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Multiple Paternity in Loggerhead Turtle Clutches

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Microsatellite DNA was used to determine paternity in loggerhead turtle clutches. Hatchlings from three clutches were genotyped at two loci, as were their mothers and a sample of adults. A maximum-likelihood analysis determined the most likely number of fathers represented in each clutch using the genotypes and population allele frequencies. The analysis concluded that only one of the three clutches was sired by multiple males, with two fathers being more likely than three.

Observational studies of green turtles (*Chelonia mydas*) have documented both males and females mating multiple times in the wild (Booth and Peters, 1972; Limpus, 1993) as well as in captivity (Ulrich and Parkes, 1978). Using allozyme electrophoresis, Harry and Briscoe (1988) found multiple paternity in clutches of eight of 21 loggerheads (*Caretta caretta*) sampled in Queensland, Australia. Multiple mating may be advantageous to female sea turtles to ensure fertilization or to allow sperm competition, thereby increasing hatching success or offspring quality.

Approximately 35,000 loggerhead females nest along the southeast coast of the United States with 90% of the nesting occurring in Florida (Murphy and Hopkins, 1984). In this study, we used microsatellite DNA loci to determine the number of fathers represented in three loggerhead clutches from Florida.

MATERIALS AND METHODS

Loggerheads nesting on Melbourne Beach, Brevard County, Florida, were sampled during the summer of 1994. Blood samples of 50–100 μl were taken from the femoral vein of 26 adult females and stored in 1 ml of lysis buffer (Longmire et al., 1988). Nests of three of these females were monitored during their incubation period. At hatching, smaller blood samples (10–20 μl) were taken from the dorsal cervical sinus of a sample of hatchlings (n = 20, 20, and 22) as they emerged.

Half the volume of each sample was incubated at 65 C with 30 μl of Proteinase K (10 μg/μl) for 4–12 h. DNA was extracted using phenol and chloroform:isoamyl alcohol. Samples were then dialyzed 4–12 h at 4 C in TNE_2 (10 mM Tris, pH 7.9, 10mM NaCl, 2 mM EDTA). DNA concentration was estimated spectrophotometrically.

Two microsatellite loci, *Cc*117 and *Ei*8, were amplified using primer sets developed from two sea turtle species (*C. caretta* and *Eretmochelys imbricata*; FitzSimmons et al., 1995). Polymerase chain reactions (PCR) were carried out in 15 μl volumes made up of 40–50 ng of template DNA, 1X PCR buffer, 1 mM dNTPs, 0.5 mM each primer, 0.03 U/μl *Taq* polymerase, and 3 mM $MgCl_2$. Reactions ran at 95 C for 2.5 min, followed by 30 cycles comprising the following: 95 C for 45 sec, 1 min annealing phase beginning at 62 C and declining one degree per cycle until 55 C where it remained for the final 23 cycles, and 72 C for 1 min. This was followed by a final 72 C extension phase for 5 min. PCR products were separated on 7.5% polyacrylamide gels at 20 watts for 2–3 h. Bands were visualized using ethidium bromide and photographed over an ultraviolet light box.

Genotypes of the 26 adult females were determined by characterizing all alleles at each locus by size relative to standard molecular weight markers and other alleles in the sample. Genotypes of hatchlings were determined by comparing their alleles to those characterized across all females. Paternal alleles were identified by eliminating the mother's allele from each genotype. The software package Arlequin (S. Schneider, J.-M. Kueffer, D. Roessli, and L. Excoffier, 1996, unpubl.) was used to test for Hardy-Weinberg equilibrium and linkage disequilibrium.

To examine the possible number of fathers for each clutch, the likelihood of observed genotypic data was calculated for one, two, and three fathers. For a hypothesized number of fathers, the likelihood calculation considered all possible combinations of assigning hatchlings to all possible combinations of potential paternal genotypes. For each possible distribution, the probability of drawing the observed hatchling genotypes from that clutch was calculated. The likelihood for a given number of fathers was the average of the probabilities for all combinations with that number of fathers. Equations used were derived from the Elston-Stewart peeling algorithm (Elston and Stewart, 1971; Lange and Elston, 1975). It was assumed that allele fre-

TABLE 1. SUMMARY OF ALLELIC INFORMATION AT TWO LOCI FOR THREE FAMILIES. Frequencies of alleles 258–242 at Locus *Cc*117 were 0.038, 0.173, 0.038, 0.308, 0.038, 0.077, and 0.328, respectively. Frequencies of alleles 203–181 at locus *Ei*8 were 0.058, 0.365, 0.135, 0.019, 0.038, and 0.385, respectively.

	Maternal genotype		Hatchling genotypes and frequencies				Paternal genotype	
	Locus		Locus				Locus	
Clutch	*Cc*117	*Ei*8	*Cc*117		*Ei*8		*Cc*117	*Ei*8
94α	242/242	199/199	242/242	20/20	199/203	20/20	242/242	203/203
94Q	250/246	197/181	246/248	9/20	181/181	4/20	258/248	199/181
			246/258	3/20	197/181	3/20		
			250/258	3/20	197/199	7/20		
			250/248	5/20	181/199	6/20		
							Paternal alleles	
94U	256/250	199/181	256/254	6/22	181/181	12/22	254	181
			250/250	4/22	199/197	2/22	250	197
			250/242	6/22	199/181	6/22	242	181 or 199
			256/242	5/22	181/197	2/22	242	197
			256/250	1/22			256 or 250	

quencies at both loci in potentially breeding males were the same as frequencies observed in the sample of adult females. For ease of interpretation, likelihood ratios (not actual likelihood values) are reported, using the likelihood for the most likely number of fathers as the standard.

Because only a sample of hatchlings from each clutch and a limited number of primers were available for genetic analysis, we ran two simulations to estimate our power in determining the correct number of fathers for each clutch. One simulation determined the chance of selecting two fathers when, in fact, only a single male sired a clutch; the other simulation determined the chance of selecting one father when there were actually two. For the second simulation, we set the proportion of offspring sired by first and second males. Three sets of values allocating paternity between males were examined: 0.9–0.1; 0.8–0.2; and 0.5–0.5. For clutch sample sizes of 15 and 20 offspring (sampled from larger clutches), 1000 datasets were simulated for each study. The power to detect two fathers in each case is approximated by the fraction of datasets in which the likelihood for two fathers is greater than the likelihood for one father. For both simulation studies, we used the actual allele frequencies drawn from microsatellite data.

TABLE 2. LIKELIHOOD RATIOS FOR ONE, TWO, AND THREE FATHERS IN EACH OF THREE CLUTCHES.

	Number of fathers		
Clutch	1	2	3
94α	1.00	4.47×10^{-4}	8.76×10^{-7}
94Q	1.00	2.95×10^{-2}	3.95×10^{-4}
94U	0.00	1.00	9.09×10^{-2}

RESULTS

There was sufficient polymorphism at the two loci to detect multiple fathers if they occurred. Among 26 adult females sampled, seven alleles were characterized at *Cc*117, with allele frequencies ranging from 0.038 to 0.328; six alleles were present at *Ei*8, with frequencies ranging from 0.019 to 0.385 (Table 1). Both loci were found to be in Hardy-Weinberg equilibrium (*Cc*117: $\chi^2 = 17.751$, $P = 0.665$, df = 21; *Ei*8: $\chi^2 = 21.185$, $P = 0.131$, df = 15). Tests for linkage disequilibrium also were nonsignificant ($P = 0.312$; $\chi^2 = 28.775$, df = 30).

Likelihood analyses indicated two of the three clutches (94α and 94Q) exhibited single paternity, with one father being over 2200 times more likely than two in 94α and over 34 times more likely in 94Q (Table 2). Single paternity also was suggested by there being only one or two paternally derived alleles at each locus across hatchlings within each family (Table 1). For the third family, 94U, one father was impossible because at least three paternal alleles were present at *Cc*117 (Table 1). In this clutch, two fathers were 11 times more likely than three (Table 2).

The chance of deciding that two males sired a clutch, when a single male is actually the father, is small. For a sample of 15 offspring, the

error rate is 2.4% and falls to 2.2% for a sample of 20. This error probability continues to drop, though not to zero, as the sample clutch size increases. When there are actually two sires, our analysis has moderate to high power to detect the second male. When one male sires 90% of the offspring in a clutch, the power to detect the second male is estimated to be 64.9% for a sample of 15 and 73.2% for a sample of 20 hatchlings. When the proportion fertilized by the first male drops to 80%, the power to detect the second male rises to 83.4% for 15 and 87.4% for 20 offspring. When the two males have an equal chance of siring each offspring, the estimated power is 91.2% for 15 and 92.6% for 20 offspring samples. Because our smallest sample contained 20 hatchlings, we are confident that we can detect multiple paternity except when success is extremely skewed between fathers. The power to detect the second male does not increase to 100% even if clutch size is infinitely large, because it is possible for two males to have identical genotypes.

Discussion

One of three loggerhead clutches was determined to have been sired by multiple males when analyzed with microsatellites, whereas two of the clutches were singly sired. It is possible that multiple paternity may not be all that common in loggerheads, as was suggested by the level of multiple mating Harry and Briscoe (1988) found in a much larger sample of loggerheads. A larger sample size and more loci would be needed to better characterize the proportion of multiple paternity in the Florida loggerheads.

The rate at which multiple paternity is observed in any study may also be influenced by the population sex ratio. Because sex in sea turtles is determined by the temperature at which the eggs incubate, in unusually warm seasons, the sex ratio of hatchlings is skewed in favor of females (Standora and Spotila, 1985). Hatchling and juvenile loggerhead sex ratios have been found to be strongly female-biased along the coast of Florida (Wibbels et al., 1991; Mrosovsky and Provancha, 1992), but the sex ratio of breeding adults is unknown.

To calculate the likelihood ratios, we assumed that males and females come from the same population. Female sea turtles (Meylan et al., 1990; Bowen et al., 1993) and male green turtles (FitzSimmons et al., 1997) exhibit natal philopatry. However, male-mediated gene flow (Karl et al., 1992) could occur at feeding grounds or along migration routes where individuals from different rookeries mix (or "overlap"). This could result in male allele frequencies that are different from those of females. The degree of gene flow between loggerhead populations along the Atlantic coast is unknown. The small sample of paternally derived alleles in this study is a subset of those described in the 26 females sampled; thus, we have no evidence that the females mated with males from another population. The sample size of paternal alleles needs to be much larger before we can test this assumption.

As many sea turtle populations decline, it becomes increasingly important to improve our understanding of their population dynamics to take appropriate conservation measures. Particularly important for understanding the relationship between sex ratio, mating systems, and population viability for sea turtles is to examine the relationship between multiple paternity and fitness for multiple species and to compare mating systems and productivity for populations with different sex ratios.

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