

Multiple paternity and female-biased mutation at a microsatellite locus in the olive ridley sea turtle (*Lepidochelys olivacea*)

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Multiple paternity in the olive ridley sea turtle (*Lepidochelys olivacea*) population nesting in Suriname was demonstrated using two microsatellite loci, *viz.*, Ei8 and Cm84. The large number of offspring sampled per clutch (70 on average, ranging from 15 to 103) and the number of alleles found at the two loci (18 and eight alleles, respectively) enabled unambiguous assessment of the occurrence of multiple paternity. In two out of 10 clutches analysed, the offspring had been sired by at least two males, which was confirmed at both loci. In both clutches, unequal paternity occurred: 73% and 92% of the offspring had been sired by the primary male. The probability of detecting multiple paternity was 0.903, and therefore there is a small chance that multiple

paternity occurred but remained undetected in some of the eight clutches that appeared to be singly sired. Analysis of 703 offspring revealed a high mutation rate for locus Ei8 ($\mu = 2.3 \times 10^{-2}$) with all 33 mutations occurring in maternal alleles. In particular, one allele of 274 bp mutated at a high frequency in a clutch to which the mother contributed the allele, but in another clutch where the father contributed the same allele, no such mutations were observed. Inferred allele-specific mutation rates for Ei8 and expected numbers of mutations per clutch confirmed that maternal alleles for Ei8 are more likely to mutate in the olive ridley sea turtle than paternal alleles. Possible explanations are discussed. *Heredity* (2002) 89, 107–113. doi:10.1038/sj.hdy.6800103

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Introduction

Small or declining populations are vulnerable to inbreeding depression and random loss of genetic variation through genetic drift. Since genetic variation is important for adaptation to changing environmental conditions, the level of genetic diversity and the factors influencing the maintenance of genetic diversity have been a primary concern of conservation biologists. An important factor that affects the amount of genetic diversity in a population is the effective population size (N_e), which in turn is greatly influenced by the mating system of a species. In a polyandrous mating system, females mate with several males within a single reproductive cycle. This may result in multiple paternity of clutches, which occurs when several males contribute to the offspring in a single clutch. In such a mating system, N_e increases, and as a result, more genetic variation can be maintained than in a monogamous mating system (Sugg and Chesser, 1994; Moran and Garcia-Vazquez, 1998).

Populations of marine turtles (order Testudines) have decreased in many parts of the world due to human factors. Until recently, the Surinam olive ridley (*Lepidochelys olivacea*) population was considered to be the largest in

the Atlantic, but the number of nesting females has declined rapidly (Reichart and Fretey, 1993). In the late 1960s, the population nesting in the Galibi Nature Reserve in East Suriname was large enough to produce arribadas (mass nesting events) (Schulz, 1975), but the estimated number of clutches laid has declined continuously from 3063 in 1968 (when counting started) to 335 in 1995 (Schulz, 1975; Reichart and Fretey, 1993; Hoekert *et al.*, 1996).

In recent years, the occurrence of multiple paternity has been investigated using allozymes, minisatellites or microsatellites in several marine turtle species from the Cheloniidae and Dermochelyidae. Several studies indicated the occurrence of multiple paternity in loggerhead turtles (*Caretta caretta*) (Harry and Briscoe, 1988; Bollmer *et al.*, 1999), the Kemp's ridley (*Lepidochelys kempi*) (Kichler *et al.*, 1999), and green turtles (*Chelonia mydas*) (Parker *et al.*, 1996). In most cases however, multiple paternity was either observed at only one of several loci, indicated by deviations from Mendelian expectations, or the number of offspring sampled per clutch was low. In other studies, some of which were extensive, multiple paternity was not observed, or only at a very low level in green turtles (FitzSimmons, 1998) or leatherback turtles (*Dermochelys coriacea*) (Rieder *et al.*, 1998; Curtis *et al.*, 2000; Dutton *et al.*, 2000).

The highly polymorphic nature of microsatellite markers, caused by high mutation rates, makes them attractive tools in paternity studies. However, the occurrence of

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mutations may lead to the false conclusion of multiple paternity, if more than two paternal alleles are detected among the progeny. Therefore, in order to rule out this possibility, multiple loci should be analysed to test whether multiple paternity can be confirmed at different loci. The exact mechanism of microsatellite mutation is not yet known, but it is generally accepted that DNA polymerase slippage is the predominant mechanism creating new alleles (Tautz and Schlötterer, 1994), usually resulting in the gain or loss of a single repeat unit.

The aim of this study was to investigate whether multiple paternity occurs in the olive ridley sea turtle population nesting in Suriname using microsatellite markers. The extensive sampling also provided a good opportunity to collect data on microsatellite mutation.

Materials and methods

Fieldwork and sampling

Blood samples were collected from nesting olive ridley females in the Galibi Nature Reserve in East Suriname at the beginning of the 1995 nesting season. Ten females were sampled and immediately after egg deposition their clutches were transferred to a hatchery located on a nearby beach. In addition, blood samples from two other nesting females were taken. A sterile syringe (0.6 × 25 mm) was used to take 0.5 ml of blood from the dorsal cervical sinus (Bennet, 1986). Several days before the expected hatching date, screens were placed around the nests to collect the hatchlings. After hatching, blood samples (<0.2 ml) were taken from most of the emerged hatchlings, again from the dorsal cervical sinus (Bennet, 1986) before releasing the hatchlings. A total of 703 offspring from 10 clutches, ranging from 15 to 103 offspring per clutch, were sampled (Table 1). Blood samples were stored at ambient temperature in lysis buffer (100 mM Tris (pH 8), 100 mM EDTA, 10 mM NaCl, and 1% SDS). The nests were dug up after hatching and the hatching success (defined as the proportion of emerged offspring) was determined.

Microsatellite analysis

Nuclear DNA was extracted from the blood samples by proteinase K digestion (overnight) followed by ethanol precipitation (FitzSimmons *et al*, 1995). The occurrence of multiple paternity was investigated using Ei8 and Cm84, two dinucleotide microsatellite loci obtained from *Eretmochelys imbricata* (the hawksbill turtle) and *C. mydas*, respectively (FitzSimmons *et al*, 1995). For Cm84, a re-designed primer was used that resulted in smaller, better scorable alleles (K Kichler, personal communication). PCR was carried out in 10 µl reactions that contained 50–100 ng of nuclear DNA, 0.4 pmol of forward primer, end-labelled with $\gamma^{32}\text{P}$ -ATP, 1.6 pmol of unlabelled forward primer, 2 pmol of reverse primer, 0.1 mM dNTPs, 0.4 U Super Taq (Sphaero Q, Leiden, The Netherlands) and 1 µl Super Taq buffer. A 4 min denaturing step at 94°C was followed by 25 (Ei8) or 30 (Cm84) cycles of 30 sec denaturation at 94°C, 30 sec annealing at 56°C (Ei8) or 57°C (Cm84), and 25 sec extension at 72°C followed by a final extension of 5 min at 72°C. The PCR products were run on a 6% polyacrylamide gel (Sequagel) at 60 W for 4–5 h and visualised by autoradiography. The size of the alleles was determined by running them next to sequenced plasmid DNA with known sequence.

Data analysis

The genotypes of 25 unrelated adult individuals were determined in order to estimate the allele frequencies for Ei8 and Cm84 in the breeding population. These individuals consisted of the 12 nesting females sampled in 1995, 12 breeding males whose genotypes were inferred from the offspring data and one nesting female sampled in 1997. It was assumed that all the sampled females and the inferred males were unrelated. Allele frequencies at Ei8 and Cm84 were tested for deviation from Hardy-Weinberg equilibrium using a Markov chain method in GENEPOP 3.1 (Raymond and Rousset, 1995). The same method was used to test for linkage disequilibrium between the two loci. The probability of two unrelated individuals sharing the same genotype at both loci was calculated following Hanotte *et al* (1991). The probability

Table 1 Analysis of paternal contribution in 10 olive ridley clutches using microsatellite loci Ei8 and Cm84. Clutch ID, clutch size (in parentheses), hatching success (in %), and the number of offspring analysed per clutch (in parentheses) are shown. Maternal and paternal alleles are given in bp for each locus. For paternal alleles, the numbers of observations are shown in parentheses

Clutch ID (Clutch size)	Hatching % (Analysed)	Locus Ei8				Locus Cm84			
		Maternal alleles		Paternal alleles		Maternal alleles		Paternal alleles	
				Father 1	Father 2			Father 1	Father 2
Lo3 (108)	36% (38)	208	234	198 (24)	274 (14)	187	192	187 (16)	188 (22)
Lo6 (126)	83% (86)	208	238	198 (40)	208 (46)	187	190	187 (86)	
Lo7r (140)	79% (103)	206	274	194 (57)	198 (46)	190	194	187 (50)	188 (53)
Lo7w (111)	50% (56)	198	198	206 (31)	244 (25)	188	194	187 (24)	188 (32)
Lo9 (111)	68% (75)	208	238	240 (32)	244 (37)	188	194	187 (69)	187 (2)
Lo11 (121)	79% (94)	198	266	206 (57)	238 (37)	187	188	187 (94)	197 (4)
Lo13 (105)	81% (85)	198	264	244 (35)	260 (50)	186	188	192 (43)	
Lo15 (122)	14% (15)	206	206	198 (5)	206 (10)	187	197	187 (15)	
Lo17 (131)	57% (71)	198	206	198 (21)	270 (31)	188	194	194 (27)	189 (6)
Lo19 (104)	79% (80)	254	266	198 (35)	208 (45)	187	187	187 (37)	192 (43)

of detecting multiple paternity (D) using Ei8 and Cm84 in the Surinam olive ridley population was calculated according to Westneat *et al* (1987). This value (D) depends on the allele frequencies in the breeding population and takes into account all genotypic combinations that will not allow the detection of multiple paternity (for instance, two males sharing the same genotype, or two males homozygous for different alleles). In order to detect with 95% confidence any male fathering at least five offspring, it is required to sample 50% of a clutch, regardless of the clutch size (Galbraith *et al*, 1989). Except for two clutches with a low hatching success, 50% to 81% of the eggs in each clutch was analysed. All offspring ($n = 703$) were screened at both loci, and paternal alleles were inferred for each clutch by comparing the progeny to their mother. Chi-square tests were used to test whether the distribution of genotypes in the progeny followed Mendelian expectations. To this end, mutant alleles were grouped with the allele they were presumed to have originated from, which is the allele that is closest in size to the mutant allele. The occurrence of an unexpected maternal allele in a descendant, ie, an allele that was not present in the mother, was designated a mutation, while the occurrence of more than two paternal alleles among the offspring in a clutch could either be explained by mutation or by multiple paternity. Multiple paternity was concluded when (i) more than two paternal alleles at a locus were found among the progeny, or if deviations from Mendelian inheritance were observed, and (ii) multiple paternity was supported at both loci in individual offspring, making the possibility of mutation highly unlikely.

In order to calculate allele-specific mutation rates at Ei8, the alleles that were found among the 703 offspring were grouped in size classes of 10 bp, and the number of mutations in each size class was divided by the number of observations of each size class. It was assumed that the alleles differed only in the number of repeat units, and that the mutation rate depended on the number of repeat units. The expected number of mutations per clutch was calculated as follows: for each allele in a clutch, the expected number of mutations was calculated by multiplying the number of offspring carrying the allele with the corresponding allele-specific mutation rate. The expected number of mutations per clutch was then calculated by summing the expected values for all the alleles in a clutch.

Results

Population variability

Among 25 unrelated individuals, 18 and eight alleles were found at microsatellites Ei8 and Cm84, respectively (Figure 1). Despite the fact that Cm84 is a dinucleotide repeat, most alleles for this locus differed by only one bp (Figure 1), which was also supported by two additional alleles (193 and 196 bp) that were found in unrelated olive ridley hatchlings sampled in 1997 (WEJ Hoekert *et al*, unpublished data). Allele frequencies at both loci were within expectations of Hardy-Weinberg equilibrium ($P > 0.05$), and no evidence for linkage disequilibrium between Ei8 and Cm84 was found ($P > 0.05$). The expected heterozygosity was 0.89 for Ei8 and 0.75 for Cm84. Observed heterozygosities were slightly higher:

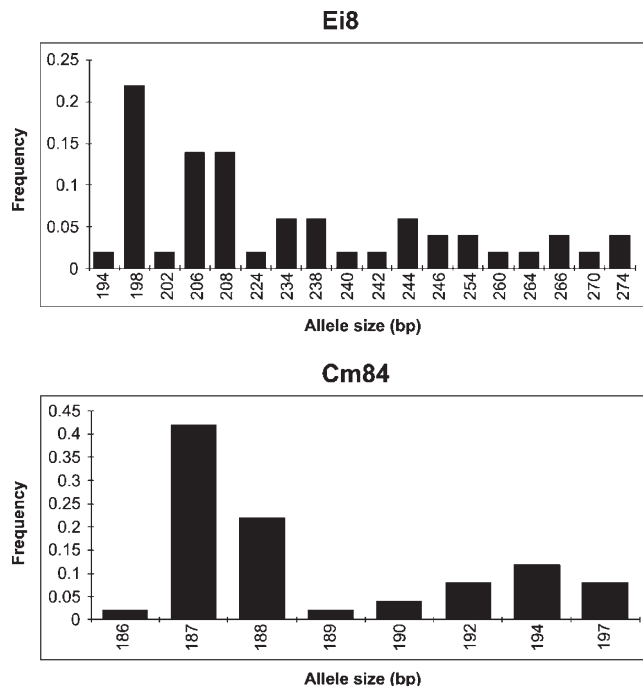


Figure 1 Allele frequencies at Ei8 and Cm84 determined in 25 unrelated individuals from the Surinam olive ridley population.

0.92 and 0.80 for Ei8 and Cm84, respectively. The probability of two different males sharing the same genotype at Ei8 as well as Cm84 was 1.9×10^{-3} , and the probability of detecting multiple paternity across both loci in the Surinam olive ridley population was 0.903.

Multiple paternity

The hatching success of the 10 clutches ranged from 14% to 83% (average 63%, Table 1). Maternal and inferred paternal alleles for each clutch are given in Table 1. Multiple paternity was found in two clutches, as shown by the number of paternal alleles and the distribution of paternal alleles (Table 1). Among the offspring in clutch Lo17, four paternal alleles were found at Ei8 and four paternal alleles were found at Cm84, strongly suggesting that this clutch had been fertilised by at least two males (Tables 1 and 2). Among the offspring in clutch Lo9, four paternal alleles were found at Ei8, and a strong deviation from Mendelian expectations was found at Cm84, both indicating multiple paternity. Of the two paternal alleles that were found at Cm84 in clutch Lo9, one (197 bp) was found at a very low frequency: only four out of 75 progeny contained this allele (Table 1). This considerable deviation from Mendelian expectations, under the assumption of a single father, strongly suggested the involvement of a second father that sired only a small proportion of this clutch. This was confirmed by the fact that the 197 bp allele was only present in offspring that had a 254 bp or 234 bp allele at Ei8 (Tables 1 and 2). Therefore, it was highly unlikely that the 197 bp alleles at Cm84 were the result of mutation, and this was also supported by the absence of mutations at Cm84 in the remaining progeny (see below).

All offspring in Lo9 and Lo17 that showed unexpected alleles at Ei8 also showed unexpected alleles at Cm84, meaning that multiple paternity was confirmed at both

Table 2 Inferred paternal alleles for Ei8 and Cm84 in the two olive ridley clutches that were multiply sired, illustrated by a representative subset of 16 offspring. The subsets were composed in order to contain all possible allelic combinations for both fathers (if possible). For clutch Lo9, all offspring sired by the secondary male are shown. The genotypes of the remainder of the offspring of both clutches displayed a similar pattern. The genotypes of the different males for Ei8 and Cm84 are indicated at the top. Allele sizes are shown in bp

Hatchling number	Clutch Lo17				Hatchling number	Clutch Lo9			
	Father 1		Father 2			Father 1		Father 2	
	Ei8 198–270	Cm84 194–197	Ei8 234–246	Cm84 189–192		Ei8 240–244	Cm84 187–187	Ei8 234–254	Cm84 187–197
29			234	189	17			254	197
30			246	192	18	240	187		
31	270	194			31	244	187		
32	270	197			32			254	197
33			246	189	33			234	197
34	270	197			34	240	187		
35	270	194			42	240	187		
36			234	192	43	244	187		
37	198	197			44	244	187		
38			246	192	45			254	187
39	198	194			61	244	187		
40			234	192	62			234	197
41	198	194			63	244	187		
42			234	189	67	244	187		
43	198	197			68			254	187
44			246	189	72	240	187		

loci (Table 2). By combining data for both loci, unique genotypes could be assigned to each father, enabling the determination of the number of progeny sired by each male. Of the 71 offspring in clutch Lo17, 52 were sired by the primary male (defined as the male that sired most of the offspring) and 19 by the secondary male. Of the 75 offspring in Lo9, 69 were sired by the primary male and six by the secondary male.

In all 10 clutches, genotype frequencies in the offspring were in accordance with Mendelian expectations. When taking into account the two different fathers in clutches Lo9 and Lo17, Mendelian expectations for each father were met at both loci. Assuming null alleles, if the mother was heterozygous and the father was heterozygous for a null allele and an allele different from both maternal alleles, then three paternal alleles would be detected among the progeny, and a false conclusion of multiple paternity could be reached (Kichler *et al*, 1999). This scenario did not occur in the two clutches displaying multiple paternity (Tables 1 and 2).

Mutation rate

Analysis of 703 offspring provided information on the mutation rate at both microsatellite loci. At Ei8, 15 different mutant alleles were found in 33 out of 703 offspring (Table 3), giving a mutation rate of 2.3×10^{-2} . At Cm84, no mutations were found. The unexpected alleles at Ei8 were identified as mutations by two observations: (i) none of the 33 progeny had unexpected alleles at Cm84, and (ii) each mutation occurred in the maternal allele, as shown by the absence of a maternal allele and the presence of a paternal allele in each of the 33 offspring. The second observation confirmed that the 33 unexpected alleles were caused by mutation and not by multiple paternity.

Most mutations ($n = 23$) resulted in the gain of one repeat unit (two base pairs). In only one case, one repeat

Table 3 Observed mutations at microsatellite locus Ei8 in 33 olive ridley offspring. The size of the allele in which the mutation occurred is shown (original allele). The number of offspring in which a particular mutation occurred is shown for each type of mutation. No mutations were observed in nine other alleles (194, 206, 208, 234, 240, 244, 246, 260, and 270 bp). For the remaining three alleles (202, 224, and 242 bp), no data are available since offspring from parents exhibiting these alleles were not analysed

Original allele	Clutch	+2 bp	-2 bp	+4 bp	+6 bp	-6 bp	-10 bp	-12 bp
198 bp	Lo11	1	-	-	-	-	-	-
238 bp	Lo6 and Lo9 ^a	4	-	-	-	-	-	-
254 bp	Lo19	2	-	-	-	1	-	1
264 bp	Lo13	3	-	-	-	-	1	-
266 bp ^b	Lo11	1	-	1	-	-	1	-
	Lo19	3	-	-	-	-	-	-
274 bp	Lo7r	9	1	3	1	-	-	-

^aOne mutation occurred in clutch Lo6 and three mutations occurred in clutch Lo9.

^bThe 266 bp allele mutated in clutch Lo11 and clutch Lo19. Data for each clutch are shown separately.

unit had been lost. In the other nine cases, two to six repeat units had been lost or gained (Table 3). Mutations were not evenly distributed among the clutches. Some clutches did not contain mutations at all, while in clutch Lo7r, 14% of the progeny (*viz.*, 14 out of 103 individuals) inherited a mutation. Large alleles (more repeat units) were more sensitive to mutation than small ones (Tables 3 and 4).

All mutations occurred in maternal Ei8 alleles. In order to analyse this unequal distribution of mutations, allele-specific mutation rates were determined for all the Ei8 alleles observed in the 10 clutches (Table 4). Using the allele-specific mutation rates, the expected number of mutations could be calculated for each clutch. The

Table 4 Allele-specific mutation rates at microsatellite locus Ei8 in 703 olive ridley offspring. Alleles were grouped in size classes of 10 bp and for each size class the number of observations and the number of mutations are shown. Allele sizes are shown in bp

Size class	No. of observations	No. of mutations	Allele-specific mutation rate
189–198	419	1	0.0024
199–208	388	0	0
229–238	151	4	0.0265
239–248	137	0	0
249–258	52	4	0.0769
259–268	162	10	0.0617
269–278	97	14	0.1443

Table 5 Expected numbers of mutations at microsatellite locus Ei8 in the two olive ridley clutches that showed a significant difference ($P < 0.05$, $df = 1$) between observed and expected values. For each clutch, the maternal (M) and paternal (P) alleles and the total number of expected mutations (Total) are given. For each allele, the number of observations, and the observed (N_{obs}) and expected (N_{exp}) number of mutations are shown. Expected values were calculated using the allele-specific mutation rates from Table 4. Allele sizes are shown in bp

Clutch	Allele	No. of observations	N_{obs}	N_{exp}	Total	χ^2
Lo7r	206 (M)	51	0	0	7.75	5.45
	274 (M)	52	14	7.50		
	194 (P)	57	0	0.14		
	198 (P)	46	0	0.11		
Lo17	198 (M)	36	0	0.09	4.90	5.27
	206 (M)	35	0	0		
	198 (P)	21	0	0.05		
	270 (P)	31	0	4.47		
	234 (P)	11	0	0.29		
	246 (P)	8	0	0		

expected number of mutations per clutch differed significantly from the observed number of mutations in clutch Lo7r ($\chi^2 = 5.45$, $df = 1$, $P < 0.05$) and clutch Lo17 ($\chi^2 = 5.27$, $df = 1$, $P < 0.05$) (Table 5). In clutch Lo7r, the difference was caused by an excess of mutations in the maternal 274 bp allele: only seven mutations were expected to occur in the 274 bp allele, yet 14 mutations occurred. In clutch Lo17, the difference was caused by an absence of mutations in the paternal 270 bp allele: at least four mutations were expected in the 270 bp allele, whereas none occurred (Table 5). Grouping of alleles in other size classes such as classes of 5 bp, 15 bp, or 20 bp, and/or shifting the size classes to different ranges yielded similar results: a significant difference between expected and observed mutations in clutches Lo7r and Lo17 (data not shown).

Discussion

Multiple paternity

The microsatellite data presented in this study unambiguously show that multiple paternity occurs in the olive ridley sea turtle. Clutches Lo9 and Lo17 were

shown to be fertilised by at least two males, and in both cases, multiple paternity was confirmed at both loci (Tables 1 and 2). Furthermore, unequal paternal contribution occurred; 92% (Lo9) and 73% (Lo17) of the progeny had been sired by the primary male. The probability of detecting multiple paternity was 0.903, which means that for each clutch that was sired by one male, there is a chance of about 10% that multiple paternity did occur but remained undetected. Therefore, the level of multiple paternity in the Surinam olive ridley population might be slightly higher than appeared from this study.

We cannot tell whether the observed multiple paternity resulted from multiple matings within one breeding season, or whether sperm stored from a previous nesting season fertilised part of the eggs. During the nesting season, Pacific olive ridleys assemble into the so-called reproductive patch (RP), a large dynamic aggregation of mature reproductive males and females engaged in courtship and mating or waiting for the next arribada (Kalb *et al*, 1992). Under these conditions, multiple matings could easily occur, and thus promiscuity could be common in olive ridleys. However, in the Surinam olive ridley population, there is no evidence for the existence of a RP, and arribadas did not occur anymore since the late 1960s. Alternatively, sperm stored from a previous nesting season could have contributed to the progeny. Sperm storage tubules containing sperm have been identified within the oviduct of several turtle species including the olive ridley (Gist and Jones, 1989), and in box turtles (*Terrapene carolina carolina*), sperm storage for up to 4 years has been reported (Ewing, 1943). This is much longer than the average internesting period for Surinam olive ridleys which nest on average every 1 or 2 years (Schulz, 1975). In copperhead snakes, multiple paternity resulting from autumn- and subsequent spring copulations was demonstrated, and in two out of three cases, most or all offspring were sired by the first male (Schuett and Gillingham, 1986). However, no multiple paternity occurred when females mated only once a year, in spring, at the time of ovulation. Extending these results to the olive ridley, and considering the relatively high proportion of offspring (27%) sired by the secondary male in clutch Lo17 suggests that the matings in both multiply sired clutches might have occurred within the same nesting season. If true, the observed unequal paternity could be explained by sperm competition, cryptic female choice (Jennions and Petrie, 2000; and references therein), or differences in genetic compatibility between certain males and females (Zeh, 1997).

The level of multiple paternity found in this study is lower than the one found in Kemp's ridleys, for which it was suggested that probably all clutches might have been fertilised by multiple males (Kichler *et al*, 1999). It should be noted that the Surinam population is not representative of a stable olive ridley population. Numbers have been, and still are, declining rapidly (Van Tienen, personal communication) and arribadas have not been observed in the last 30 years. Therefore, levels of multiple paternity could differ considerably in other, larger populations.

Mutation rate

The mutation rate at locus Ei8 ($\mu = 2.3 \times 10^{-2}$) contrasted greatly with the one at Cm84, for which no mutations were observed. The high mutation rate for Ei8 differs

from values reported in a study on the closely related Kemp's ridley (Kichler *et al*, 1999). In that study, maximum likelihood analyses were performed on data for three microsatellite loci (among which Ei8 and Cm84) in 161 offspring from 36 Kemp's ridley clutches. It was concluded that the maximum likelihood estimate of the overall mutation rate was 0, although rates as high as 0.01 could not be rejected (Kichler *et al*, 1999). The mutation rate at Ei8 is relatively high compared to values found in green turtles, which ranged from $<5.7 \times 10^{-4}$ to 9.6×10^{-3} (FitzSimmons, 1998), and comparable to the highest mutation rate observed so far for a microsatellite locus (3.6×10^{-2} , Primmer and Ellegren, 1996). The fact that large alleles mutated more often than small ones (Tables 3 and 4) is in agreement with the hypothesis that microsatellite alleles with a larger number of repeats tend to have higher mutation rates (Weber, 1990), and with results from other studies (eg, Primmer and Ellegren, 1996; Schlötterer *et al*, 1998). Most of the mutations (85%) involved an increase in allele size (Table 3), which is also in agreement with other studies (Weber and Wong, 1993; Primmer and Ellegren, 1996; Cooper *et al*, 1999).

Surprisingly, all 33 mutations occurred in maternal Ei8 alleles. It is generally believed that mutation rates are much higher in males than in females because of the many more cell divisions in spermatogenesis than in oogenesis (Crow, 1993). Indeed, Weber and Wong (1993) observed a bias towards paternal mutation events in their study on human STRPs, and a strong tendency for mutations to occur in male germlines was found in a number of VNTRs (eg, Vergnaud *et al*, 1991). In birds, a male-biased mutation rate, manifested as faster evolution of the Z chromosome than of the W chromosome has been demonstrated (Carmichael *et al*, 2000, and references therein). On the other hand, no sexual bias was observed in mutation rates at microsatellite loci in green turtles (FitzSimmons, 1998) and leatherback turtles (Dutton *et al*, 2000), nor at particular human VNTR loci (eg, Jeffreys *et al*, 1991). One example, however, of female biased mutation is found at the fragile X mutation site, an exon in the human FMR-1 gene containing a highly polymorphic trinucleotide repeat (Fu *et al*, 1991, and references therein).

Most of the mutations ($n = 21$) occurred in two of the largest clutches, *viz.*, Lo7r ($n = 103$) and Lo19 ($n = 80$). In these clutches, the mothers contributed large alleles and the fathers contributed small alleles (Table 1). Since larger alleles mutated at a higher rate, this may have contributed to the observed female-biased mutation. However, the fathers in other clutches had large alleles as well, and mutations in these alleles should have occurred if we assume no sexual bias. A conspicuous example of female-biased mutation at locus Ei8 is the absence of mutations in the paternal 274 bp allele among 14 offspring in clutch Lo3, while the maternal 274 bp allele in clutch Lo7r mutated in 14 out of 52 offspring (Tables 3 and 5). The observed number of mutations in clutch Lo7r differed significantly from the expected number of mutations ($\chi^2 = 5.45$, $df = 1$, $P < 0.05$), which was caused by an excess of mutations in the maternal 274 bp allele (Table 5). This observation suggests that maternal alleles for Ei8 are more prone to mutation than paternal alleles of the same size. The discrepancy might also be explained by the occurrence of allelic homoplasy due to variable flanking sequences. In a study on a human microsatellite locus,

sequence data revealed that alleles of the same size could differ considerably in internal structure, and that a high degree of variability was due to variation in the flanking sequences (Grimaldi and Crouau-Roy, 1997). It is possible that the number of repeats in the maternal 274 bp allele in clutch Lo7r is higher than that of the paternal 274 bp allele in clutch Lo3 and that the difference in repeat number is masked by size differences in the flanking sequences. Indeed, sequence data from a Pacific olive ridley individual indicated that a 198 bp allele of locus Ei8 probably contains a CA₁₆ repeat (FitzSimmons *et al*, 1995; N FitzSimmons, personal communication), leaving more than 160 bp of flanking sequences. Although allelic homoplasy could explain the observed results for a given allele, we regard it as unlikely that the overall absence of paternal mutations was caused by allelic homoplasy, because that would require the studied females and males to come from two distinct populations. Another explanation for the 274 bp alleles might be that the paternal 274 bp allele has a point mutation that interrupts the repeat, thus reducing the chance of DNA slippage.

Large size differences between the two microsatellite alleles in an individual might result in an increased mutation rate (Amos *et al*, 1996; Schlötterer *et al*, 1998). However, although the mother of clutch Lo7r did indeed have one of the largest size differences (206 and 274 bp), the size difference was even more pronounced in the fathers of clutch Lo3 (198 and 274 bp) and Lo17 (198 and 270 bp), two clutches in which no mutations were found. Moreover, the size difference in the mother of clutch Lo19 (254 and 266 bp) was very low, yet seven mutations occurred. Therefore, the observed bias towards maternal mutations cannot be explained by this mechanism. On the contrary, if this mechanism really exists, it confirms that mutation is restricted to females. The mutational pattern observed at Ei8 needs further study. Sequencing of alleles will be an important step in explaining the results, providing more insight in the process of microsatellite mutation and evolution in general, and in the olive ridley sea turtle in particular.

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