loci, number of alleles per locus, expected heterozygosity (He), probability of exclusion, and probability of identity for each locus, and combinations of the seven loci. PISibs 1 – Probability of identity for each locus; PX3 – Probability of exclusion (excluding both parents) for each locus; PISibs 2 – Probability of sibling identity for locus combinations; P3Max – Probability of exclusion (excluding both parents) for locus combinations.

Locus	Number of alleles	He	Probabilities of each locus		Locus combinations	Probabilities of locus combinations	
			PISibs 1	PX3		PISibs 2	P3Max
Puni_1B10	8	0.682	0.372	0.831	Puni_1B10	0.372	0.831
Puni_1B11	10	0.858	0.449	0.676	Puni_1B10+Puni_1B11	0.167	0.945
Puni_1D9	1	0.000	1.000	0.000	Puni_1B10+Puni_1B11+Puni_1D9	0.167	0.945
Puni_1D12	6	0.742	0.629	0.281	Puni_1B10+Puni_1B11+Puni_1D9+Puni_1D12	0.105	0.960
PE_344	7	0.827	0.345	0.870	Puni_1B10+Puni_1B11+Puni_1D9+Puni_1D12+PE_344	0.036	0.994
Puni_1E1	2	0.357	0.751	0.281	Puni_1B10+Puni_1B11+Puni_1D9+Puni_1D12+PE_344+Puni_1E1	0.027	0.996
Puni_2E7	3	0.646	0.472	0.518	Puni_1B10+Puni_1B11+Puni_1D9+Puni_1D12+PE_344+Puni_1E1+Puni_2E7	0.012	0.996

tles hatched and three eggs were infertile. At the PP-SDR, the mean number of eggs per nest is 13 and mean hatching success is approximately 11 hatchlings per nest, which is similar to data reported for *P. sextuberculata* from other locations of the Brazilian Amazons (Haller & Rodrigues 2006, Pantoja-Lima et al. 2009).

One to ten alleles were found per locus. Expected heterozygosity (He) ranged from 0.000 (Puni_1D9) to 0.858 (Puni_1B11). The probability of genetic identity per locus ranged from 0.345 (locus PE_344) to 1.000 (locus Puni_1D9). When all loci were combined, the joint probability of genetic identity reached 0.012. The probability of paternity exclusion per locus ranged from 0.000 (locus Puni_1D9) to 0.831 (locus Puni_1B10). When all loci were combined, the joint probability of paternity exclusion reached 0.996. Table 2 provides a summary of the number of alleles, expected heterozygosity, and probabilities of genetic identity and paternity exclusion.

The findings indicate multiple paternity for *P. sextuberculata,* as each nest had the allelic contribution of two males. In Nest 1, the primary male accounted for 80% of the offspring. In Nest 2, the primary male accounted for 92% of the offspring (Tab. 3). As the program does not accept missing data, the offspring that could not be genotyped at all loci were excluded (2 offsprings in Nest 1 and 1 offspring in Nest 2). The program also makes no concessions for genotyping errors or mutations, but detects incompatibilities between parents and known offspring, which gives and indication of the rate of genotyping errors in the dataset, thereby allowing these data to be removed from the analyses. These findings are in agreement with data reported for other species of the genus, such as *P. expansa*, *P. unifilis*, and *P. erythrocephala,* for which two or three contributing fathers have been found in each nest (Valenzuela 2000, Pearse et al. 2006, Fantin et al. 2008, 2010). The genotypes of the offspring analysed and possible genotypes of the parents (fathers and mothers) reconstructed in Gerud 2.0 are listed in the Appendix.

Many difficulties are encountered when studying the behaviour of chelonians, especially those that inhabit Amazonian aquatic ecosystems, as has been reported by Ferrara et al. (2009) and Schneider et al. (2010) who conducted studies on *P. erythrocephala* in captivity. Therefore, paternity analyses provide an indirect understanding of the reproductive biology of chelonians (Valenzuela 2000, Ireland et al. 2003, Moore & Ball 2002, Roques et al. 2006, Fantin et al. 2008, 2010, Uller & Olsson 2008). The sex ratio in a given population, cohort, sexual selection, sexual signalling, and sperm competition (Gist & Jones 1989, Palmer et al. 1998, Simmons 2005, Roques et al. 2006, Zuffi et al. 2007, Lovich et al. 2010, Halámková et al. 2013) are considered determinants of a greater incidence of multiple paternity. To ensure reproductive success, females will mate with different males due to the restrictions to which these females are subjected (Rowe 1994, Wright et al. 2012).

Table 3. Inference of the minimum number of fathers contributing to each clutch, relative contribution of each father, and rank of solutions by likelihood.

Code	No. of progeny in sample	Minimum No. of fathers	% Contribution by fathers	Ranking by likelihood
Clutch 1				
Father 1	8	2	6 (80%)	<0.01
Father 2			2 (20%)	
Clutch 2				
Father 1	11	2	10 (92%)	<0.01
Father 2			1 (8%)	

Hunting pressure is another factor that may determine whether a species adopts promiscuous mating, as the low abundance of one sex may make the search for the opposite sex and intra-sexual competition more intensive in a population (J. Erickson pers. obs.). The lower Purus River is an example of the depletion of natural chelonian populations. Intensive exploitation of chelonians and eggs of the species of *Podocnemis* occurs historically in the region for subsistence or commercial poaching purposes (Kemenes & Pezzuti 2007, Waldez et al. 2013, J. Erickson unpubl. data).

The community-based monitoring programme focusing on the protection of breeding areas provides important knowledge on wild populations. Despite the lack of information on the population structure of *P. sextuberculata* in the region and the fact that this is the first and only record of nesting in the PP-SDR, the present findings indicate that this species has a polyandrous mating system similar to that of other species of the genus, as evidenced through the analysis of the paternal allelic contribution in the offspring. This is particularly important for the overexploited species *P. sextuberculata,* a species that is currently classified as Vulnerable by the International Union for Conservation of Nature and Natural Resources (IUCN 2014). However, further studies are necessary to gain a better understanding of the reproductive biologies of the remaining majority of chelonian species, especially in the context of conservation and management.

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Appendix

Genotypes of mothers and offspring from each nest genotyped (Nests 1 and 2) and most likely inferred genotypes of each father from each nest. Tissues are deposited in the CTGA collection of LEGAL/UFAM.

Clutch	Blood voucher	Locus													
		Puni_1B10		Puni_1B11		Puni_1D9		Puni_1D12		PE_344		Puni_1E1		Puni_2E7	
		Mother/Father/Offspring genotype													
Clutch 1															
Mother clutch 1		244	252	265	273	137	137	182	182	200	204	213	213	256	278
Father 1		242	266	265	265	137	137	170	170	190	194	213	213	256	278
Father 2		244	244	273	275	137	137	170	170	194	210	213	213	270	270
Offspring															
n.1	CTGA_Q_05264	170	182	242	252	137	137	265	273	194	204	213	213	278	278
n.2	CTGA_Q_05265	170	182	244	266	137	137	265	273	194	200	213	213	0	0
n.3	CTGA_Q_05266	170	182	242	244	137	137	265	265	194	200	213	213	0	0
n.4	CTGA_Q_05267	170	182	244	244	137	137	265	273	194	200	213	213	270	278
n.5	CTGA_Q_05268	170	182	242	252	137	137	265	273	190	200	213	213	256	256
n.6	CTGA_Q_05269	170	182	244	266	137	137	265	265	194	200	213	213	256	256
n.7	CTGA_Q_05270	170	182	244	244	137	137	265	275	200	210	213	213	270	278
n.8	CTGA_Q_05271	170	182	244	266	137	137	265	273	194	200	213	213	256	278
n.9	CTGA_Q_05272	170	182	252	266	137	137	265	273	190	200	213	213	256	278
n.10	CTGA_Q_05461	170	182	252	266	137	137	265	273	190	204	213	213	256	256
Clutch 2															
Mother clutch 2		252	254	265	267	137	137	182	182	204	206	213	219	256	278
Father 1		250	252	265	267	137	137	170	170	194	208	213	219	270	278
Father 2		254	254	267	267	137	137	170	170	206	208	213	213	256	256
Offspring															
n.1	CTGA_Q_05273	170	182	250	254	137	137	265	267	194	206	213	219	256	270
n.2	CTGA_Q_05274	170	182	252	254	137	137	265	265	204	208	213	219	256	270
n.3	CTGA_Q_05275	170	182	250	252	137	137	265	265	194	206	213	219	256	270
n.4	CTGA_Q_05276	170	182	250	254	137	137	265	267	194	206	213	213	256	278
n.5	CTGA_Q_05277	170	182	252	252	137	137	267	267	194	204	213	219	256	278
n.6	CTGA_Q_05278	170	182	250	252	137	137	265	267	206	208	219	219	278	278
n.7	CTGA_Q_05279	170	182	252	254	137	137	265	267	206	208	219	219	270	278
n.8	CTGA_Q_05280	170	182	252	254	137	137	267	267	194	204	213	213	270	278
n.9	CTGA_Q_05281	170	182	250	254	137	137	267	267	204	208	213	219	0	0
n.10	CTGA_Q_05282	170	182	252	254	137	137	267	267	204	208	213	213	270	278
n.11	CTGA_Q_05283	170	182	252	254	137	137	265	267	206	208	213	219	256	278
n.12	CTGA_Q_05284	170	182	252	254	137	137	265	267	204	206	213	219	256	256