Paternity in an Orinoco Population of Endangered Arrau River Turtles, *Podocnemis expansa* (Pleurodira; Podocnemididae), from Venezuela

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ABSTRACT. – We used genetic data from 7 microsatellite loci to determine the frequency of multiple paternity in clutches of giant Amazon river turtles, *Podocnemis expansa*, from the Orinoco River in Venezuela. Among hatchlings sampled from 32 clutches, paternity analysis found that a minimum of 10.3% could conclusively be shown to have been sired by more than one male. We contrast this result with those from another population of this species, as well as other species of turtles, and discuss the importance of documenting patterns of paternity in different populations of a given species and considering the effects of ecological differences among populations on female mating behavior.

KEY WORDS. – Reptilia; Testudines; Podocnemididae; Podocnemis expansa; turtle; genetics; multiple paternity; microsatellites; Venezuela

The use of molecular genetic methods has shown that multiple paternity occurs commonly in many animal species (Birkhead and Møller 1998; e.g., snakes, Höggren and Tegelström 1995; turtles, Pearse and Avise 2001), and hypotheses such as protection against infertility and indirect offspring genetic benefits have been proposed to explain the occurrence of multiple mating by females (Thornhill and Alcock 1983; Madsen et al. 1992; Gowaty 1997). However, relatively few studies have examined paternity in more than one population of a single species (Kelly et al. 1999; Jones et al. 2001; Garner et al. 2002), therefore, little is known about within-species variation in the frequency of multiple paternity (the proportion of clutches that have mixed paternal ancestry). For example, there are few available data on the effects of physical or biological variables (e.g., resource availability, population density, predation risk, genetic variability) on females' mating decisions. An understanding of the effects of these factors will lead to an appreciation of the range possible in the mating system of a species, rather than characterization of a species' mating system from a single population, as is typically done. Thus, basic information on variation in the frequency of multiple paternity in multiple populations of a given species is needed to evaluate the potential importance of physical and biological factors that affect female mating patterns.

Because they lack parental care beyond nesting, mate choice decisions in turtles must be primarily influenced by fertilization and/or genetic factors, and females' ability to store sperm for long periods provides a clear mechanism by which multiple paternity and sperm competition may be facilitated (Galbraith 1993; Palmer et al. 1998; Pearse et al. 2002). Parentage analyses on a variety of turtle species have

documented multiple paternity in from 0% to 100% of the assayed clutches (Pearse and Avise 2001). However, the extent of this variability that can be attributed to among-species differences vs. within-species has not been explored.

The giant Amazon (or arrau) river turtle, *Podocnemis expansa*, is a large, freshwater turtle that occurs throughout the Amazon and Orinoco river basins. Human hunting pressure over the past 200+ years has lead to extirpation of many local populations and the reduction of many others (Pritchard and Trebbau 1984; Cantarelli 1997). The species is now listed in Appendix II of CITES, and is the focus of several ongoing efforts to protect nesting populations (Cantarelli 1997; Licata and Elguezabal 1997; Von Hildebrand et al. 1997). Clutch sizes are large (often > 100 eggs, Pritchard and Trebbau 1984), which permits the accurate assessment of multiple paternity. This has potentially important conservation ramifications given the relation between multiple paternity and effective population size (Sugg and Chesser 1994). A previous genetic study of paternity in clutches of *P. expansa* from Colombia (Valenzuela 2000) found that 100% of the assayed clutches showed evidence of multiple paternity (the highest value ever reported for a turtle; Pearse and Avise 2001). However, Valenzuela (2000) examined only 2 clutches, so it is difficult to evaluate these results in the larger context of mating systems research, and, at best, we can conclude that multiple paternity has been documented in the Colombian population of *P. expansa*. Here, we present paternity data from 32 clutches of *P. expansa* from the Orinoco River in Venezuela. Results from this study add to the growing body of knowledge on turtle mating systems, and greatly extend previous paternity research on this endangered species.
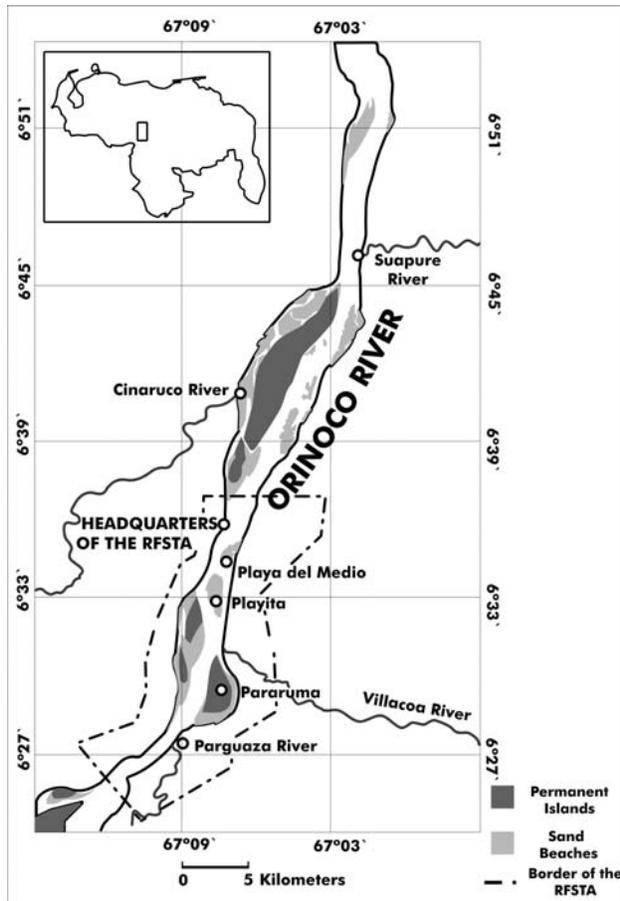
Figure 1. Map of the Refugio de Fauna Silvestre de la Tortuga Arrau, showing its location on the Orinoco River in Venezuela. The permanent islands are vegetated and are not covered by water during the rainy season, whereas the sand islands on which the turtles nest are exposed only during the dry season in most years.

METHODS

Sampling and DNA Procedures

Blood samples were obtained from 886 hatchlings from 32 clutches and were stored in Queen's lysis buffer (Seutin et al. 1991). All hatchlings were collected in April 2000 from nests from Isla Playita, Refugio de la Tortuga Arrau, in the Medio Orinoco (06°33'37" N, 67°07'36" W;

Fig. 1), Venezuela. Upon collection, hatchlings were marked with clutch-specific shell notches and kept in common tanks until blood samples could be taken. The clutches were divided into 2 categories based on number of hatchlings sampled; 28 clutches with a mean sample size of 21.36 (range, 9–25), and 4 clutches with a mean sample size of 71.75 (range, 70–76). The selection of the 4 clutches for the more extensive sampling in the latter category was arbitrary. For some clutches ($n = 15$), additional information was recorded on the total clutch size, number of infertile and inviable eggs, and number of live hatchlings. Mean total clutch size based on these 15 clutches was 95.1 ($\pm$ 25.5 SD; range, 61–156 eggs) and did not differ between the 2 sampling categories.

Deoxyribonucleic acid (DNA) was extracted from the hatchling blood/buffer samples by using the method of Fetzner (1999). After resuspension in dH_2O, the DNA was visualized in a 2% agarose gel stained with ethidium bromide and then used as the template for the amplification of 7 previously characterized microsatellite loci (Table 1) (Sites et al. 1999; Valenzuela 2000). Microsatellite genotypes were obtained by using ABI 3700 and 3100 genetic analyzers and the software GENOTYPER (Applied Biosystems). Although these 2 genetic analyzers use similar allele size standards, individual allele calls were not equivalent (alleles scored on one machine were not directly comparable with those scored on the other machine). As a result, statistical parentage analysis by using allele frequency data across all clutches was not possible. Thus, parentage was assessed on each clutch independently based strictly on the number of alleles observed within that clutch.

Parentage Analysis

Clutches were individually evaluated to assess multiple paternity. No DNA samples were available from the mothers, so parental inferences were made in the following manner. First, each clutch was checked to determine if all hatchling genotypes were consistent with single maternity (indicated by the presence of 1 of 2 alleles in each hatchling, at each locus). In many cases, the maternal genotype at some or all loci could be deduced from the segregation of offspring genotypes and/or the

Table 1. Microsatellite Loci Used in the Paternity Analysis.

Locus	No. Alleles	Heterozygosity	Exclusion Probability[a]	Reference
Pod1	13	0.727	0.486	Sites et al. 1999
Pod62	7	0.728	0.274	Sites et al. 1999
Pod91	10	0.586	0.199	Sites et al. 1999
Pod147	8	0.705	0.252	Sites et al. 1999
Pe344	5	0.687	0.323	Valenzuela 2000
Pod79	12	0.819	0.533	Sites et al. 1999
Pod128	7	0.737	0.292	Sites et al. 1999
Mean	**8.86**	**0.713**	**Overall: 0.950**	

[a] The exclusion probability was calculated by using allele frequencies estimated from the genotypes of 1 hatchling from each assayed clutch. See text for details.

presence of homozygous offspring. Individual hatchlings that did not share 1 of the 2 maternal alleles with the rest of the clutch were considered to originate from another clutch (see Results).

Second, clutches were evaluated for evidence of multiple paternity by using the minimum method (e.g., Meyers and Zamudio 2004). Each offspring was assumed to have received 1 maternal and 1 paternal allele, the maternal allele randomly coming from one of the mother's 2 alleles, and the paternal allele derived from a variably sized pool of alleles, depending on the number of contributing fathers. The null hypothesis of single paternity was rejected when 5 or more alleles were detected at 2 or more loci in a single clutch. Homozygous hatchling genotypes indicate that the mother and at least 1 father share an allele, and, in such cases, that allele was counted twice. Note that detection of 5 alleles in a clutch at a single locus was not considered sufficient proof of multiple paternity, because it is impossible to determine if the extra, unexplained, allele was because of the presence of a mutation at that microsatellite locus or to an additional contributing father. This stringent criterion is useful because the probability of simultaneous mutations at independent loci is extremely low, so unique alleles at 2 or more loci provides strong evidence for an additional contributing father.

To determine the power of our samples to detect multiple paternity, we used 2 methods. First, we estimated the microsatellite allele frequencies in the population by using 1 offspring from each clutch, because these represent independent genetic samples. These allele frequency estimates were then used to calculate the exclusion probability, or the probability of correctly excluding a male who is not the parent of an offspring based on its genotype at the 7 microsatellite loci (Jamieson and Taylor 1997). Because this probability ignores information gained from having multiple full- or half-sibling offspring, sampled together, it provides a conservative estimate of the power of our markers to detect multiple paternity. Second, simulations were run by using the program BROOD (DeWoody et al. 2000) to evaluate the adequacy of our offspring sample sizes. This program simulates genotypic data for parents and offspring to estimate *n*, the minimum number of offspring samples needed to detect all contributing fathers given a specified set of parameters. Simulations run by assuming a high potential number of contributing fathers and/or a high skew in paternal contributions will raise the estimated number of offspring needed and thus provide a conservative estimate of *n*.

RESULTS

The overall combined exclusion probability for the 7 microsatellite loci used in the study was 0.950 (Table 1), indicating that they provide sufficient power to detect additional males contributing to the offspring in a given clutch. BROOD simulations were run under the assump- tion of 5 potential fathers contributing to each clutch, and resulted in and estimated *n* of 28.6 ± 8.8 hatchlings (95% confidence interval [CI]; A. Fiumera, *pers. comm.*). This estimate is conservative, because if fewer than 5 fathers contributed to a nest, as is likely, fewer offspring samples would be required to detect all parents. Thus, under conditions of extreme multiple paternity, we would expect to have slightly better power to detect multiple paternity in the 4 more extensively sampled clutches than in the other 28 clutches.

A total of 11 scored alleles were determined to be evidence of mutation events. Eleven mutations in ca.12,404 allelic transmissions (886 hatchlings × 2 alleles × 7 loci) is equivalent to 1 mutation for every 1128 meiotic events, which is a typical rate reported for microsatellite loci (8.9×10^{-4}). In addition, the genetic data indicated that 30 of the 886 hatchlings genotyped (3.4%) had been grouped with the wrong clutch at some point during the sampling process, based on their not sharing any possible maternal genotype at multiple loci (Table 2). Of these, 15 were reassigned to another sampled clutch where their genotype fit perfectly with the other hatchlings at all loci under single maternity and without requiring the inference of any additional fathers. Over half (*n* = 8) of the reassigned individuals appeared to belong to a clutch numbered immediately adjacent to their originally assigned clutch, suggesting that a mix-up when the shell was notched or read during blood collection may have been responsible for their misassignment. The other 15 of these mislabeled hatchlings could not be assigned to any clutch and were excluded from the analysis.

Of the 32 clutches, 26 appeared to be singly sired, 3 were found to be multiply sired, 1 (c12) was excluded because of scoring difficulties, and 2 had equivocal paternity based on the criteria outlined above. These results provide us with a minimum estimate of 10.3% multiple paternity in this sample (3/29; Table 3). Of the multiply sired clutches, 2 clutches (c22 and c40) showed clear evidence of 2 sires, whereas the third clutch (c31) was sired by a minimum of 2 males and showed evidence of a third contributing father at a single locus (*Pod1*, Table 3). Two of the 3 confirmed multiply sired clutches, c31 and c40, were from the larger sampling category, with 71 and 70 genotyped hatchlings, respectively. The single clutch excluded because of scoring problems (c12) was either a case of multiple paternity with extensive allele sharing between males, mutations at multiple loci, or several misassigned individuals, and we could not clearly deter- mine which of these possibilities was the true reason. For 2 clutches (c8 and c25), we were not able to unequivocally determine paternity under our criteria. In clutch c8, no locus displayed 5 alleles, but a single hatchling had unique alleles at 2 loci, which would imply a nonindependent segregation ratio of 19:1 if explained by a single heterozygous father. The other equivocal clutch (c25) did display 5 alleles at 2 loci, as well as highly skewed segregation at 2 other loci, and therefore strictly qualifies as

Table 2. Details of Number of Offspring Sampled and Final Paternity Analysis Results in Each Clutch.

Clutch	No. Individuals Genotyped	No. Exported[a]	Moved To[b]	No. Imported[a]	Source Clutch	Final No.	Result[c]
1	20	1	c6			19	SP
2	23	4	c6			19	SP
3	21			1	c4	22	SP*
4	25	2	c3, nowhere			23	SP
5	13			1	c20	13	SP
6	23	1	c7	5	c2(4)[d]; c1(1)[d]	27	SP
7	23			1	c6	24	SP
8	22					22	MP?
9	21			1	c10	22	SP*
10	9	1	c9			8	SP
11	22	1	Nowhere	1	c12	22	SP
12	12	1	c11			12	Excluded
13	23	1	Nowhere			22	SP*
14	22			2	c15, c27	23	SP
15	25	1	c14			24	SP*
16	19	1	Nowhere			18	SP
17	24	3	c18, nowhere (2)[d]			21	SP*
18	20	1	Nowhere	1	c17	20	SP
19	25	1	Nowhere			24	SP*
20	22	1	c5	1	c21	22	SP
21	23	1	c20			22	SP
22	20					20	MP
24	25	1	Nowhere			24	SP
25	22					22	MP?**
26	23					23	SP*
27	23	1	c14			22	SP
28	24					24	SP
29	24	1	Nowhere			23	SP
30	76			1	c31	76	SP
31	71	1	c30			70	MP
34	70	2	Nowhere			68	SP**
40	70	3	Nowhere			67	MP
		30		15			

[a] "No. Exported" and "No. Imported" refer to offspring moved to an alternate clutch based on mismatching at inferred maternal alleles in their original clutch.
[b] "Moved To" refers to the clutch that an offspring was reassigned to based on matching both maternal and paternal alleles present in that clutch.
[c] SP = single paternity; MP = multiple maternity. An asterisk indicates the presence of a mutation in that clutch.
[d] Numbers in parentheses indicate movement of multiple offspring.

an additional case of multiple paternity under our criteria. However, extensive apparent allele sharing between the males at the other loci made it impossible to clearly identify the paternal contributions. If, as it appears, these 2 clutches indeed represent cases of multiple paternity, then our overall estimate would be higher (5/31 [16.1%]).

Finally, 3 clutches each appeared to contain a single hatchling whose genotype was not like the other offspring in the clutch. Like the single individual in c8, these 3

Table 3. Clutches with Evidence of Multiple Paternity.[a]

Clutch	1	62	91	147	344	79	128
22	2	**5**	3	4	**5**	**5**	4
31	**7**	**6**	3	**5**	**6**	**5**	**6**
40	**5**	**5**	3	4	**5**	**6**	3
8[b]	3	4	4	3	3	4	4
25	**5**	4	3	4	**5**	4	4

[a] The total number of alleles found at each locus is shown (bold when > 4).
[b] Although no locus displayed 5 alleles in this clutch, at 2 loci, *Pod62* and *Pod91,* a single hatchling was responsible for the presence of the unique fourth allele, supporting a hypothesis of an additional sire for that individual. See text for details.

singletons each appeared to have a possible matching maternal genotype but required a different paternal genotype than all the other offspring in their clutch at multiple loci. However, in these 3 cases, the hatchling's genotype also fit the maternal and paternal alleles in another clutch, without necessitating the inference of an additional father in that clutch. Because of the uncertainty over clutch assignments during sampling discussed above, we chose to reassign these 3 hatchlings to the alternate clutches, and no multiple paternity was concluded in these cases. However, had these 3 individual reassignments not been made, and these hatchlings were instead considered as single offspring of additional fathers in their original clutches, then the overall frequency of multiple paternity would again have been greater (8/31 [25.8%]).

DISCUSSION

Although multiple paternity has been found in a wide variety of turtles (Pearse and Avise 2001), small numbers of sampled hatchlings per clutch (Kitchler et al. 1999) and/ or small numbers of total clutches assayed (Valenzuela 2000) have often hampered accurate evaluation of the

frequency of multiple paternity within a population. Nonetheless, some striking patterns have emerged, notably the large range in the frequency of multiple paternity recorded between species (0%–100%; Pearse and Avise 2001). To date, only a few species of turtles have been examined in more than a single population (and these are primarily marine species), therefore, little information is available on within-species variation in the frequency of multiple paternity. Such information may be important, however, if life-history traits (e.g., population densities or sex ratios) differ substantially among populations. In *P. expansa*, for example, mark-recapture data of nesting females from the Rio Caquetá beaches studied by Valenzuela (2000) showed that the majority of females nest on different beaches in successive seasons (Von Hildebrand et al. 1997), whereas the majority of nesting females in the Medio Orinoco regions appear to return to the same beach in successive seasons (Ojasti 1967). If life-history trait differences among populations are widespread, then either ecological or genetic mechanisms may lead to different frequencies of multiple fertilization in *P. expansa* (see Garner et al. 2002, for an example, of geographic variation in multiple paternity in the snake *Thamnophis sirtalis*).

The results of the present study, which suggest that the frequency of multiple paternity in the Rio Orinoco population of *P. expansa* is between 10.3% and 25.8%, differ from those of Valenzuela (2000), who found evidence of a high incidence of multiple paternity (100%) in *P. expansa* from the Rio Caquetá of Colombia. However, the small number of clutches examined in that study (n = 2; Valenzuela 2000) makes direct comparisons difficult. Nonetheless, the large difference in detected rate of multiple paternity suggests that ecological or biological differences in the populations at these 2 sites may have a significant effect on females' mating behavior and influence patterns of offspring paternity. The Venezuelan population has suffered large reductions in population size because of hunting in the last 200+ years; von Humboldt (1814; cited in Pritchard and Trebbau 1984) estimated that the annual reproductive output of ca. 330,000 adult female turtles was removed annually from 3 major Medio Orinoco beaches in the early 1800s. Ojasti (1967) estimated a reduction of nests in this same region from 34,300 (1963) to 13,800 (1965), and this may have further declined by an order of magnitude, although current protection efforts may have stabilized the nesting population (Licata and Elguezabal 1997). Currently, the Medio Orinoco population is characterized by very low genetic diversity relative to most other *P. expansa* populations, including the Rio Caquetá population in Colombia (Pearse et al. 2006). The results of the present study are important, because, although the incidence of multiple paternity reported here is low, any polyandrous breeding system is expected to have an increased effective population size relative to a strictly monogamous population because of the decreased

variance in male reproductive success (Sugg and Chesser 1994).

Recently, sophisticated approaches to statistical parentage analysis have been developed to improve the accuracy of genetic parentage inference (DeWoody et al. 2000; Neff et al. 2000a, 2000b, 2002; Emery et al. 2001). However, in most cases, these methods are not applicable to our data because of the requirement of having sampled the mother, potential fathers, or both. The method of Emery et al. (2001) is potentially appropriate, but we were unable to use any allele frequency based statistical methods because of the discrepancies in the scores from the 2 ABI machines described in the Methods. However, Emery et al. (2001) found that both their method and the minimum method we used here produce concordant results, particularly when all the offspring in the progeny array share one parent (the mother, in the case of turtles). Thus, it is likely that additional statistical analysis would not substantially affect our basic conclusion that the Medío Orinoco population of *P. expansa* has a low to moderate frequency of multiple paternity. Nonetheless, the fact that 2 of the 3 clutches with strong evidence for multiple paternity had large sample sizes, despite there being only 4 such extensively sampled clutches, suggests that we may have failed to detect multiple paternity in some of the less completely sampled clutches. Based on only the 4 clutches with large sample sizes the detected frequency of multiple paternity would be 50%.

A moderate frequency of multiple paternity, similar to that found in the Venezuelan population of *P. expansa*, appears to be typical of many turtle species (e.g., green turtles (*Chelonia mydas*): Parker et. al. 1996; Fitzsimmons 1998, painted turtles (*Chrysemys picta*): Pearse et al. 2002; McTaggart 2000, reviewed by Pearse and Avise 2001). However, we have little information on the ecological factors that influence females' mating decisions, and the relative importance of multiple mating vs. sperm storage in turtles is not known (Pearse et al. 2002). Thus, additional studies of paternity patterns of a variety of turtle species in multiple populations and over multiple nesting seasons will be useful in determining the extent to which the frequency of multiple mating is influenced by biological and/or ecological differences among different populations for a given species.

RESUMEN

Se utilizaron datos de 7 loci de microsatélites de AND para deteminar frecuencia de paternidad múltiple en nidadas de tortuga arrau, *Podocnemis expansa*, procedentes del Orinoco Medio en Venezuela. El muestreo se realizó en crías con nueve meses de edad provenientes de 32 nidadas. A través del análisis de paternidad se encontró de manera concluyente que solo tres de las nidadas (10,3%) habían sido engendradas por más de un padre. Este estudio demuestra la importancia de documentar patrones de paternidad en diferentes poblaciones de una

especie, y considera los efectos de las diferencias ecológocas entre poblaciones por el comportamiento de las hembras al aparearse.

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