Phyllomedusa 14(2):89–97, 2015

ISSN 1519-1397 (print) / ISSN 2316-9079 (online)
doi: http://dx.doi.org/10.11606/issn.2316-9079.v14i2p89-97

Evidence of multiple paternal contribution in *Podocnemis sextuberculata* (Testudines: Podocnemididae) detected by microsatellite markers

Cleiton Fantin[1], Dorothy Ivila Melo Pereira[1], Jorge Frank Ferreira[1], Paulo Cesar Andrade Machado[2], Tomas Hrbek[2], Luiz Alberto Santos Monjeló[2], and Izeni Pires Farias[2]

[1] Departamento de Biologia, Universidade do Estado do Amazonas, Av. Djalma Batista, 2470, 69050-010, Manaus, AM, Brazil. E-mail: cleitonfantin@hotmail.com.

[2] Departamento de Biologia, Laboratório de Evolução e Genética Animal, Universidade Federal do Amazonas, Av. Rodrigo Octávio Jordão Ramos, 3000, 69077-000, Manaus, AM, Brazil.

Abstract

Evidence of multiple paternity in *Podocnemis sextuberculata* (Testudines: Podocnemididae) detected by microsatellite markers. We found evidence of multiple paternity in a sample of 12 *Podocnemis sextuberculata* nests including seven nests (80 hatchlings) collected along the Amazonas River, in the municipality of Barreirinha, AM, Brazil and five nests in the Abufari Biological Reserve, Tapauá, AM, Brazil (54 hatchlings). As observed in other species of the genus, *P. sextuberculata* also presented polyandric behavior. By means of allelic frequency and variation in six microsatellite loci for each location, the occurrence of multiple paternity in sampled nests of this species was inferred, even though the maternal genotype was unknown. For one of the nests, a minimum of four males contributed to the clutch, whereas for nearly all remaining nests at least two males contributed. Only one of the twelve nests did not show clear evidence for contributions from more than one male. This is the first genetic evidence of multiple paternity in *P. sextuberculata.*

Keywords: mating system, paternity, *Podocnemis*, reproduction, turtles.

Resumo

Evidência de contribuição paterna múltipla em *Podocnemis sextuberculata* (Chelonia: Podocnemididae) por meio de marcadores microssatélites. Encontramos evidências de paternidade múltipla em uma amostra de 80 recém-eclodidos de sete ninhos *de Podocnemis sextuberculata* situados ao longo do rio Amazonas, no município de Barreirinha, AM, Brasil, e 54 indivíduos recém-eclodidos de cinco ninhos na Reserva Biológica Abufari, Tapauá, AM, Brasil. Como observado em outras espécies do gênero, *P. sextuberculata* apresentou comportamento poliândrico. Por meio da frequência alélica e variação em seis locos microssatélite para cada localidade, a ocorrência de

Received 8 April 2015.
Accepted 16 September 2015.
Distributed December 2015.

paternidade múltipla em ninhos amostrados dessa espécie foi inferida, mesmo o genótipo da mãe sendo desconhecido. Para um dos ninhos, um mínimo de quatro machos contribuíram para a prole, enquanto que para quase todos os outros ninhos, pelo menos dois machos contribuíram. Apenas um dos 12 ninhos não mostrou evidência clara de contribuição de mais de um macho. Esta é a primeira evidência genética de paternidade múltipla em *P. sextuberculata*.

Palavras-chave: cágados, paternidade, *Podocnemis*, reprodução, sistema de acasalamento.

Introduction

The turtle fauna of South America is rich and varied, comprising around 20% of the world's living species (Pritchard 1975). The genus *Podocnemis* comprises six species, including *Podocnemis sextuberculata* Cornelia, 1849, popularly known as iaçá, pitiú or cambéua. The coloring of the carapace varies from light to dark brown. The adult male is smaller than the female and, in young individuals the plastron has six gray or brown tubercles, which gives the species its name (Ibama 1989). Its geographic distribution extends throughout the Amazonas River basin in Brazil, Peru and Colombia (Ernst and Barbour 1989, Iverson 1992). Nests are made on river beaches, once or twice a year, and 8–19 elliptical eggs are laid (Ferri 2002). *P. sextuberculata* meat has been intensely consumed and illegally commercialized in the Amazon region, which contributed to the inclusion of this species in the IUCN red list of threatened species as vulnerable.

Studies of mating systems of turtles have substantially advanced in the recent decades (Galbraith 1991, Galbraith *et al.* 1993, Bowen and Karl 1996, Rieder 1996, Curtis 1998, FitzSimmons 1998, Crim *et al.* 2002, Moore and Ball 2002, Jensen *et al.* 2006, Zbinden *et al.* 2007, Refsnider 2009, Theissinger *et al.* 2009, Fantin *et al.* 2010, Stewart and Dutton 2011, Lasala *et al.* 2013, Todd *et al.* 2013). However, the fact that the majority of species are aquatic makes it difficult to directly observe mating behavior in nature and accounts for the lack of information for most species (Carpenter and Ferguson 1977).

Certain aspects of chelonian reproductive behavior, such as nest site fidelity, have been investigated using molecular tools, but many others still remain unstudied (Bowen and Karl 1996). Kinship analysis of turtle nests can reveal fundamental aspects of the reproductive biology within a population, but the majority of efforts are limited by small sample sizes and/or ambiguous markers, and it remains unclear if multiple paternity is the exception or the rule.

Recent work has demonstrated multiple paternity in *P. expansa*, *P. erythrocephala* and *P. unifilis* (Pearse *et al.* 2006, Fantin *et al.* 2008, Fantin *et al.* 2010). *P. expansa* females show polyandric behavior that varies in rates of multiple paternity among different nesting localities (Valenzuela 2000, Pearse *et al.* 2006). Fantin *et al.* (2008) found multiple paternity in studies of *P. unifilis* nests; and Fantin *et al.* (2010) reported multiple paternity in *P. erythrocephala* nests. However, observations on the reproductive pattern in *P. sextuberculata* have not yet been reported.

Molecular data have been broadly used to study chelonian mating systems (DeWoody *et al.* 2000, Neff *et al.* 2000, Valenzuela 2000, Myers and Zamudio 2004, Pearse *et al.* 2006). Among the molecular techniques available, microsatellite markers have been the most frequently and successfully applied tool for determining paternity patterns in turtle species.

In this study, we used microsatellite markers to determine the mating system in two *P. sextuberculata* populations. We tested the hypothesis that the reproductive system in this species is polyandric as observed in other species

of this genus. This study provides valuable information for conservation and management programs. It provides a better understanding of the breeding biology of the species and will hopefully stimulate other studies of the biology of *P. sextuberculata* and related species.

Materials and Methods

A total of 12 nests from two localities were studied for this report. 80 recent hatchlings of *P. sextuberculata* were collected along the Amazonas River at Barreirinha-AM (03°03'25" S, 57°10'28" W) (Figure 1), and 54 hatchlings were collected in the Abufari Biological Reserve, Tapauá-AM. Permission to collect tissue samples was granted by RAN/IBAMA (#113/2006).

The collection of the hatchlings was made with the support of members of the Pé-de-Pincha project (UFAM), who selected the nests randomly. The nests were transplanted to a location known as "the nursery", protected by the local community. To avoid mixing of hatchlings from different clutches, the nests were separated from each other by small fences. Once hatching started, up to 500µL of blood was obtained through puncture of the femoral vein using a 1 mL syringe. Samples were stored in Eppendorf tubes with 500mL of absolute ethanol. The animals were then released at the sites where they had been collected. The total of nests and hatchlings per nest are listed in Table 1. The maternal blood sample was not collected, so the maternal genotype was unknown.

DNA extraction was performed using a GFX Kit (GE-Healthcare), according to manufacturer's instructions. Paternity was analyzed by using six different microsatellite loci for each location. For hatchlings from the Amazon River, Barreirinha-AM, four of the pairs of primers used for the DNA amplification were initially developed for *P. unifilis* by Fantin *et al.* (2007), the fifth one was developed for *P. expansa* by

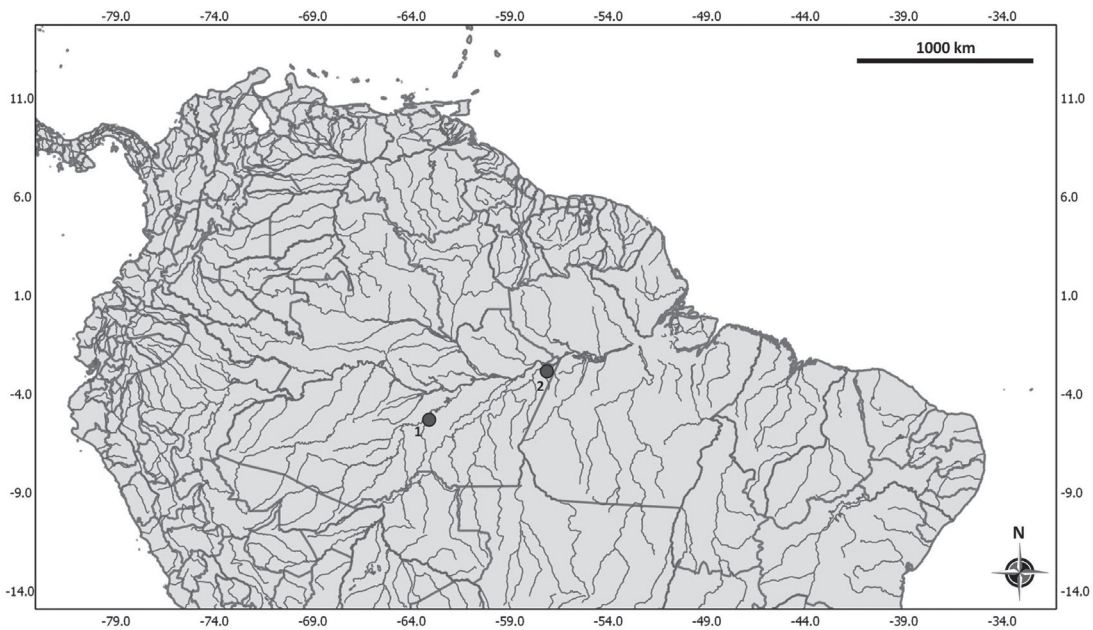

Figure 1. Map showing the location of the Barreirinha and Tapauá sites for the nests of *Podocnemis sextuberculata* studied in the present work.

Table 1. Number of maternal alleles inferred/number of alleles found for each locus in the *Podocnemis sextuberculata* nests. The maternal alleles were inferred by the presence of homozygous young, and the results in bold indicate the presence of extra-pair contribution.

Nest	Number of hatchlings	Simple allelic counting method									Kinalyser
		1B10	1D9	2D9	2E7	91	344	1B11	1D12	2A9	Number of full sib families
N1	12	0/3	1/2	1/3	**0/6**	**0/5**	1/3	–	–	–	3
N2	12	1/3	1/2	1/3	**1/6**	**2/4**	0/4	–	–	–	3
N3	12	0/1	0/1	1/3	1/3	**2/4**	1/3	–	–	–	2
N4	12	**2/3**	1/3	1/3	1/3	**1/4**	1/3	–	–	–	2
N5	12	2/2	1/2	0/4	**2/4**	**0/5**	**2/3**	–	–	–	3
N6	8	1/3	0/1	1/3	**2/3**	**1/4**	0/4	–	–	–	3
N7	12	1/3	0/1	1/2	**2/4**	**1/5**	0/4	–	–	–	3
N8	12	**1/6**	–	1/3	**1/6**	–	–	1/3	1/2	1/3	3
N9	12	0/4	–	1/3	**0/6**	–	–	1/2	1/2	**1/4**	3
N10	10	0/6	–	**1/6**	**1/8**	–	–	**1/4**	0/2	**2/5**	4
N11	10	1/3	–	1/3	**2/4**	–	–	1/2	1/2	**2/3**	2
N12	10	1/3	–	0/2	0/4	–	–	1/3	0/2	0/4	2

Sites Jr. *et al.* (1999) and the last one was developed for *P. expansa* by Valenzuela (2000). For individuals from the Abufari Biological Reserve, Tapauá-AM, all six pairs of primers were developed by Fantin *et al.* (2007). The genotyping polymerase chain reaction (PCR) followed the economic protocol described by Schuelke (2000). All loci were amplified under identical thermocycling conditions, which were adjusted for a total volume of 11 µL containing 3.7 µL H_2O, 1.0 µL 10X PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 1.5 µL $MgCl_2$ (25 mM), 1.0 µL reverse primer (2 µM), 0.5 µL forward primer (2 µM), 0.5 µL 6-FAM labeled M13 label primer (2 µM), 1.5 µL dNTP mix (0.2 mM each), 0.3 µL LGC Biotecnologia Taq DNA Polymerase (1 U/µL), and 1µL DNA (10 ng). The initial denaturation temperature was 94ºC for 1 min, followed by 25 cycles at 94ºC for 30 s, 55ºC for 30 s and 68ºC for 30 s. Then, 20 cycles at 94ºC for 30 s, 50ºC for 30 s and 68ºC for 30 s with a final extension to 72ºC for 15 min. The amplified DNA fragment was visualized on 1% agarose gel prior to genotyping.

The PCR products were diluted in the proportion 1:100, and the size marker ROX pUC-19, modified from DeWoody *et al.* (2004), was added to determine sizes of observed alleles.

The genotyping was performed in the ABI 3730xl DNA Analyzer, and the analyses of the alleles observed for each locus were made using the program GeneMapper v.4.0 (Applied Biosystems).

The observed and expected per-locus heterozygosities were calculated for each locus with Arlequin 3.1 (Excoffier *et al.* 2005). In order to determine the probability of paternity exclusion, the method described by Weir (1996) was used. The probability of paternity exclusion (*Q*) is the parameter based on exclusion of various males involved in each clutch, which can be statistically distinguished. Through this parameter, the most probable male parent is not discovered, although it is possible to exclude probable male parents in the population studied. Paternity exclusion values closer to 1 provide greater confidence to correctly exclude an individual from paternity. The probability of two unrelated individuals presenting the same genotype was calculated by the method of genetic identity (*I*) described by Paetkau *et al.* (1995). Considering all the loci analyzed together, the probability of paternity exclusion (*QC*) (Weir 1996) and the joint probability of genetic identity (*IC*) were also analyzed.

Paternity analysis was performed according to the Minimum Method of Allele counts (Myers and Zamudio 2004), which presupposes a Mendelian distribution of the alleles in the progeny. When both maternal alleles are unknown, the presence of five alleles per sampled locus among the hatchlings of each nest is considered indicative of multiple paternity. Since one or more homozygous offspring is observed in a single nest, it is possible to estimate the maternal allele and the remaining ones are counted up as the paternal contribution. In such cases, the presence of three different alleles at a given locus indicates multiple paternity.

Unfortunately, the minimum method of alleles counts does not provide the real number of contributing fathers to the offspring, given that this method does not distinguish multiple pairs with common alleles. Therefore, to estimate the most likely number of contributing males, we used the computer program Kinalyser (Berger-Wolf *et al.* 2007), which performs a multiloci analysis with the purpose of estimating the relationship among full and half-siblings in the same nest, attributing paternity and inferring mating systems (polygamic/monogamic). The inference of families of full-siblings is possible even without knowing the parental genotype, given that this program uses the information from the sharing of alleles in multiple loci among the individuals of each nest.

Results

The number of alleles per locus varied from 1–8, with an average of 7.5 ± 2.8 alleles per locus. Observed heterozygosity varied from 0.2297 (Puni_1D9) to 0.9500 (Puni_2A9) (Table 2). The probability of two unrelated individuals presenting the same genotype (*IC*) was 1.29 x 10^{-6}, and the joint probability of exclusion across all loci (*QC*) was 99.27% (Table 2). Taken together, *IC* and *QC* provide evidence for high power to detect multiple paternity using these loci for *P. sextuberculata*.

The number of alleles per locus within each nest ranged from one (Nests 3, 6, and 7) to eight (Nest 10) (Table 1).

Based on the minimum method of allele counts, multiple paternity was found in 7 of the 12 nests analyzed (Nests 1, 2, 5, 7, 8, 9, and 10) (Table 1). When counting was used with the inference of the maternal allele, all the nests, except Nest 12, showed at least two males contributing to the offspring. A minimum of two male parents contributed to nests 1, 3, 4, 5, 6, 7, and 9; at least three males contributed to Nests 2 (Puni_2E7) and 8 (Puni_B1 and Puni_2E7); and at least four males contributed to the offspring in Nest 10 (Puni_2E7). The use of the program KINALYSER (Berger-Wolf *et al.* 2007) reinforces the extra-pair contribution by means of the inference of more than one family of half siblings for each nest analyzed (Table 1).

Table 2. Number of alleles, observed heterozygosity (*Ho*), expected heterozygosity (*He*), paternity exclusion probability (*Q*) and identity probability (*I*) of the 6 microsatellite loci used in the *P. sextuberculata* paternity test.

Locus	Number of alleles	Ho	He	Q	I	Reference
Puni_1B10	7	0,5875	0,6874	0,4824	0,1381	Fantin *et al.* 2007
Puni_1B11	7	0,7500	0,6666	0,4267	0,1772	Fantin *et al.* 2007
Puni_1D9	3	0,2297	0,2062	0,0996	0,6507	Fantin *et al.* 2007
Puni_1D12	3	0,8750	0,5950	0,2672	0,3199	Fantin *et al.* 2007
Puni_2A9	11	0,9500	0,8810	0,6640	0,0355	Fantin *et al.* 2007
Puni_2D9	6	0,7948	0,6897	0,4534	0,1588	Fantin *et al.* 2007
Puni_2E7	11	0,7222	0,8276	0,6736	0,0510	Fantin *et al.* 2007
Pod91	8	0,7948	0,8383	0,6832	0,0489	Sites Jr. *et al.* 1999
PE344	10	0,8533	0,8583	0,7235	0,0365	Valenzuela 2000
All	7,5	0,6637	0,6846	$QC = 0,992716$	$IC = 1,29x10^{-6}$	

Discussion

Multiple paternity is a common mating system in natural populations of reptiles including turtles. Many studies of marine turtles (Harry and Briscoe 1988, Parker *et al.* 1996, FitzSimmons 1998, Bollmer *et al.* 1999, Kichler *et al.* 1999, Crim *et al.* 2002, Hoekert *et al.* 2002, Moore and Ball 2002, Ireland *et al.* 2003, Lee and Hays 2004, Jensen *et al.* 2006, Zbinden *et al.* 2007) have demonstrated the clear presence of multiple paternity. However, there are few studies of mating systems of freshwater species.

The existence of multiple paternity has already been reported in the Amazonian river turtle *Podocnemis* (*P. expansa*: Valenzuela 2000; *P. unifilis*: Fantin *et al.* 2008; *P. erythrocephala*: Fantin *et al.* 2010). Valenzuela (2000) reported 100% multiple paternity in 2 nests of a *P. expansa* population from Colombia using eight microsatellite loci. Pearse *et al.* (2006) studied the same species as Venezuela using seven microsatellite loci and only found 10.3% multiple paternity in 32 nests. The large difference in the rate of multiple paternity between these populations suggests that there might be variation in patterns of paternity among populations but the difference may simply reflect the very small sample size in the earlier study.

Variation in the frequency of multiple paternity has also been demonstrated in studies of the mating system of marine turtle populations including *Chelonia mydas* (92%: Alfaro-Núñez *et al.* 2015; 61%: 75%: Pearse *et al.* 2001; Lee and Hays 2004; 9%: FitzSimmons 1998) and *Caretta caretta* (31%: Moore and Ball 2002; 33%: Harry and Briscoe 1988), as well as the freshwater turtle, *Chrysemys picta* (0%: McTaggart 2000; 13%: Pearse *et al*. 2001; 33%: Pearse *et al*. 2002). Some authors suggest that the rate of multiple paternity found in populations of the same species could be influenced by

different factors, such a scarcity of resources, which is likely to reduce the population density and thus the number of copulations (Engqvist 2011). At higher density, there are higher chances of encounters that result in mating (Taylor *et al*. 2014). Sex ratio will also be an important factor; populations with male-biased sex ratios are more likely to present multiple paternity (Crim *et al*. 2002).

Considering the vulnerable status of *P. sextuberculata* populations, genetic studies focusing on reproductive behavior of this species greatly contribute to the creation of conservation programs for this species and to its appropriate management. The latter should aim to increase the number of males that will potentially fertilize the eggs (Alfaro-Núñez *et al.* 2015), to provide higher rates of multiple paternity and consequently to maintain or increase intrapopulation genetic variability (Sugg and Chesser 1994, Chesser and Baker 1996).

In our study, we analysed microsatellite data from six loci using the Minimum Method of Allele counts (Myers and Zamudio 2004) and KINALYSER (Berger-Wolf *et al.* 2007). We aimed to analyze a standard number of hatchlings per nest (varying from 8 to 12 individuals), in order to minimize or neutralize the potential effect of sample size on the rate of multiple paternity found. To resolve uncertainties such as the presence of multiple pairs with common alleles, we used highly polymorphic microsatellite loci. When compared to previous studies on the genus *Podocnemis*, we studied a greater number of microsatellite loci (9 loci across the two study sites), which were considered appropriate, and with highly efficient discriminatory power to determine the frequency of multiple paternity in *P. sextuberculata*, according to probability calculations for both *IC* and *QC*.

We conclude therefore that multiple paternity is present in *P. sextuberculata* in the majority of nests of both populations studied. The same pattern of mating behavior was observed in both populations studied, however, environmental data that would allow the comparison between localities such as sand temperature and rainfall frequency were not collected.

This study suggests that strategies for management and conservation of *P. sextuberculata* populations should consider operational sex ratios that will promote significant levels of multiple paternity. New studies focusing on other species of the genus and on different populations of this species will be very useful for elucidating strategies for the maintenance of healthy reproductive output in river turtles.

Acknowledgments

We thank "Pé-de-Pincha" turtle project for help with samples collection. This research was supported by grants #557090/2005-9 (I. Farias - PPG7/CNPq; T. Hrbek - startup funds from the University of Puerto Rico-Rio Piedras). This study is part of a Ph.D. thesis presented by Cleiton Fantin in the Biotechnology Program of Universidade Federal do Amazonas; C. Fantin was supported by a Ph.D. fellowship from SUFRAMA.

References

Alfaro-Núñez, A., M. P. Jensen, and F. A. Abreu-Grobois. 2015. Does polyandry really pay off? The effects of multiple mating and number of fathers on morphological traits and survival in clutches of nesting green turtles at Tortuguero. *PeerJ 3:* 1–22.

Berger-Wolf, T. Y., S. I. Sheikh, B. DasGupta, M. V. Ashley, I. C. Caballero, W. Chaovalitwongse, and S. L. Putrevu. 2007. Reconstructing sibling relationships in wild populations. *Bioinformatics 23:* 49–56.

Bollmer, J. L., M. E. Irwin, J. P. Reider, and P. G. Parker. 1999. Multiple paternity in loggerhead turtle clutches. *Copeia 1999:* 475–478.

Bowen, B. W. and S. A. Karl. 1996. Population structure, phylogeography, and molecular evolution. Pp. 29–50 *in* P. L. Lutz and J. A. Musick (eds.), *The Biology of Sea Turtles*. Boca Raton. CRC Press.

Carpenter, C. C. and G. W. Ferguson. 1977. Variation and evolution of stereotyped behavior in reptiles. Pp. 335–403 *in* C. Gans and D. W. Tinkle (eds.), *Biology of the Reptilia. Ecology and Behaviour*. Volume 7. New York. Academic Press.

Chesser, R. K. and R. J. Baker. 1996. Effective sizes and dynamics of uniparentally and diparentally inherited genes. *Genetics 144:* 1225–1235.

Crim, J. L., L. D. Spotila, J. R. Spotila, M. O'Connor, R. Reina, C. J. Williams, and F. V. Paladino. 2002. The leatherback turtle, *Dermochelys coriacea*, exhibits both polyandry and polygyny. *Molecular Ecology 11:* 2097–2106.

Curtis, C. 1998. *Genotyping with Polymorphic Markers Reveals Multiple Paternity in Leatherback Turtle Clutches*. Philadelphia. Drexel University. 70 pp.

DeWoody, J. A., Y. D. DeWoody, A. C. Fiumera, and J. C. Avise. 2000. On the number of reproductives contributing to a half-sib progeny array. *Genetical Research 75:* 95–105.

DeWoody, J. A., J. Schupp, L. Kenefic, J. Busch, L. Murfitt, and P. Keim. 2004. Universal method for producing ROX-labeled size standards suitable for automated genotyping. *Bio Techniques 37:* 348–352.

Engqvist, L. 2011. Male attractiveness is negatively genetically associated with investment in copulations. *Behavioral Ecology 22*: 345–349.

Ernst, C. H. and R. W. Barbour. 1989. *Turtles of the World*. Washington. Smithsonian Institution Press. 313 pp.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online 1:* 47–50.

Fantin, C., C. F. Carvalho, T. Hrbek, J. W. Sites Jr., L. A. S. Monjeló, S. Astolfi-Filho, and I. P. Farias. 2007. Microsatellite DNA markers for *Podocnemis unifilis*, the endangered yellow-spotted Amazon River turtle. *Molecular Ecology Notes 7:* 1235–1238.

Fantin, C., L. S. Viana, L. A. S. Monjeló, and I. P. Farias. 2008. Polyandy in *Podocnemis unifilis* (Pleurodira; Podocnemididae), the vulnerable yellow-spotted Amazon River turtle. *Amphibia-Reptilia 29:* 479–486.

Fantin, C., I. P. Farias, L. A. S. Monjeló, and T. Hrbek. 2010. Polyandry in the red-headed river turtle *Podocnemis erythrocephala* (Testudines, Podocnemidae) in the Brazilian Amazon. *Genetics and Molecular Research 9:* 435–440.

Ferri, V. 2002. *Turtles and Tortoises. A Firefly Guide*. 1st edition. New York. Firefly Books. 256 pp.

FitzSimmons, N. N. 1998. Single paternity of clutches and sperm storage in the promiscuous green turtle (*Chelonia mydas*). *Molecular Ecology 7:* 575–584.

Galbraith, D. A. 1991. *Studies of Mating Systems in Wood Turtles (*Clemmys insculpta*) and Snapping Turtles (*Chelydra serpentina*) Using DNA Fingerprinting*. Unpubl. PhD. Thesis. Queen's University, Kingston, Canada.

Galbraith, D. A., B. N. White, R. J. Brooks, and P. T. Boag. 1993. Multiple paternity in clutches of snapping turtles (*Chelydra serpentina*) detected using DNA fingerprints. *Canadian Journal of Zoology 71:* 318–324.

Harry, J. L. and D. A. Briscoe. 1988. Multiple paternity in the loggerhead turtle (*Caretta caretta*). *Journal of Heredity 79:* 96–99.

Hoekert, W. E. J., H. Neuféglise, A. D. Schouten, and S. B. Menken. 2002. Multiple paternity and female-biased mutation at a microsatellite locus in the olive ridley sea turtle (*Lepidochelys olivacea*). *Heredity 89:* 107–113.

Ibama. 1989. *Projeto Quelônios da Amazônia, 10 anos*. Brasília. Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. 119 pp.

Ireland, J. S., A. C. Broderick, F. Glen, B. J. Godley, G. C. Hays, P. L. M. Lee, and D. O. F. Skibinski. 2003. Multiple paternity assessed using microsatellite markers, in green turtles *Chelonia mydas* (Linnaeus, 1758) of Ascension Island, south Atlantic. *Journal of Experimental Marine Biology and Ecology 291:* 149–160.

Iverson, J. B. 1992. *A Revised Checklist with Distribution Maps of the Turtles of the World.* Richmond, VA. Printed Privately. 363 pp.

Jensen, M. P., F. A. Abreu-Grobois, J. Frydenberg, and V. Loeschcke. 2006. Microsatellites provide insight into contrasting mating patterns in arribada vs. non-arribada olive ridley sea turtle rookeries. *Molecular Ecology 15:* 2567–2575.

Kichler, K., M. T. Holder, S. K. Davis, R. Marquez-M., and D.W. Owens. 1999. Detection of multiple paternity in the kemp's ridley sea turtle with limited sampling. *Molecular Ecology 8:* 819–830.

Lasala, J. A., J. S. Harrison, K. L. Williams, and D. C. Rostal. 2013. Strong male-biased operational sex ratio in a breeding population of loggerhead turtles (*Caretta caretta*) inferred by paternal genotype reconstruction analysis. *Ecology and Evolution 3:* 4736–4747.

Lee, P. L. M. and G. C. Hays. 2004. Polyandry in a marine turtle: females make the best of a bad job. *Proceedings of the National Academy of Sciences 101:* 6530–6535.

McTaggart, S. J. 2000. Good genes or sexy sons? Testing the benefits of female mate choice in the painted turtle, *Chrysemys picta.* Unpublished M.Sc. Thesis. University of Gelph, Ottawa, Canada.

Moore, M. K. and R. M. Ball. 2002. Multiple paternity in loggerhead turtle (*Caretta caretta*) nests on Melbourne beach, Florida: a microsatellite analysis. *Molecular Ecology 11:* 281–288.

Myers, E. M. and K. R. Zamudio. 2004. Multiple paternity in an aggregate breeding amphibian: the effect of reproductive skew on estimates of male reproductive success. *Molecular Ecology 13:* 1951–1963.

Neff, B. D., J. Repka, and M. R. Gross. 2000. Parentage analysis with incomplete sampling of candidate parents and offspring. *Molecular Ecology 9:* 515–528.

Paetkau, D., W. Calvert, I. Stirling, and C. Strobeck. 1995. Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology 4:* 347–354.

Parker, P. G., T. A. Waite, and T. Peare. 1996. Paternity studies in animal populations. Pp. 413–423 *in* T. B. Smith and R. K. Wayne (eds.), *Molecular Genetic Approaches in Animal Conservation*. New York. Oxford University Press.

Pearse, D. E., F. J. Janzen, and J. C. Avise. 2001. Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles. *Heredity 86:* 378–384.

Pearse, D. E., F. J. Janzen, J. C. Avise. 2002. Multiple paternity, sperm storage, and reproductive success of female and male painted turtles (*Chrysemys picta*) in nature. *Behavioral Ecology and Sociobiology 51:* 164–171.

Pearse, D. E., R. B. Dastrup, O. Hernandez, and J. W. Sites Jr. 2006. Paternity in an Orinoco population of endangered Arrau River turtles, *Podocnemis expansa* (Pleurodira; Podocnemididae), from Venezuela. *Chelonian Conservation and Biology 5:* 232–238.

Pritchard, P. C. H. 1975. Distribution of tortoises in tropical South America. *Chelonia 2:* 3–10.

Refsnider, J. M. 2009. High frequency of multiple paternity in Blanding's turtle (*Emys blandingii*). *Journal of Herpetology 43:* 74–81.

Rieder, J. P. 1996. Mating systems and conservation biology: microsatellite analysis of paternity in leatherback sea turtle clutches. M.Sc. Thesis. Ohio State University, Columbus, USA.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology 18:* 233–234.

Sites Jr., J. W., N. N. Fitzsimmons, N. J. Silva, and V. H. Cantarelli. 1999. Conservation genetics of the giant Amazon river turtle (*Podocnemis expansa*; Pelomedusidae) - inferences from two classes of molecular markers. *Chelonian Conservation and Biology 3:* 454–463.

Stewart, K. R. and P. H. Dutton. 2011. Paternal genotype reconstruction reveals multiple paternity and sex ratios in a breeding population of leatherback turtles (*Dermochelys coriacea*). *Conservation Genetics 12:* 1101–1113.

Sugg, D. W. and R. K. Chesser. 1994. Effective population sizes with multiple paternity. *Genetics 137:* 1147–1155.

Taylor, M. L.; T. A. R. Price, and N. Wedell. 2014. Polyandry in nature: a global analysis. *Trends in Ecology and Evolution 29:* 376–383.

Theissinger, K., N. N. FitzSimmons, C. J. Limpus, C. J. Parmenter, and A. D. Phillott. 2009. Mating system, multiple paternity and effective population size in the endemic flatback turtle (*Natator depressus*) in Australia. *Conservation Genetics 10:* 329–346.

Todd, E. V., D. Blair, C. J. Limpus, D. J. Limpus, and D. R. Jerry. 2013. High incidence of multiple paternity in an Australian snapping turtle (*Elseya albagula*). *Australian Journal of Zoology 60:* 412–418.

Valenzuela, N. 2000. Multiple Paternity in side-neck turtles *Podocnemis expansa*: evidence from microsatellite DNA data. *Molecular Ecology 9:* 99–105.

Weir, B. S. 1996. *Genetic Data Analysis II: Methods for Discrete Population Genetic Data.* Sunderland. Sinauer Associates. 376 pp.

Zbinden, J. A., C. R. Largiadèr, F. Leippert, D. Margaritoulis, and R. Arlettaz. 2007. High frequency of multiple paternity in the largest rookery of Mediterranean loggerhead sea turtles. *Molecular Ecology 16:* 3703–3711.

Editor: Peter Meylan