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The leatherback turtle, *Dermochelys coriacea*, exhibits both polyandry and polygyny

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Abstract

The leatherback turtle (*Dermochelys coriacea*) is an endangered species, and world-wide populations are declining. To understand better the mating structure of this pelagic and fragile species, we investigated paternity in nearly 1000 hatchlings from Playa Grande in Parque Marino Nacional Las Baulas, Costa Rica. We collected DNA samples from 36 adult female leatherbacks and assessed allele frequency distributions for three microsatellite loci. For 20 of these 36 females, we examined DNA from hatchlings representing multiple clutches, and in some cases assessed up to four successive clutches from the same female. We inferred paternal alleles by comparing maternal and hatchling genotypes. We could not reject the null hypothesis of single paternity in 12 of 20 families (31 of 50 clutches), but we did reject the null hypothesis in two families (eight of 50 clutches). In the remaining six families, the null hypothesis could not be accepted or rejected with certainty because the number of hatchlings exhibiting extra nonmaternal alleles was small, and could thus be a result of mutation or sample error. Successive clutches laid by the same female had the same paternal allelic contribution, indicating sperm storage or possibly monogamy. None of 20 females shared the same three-locus genotype whereas there were two instances of shared genotypes among 17 inferred paternal three-locus genotypes. We conclude that both polyandry and polygyny are part of the mating structure of this leatherback sea turtle population.

Keywords: *Dermochelys coriacea*, leatherback, mating system, microsatellite, multiple paternity, polyandry, polygyny

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Introduction

The leatherback turtle, *Dermochelys coriacea*, is an endangered species that has undergone severe population declines over the past few decades (Spotila *et al*. 1996; Spotila *et al*. 2000). The population of nesting leatherbacks on Playa Grande in Parque Marino Nacional Las Baulas, Costa Rica decreased from 1367 in 1988–89 to 469 in 1994–95 and to only 117 females in the 1998–99 season (Steyermark *et al*. 1996; Spotila *et al*. 2000). World-wide leatherback population declines are a result of human poaching of eggs and of fishing practices which result in adult mortality (Spotila *et al*. 1996). A more complete knowledge of the mating system of this declining species could inform conservation plans aimed at preventing the extinction of these turtles.

The investigation into mating systems of reptiles has advanced substantially over the past decade. Harry & Briscoe (1988) reported multiple paternity in clutches of eight out of 24 loggerhead females (*Caretta caretta*) using allozyme electrophoresis. Galbraith (1991) and Galbraith *et al*. (1993) used DNA fingerprinting to detect multiple paternity in wood turtles, *Clemmys insculpta*, and snapping turtles, *Chelydra serpentina*. Various snake species, such as adders (*Vipera berus*) (Tegelström & Höggren 1994) and garter snakes (*Thamnophis sirtalis*) (Gibson & Falls 1975), produce bipaternal clutches, and multiple paternity occurs in the

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sand lizard (*Lacerta agilis*) (Gullberg *et al*. 1997). Bollmer *et al*. (1999) reported multiple paternity in a Florida loggerhead population using microsatellite analysis. FitzSimmons (1998) reported that green turtles, *Chelonia mydas*, have a low frequency of multiple paternity despite their promiscuous mating behaviour. Multiple paternity is a prevalent pattern in the Kemp's Ridley (*Lepidochelys kempi*) population near Rancho Nuevo, Tamaulipas, Mexico (Kichler *et al*. 1999), which is the only substantial breeding population of that endangered sea turtle species.

Few data are available concerning leatherback reproductive behaviours and mating systems (Carr & Carr 1986; Rieder 1996; Curtis 1998; Dutton *et al*. 1998). Rieder (1996) examined the paternal input of five leatherback clutches at Playa Grande, Costa Rica, and concluded that all clutches had single paternity. Dutton *et al*. (1998) reported a low frequency of multiple paternity in leatherback clutches at St Croix, US Virgin Islands. Curtis (1998) discovered multiple paternity in one of 12 leatherback nests at the Pacuare Reserve on the Caribbean coast of Costa Rica. However, all of these studies were limited by small sample size.

In this study we determined genotypes of females and hatchlings in the leatherback population nesting at Playa Grande in Parque Marino Nacional Las Baulas, Costa Rica to test the null hypothesis of single paternity in this population. By examining numerous clutches, as well as several clutches laid by a single female throughout a nesting season, we were able to assess the frequency and pattern of multiple paternity in this leatherback population. Inferences regarding male genotypes allowed us to estimate the number of distinct males contributing to this population.

Materials and methods

Collection of samples

We collected blood samples from adult female leatherbacks and their hatchlings on Playa Grande, Costa Rica during the 1998/1999 nesting season (October to February). Playa Grande is a 3.5-km beach located on the Pacific coast of Costa Rica in Parque Marino Nacional Las Baulas in Guanacaste (10°20′ N, 85°51′ W). We collected blood samples of approximately 50–100 μL from 36 adult females while they nested. We obtained these samples by pricking the wrist area with a lancet and drawing pooled blood from the skin with a 27-gauge needle on a 1-cm^3 insulin syringe. To minimize contamination, we cleansed the wrist area with an alcohol swab before taking blood. Individual females were identified by the presence of PIT (Passive Integrated Transponder) tags. If no tag was present, we administered one tag into each shoulder of the animal. Fifteen of the 36 females were remigrants. For 20 of these 36 females (10 remigrants), we collected at least four successive clutches and relocated them to hatchery nests (depth = 1 m) within 5 h of the time of laying. We protected the nests from fly infestation and restrained hatchlings after emergence by placing wire corrals covered with mosquito netting over each nest. For each clutch included in the data set, we took blood samples of approximately 20 μL from the dorsal cervical sinus of up to 28 hatchlings using a 27-gauge needle on a 1-cm^3 insulin syringe. The sampling effort ranged from 29% to 100% of emerged hatchlings in each nest. Immediately after blood sampling, we released all hatchlings to the beach. In clutches with low hatching success, we took blood samples from dead stage-three embryos (embryos that died during the last stages of development) in the same manner described above (n = 25). We spotted all blood samples on Isocode PCR DNA Isolation Papers (Schleicher & Schuell, Inc.), allowed them to dry in a desiccation chamber for 3–5 days, and then stored the papers at room temperature until use.

Microsatellite amplification and analysis

We extracted DNA from the blood spot according to the manufacturer's protocol (Schleicher & Schuell, Inc.). In brief, we cut a circle (diameter = 0.3 cm) from the paper and vortexed it in 500 μL water (Eppendorf 5-Prime, Inc.) for 5 s. We then transferred the paper to 50 μL water and incubated for 30 min at 95 °C. After incubation, we removed the sample paper and used the remaining solution, which contained the DNA, as template in the following analyses. We stored all DNA solutions at –20 °C.

Polymerase chain reactions (PCR) amplified microsatellite DNA loci for the 36 adult samples, hatchlings from the first and last clutches laid by 13 females (n = 26 clutches), and hatchlings from three or four clutches laid by the remaining seven females (n = 24 clutches). We used primer sets Cc117 (*Caretta caretta*), Ei8 (*Eretmochelys imbricata*), Cm3, Cm84 (*Chelonia mydas*) (FitzSimmons *et al*. 1995) and Dc99 (*Dermochelys coriacea*) (Dutton 1995) to assess genotypes in the 36 adult females. We used primer sets Cc117, Ei8 (FitzSimmons *et al*. 1995) and Dc99 (Dutton 1995) to obtain genotypic profiles for hatchlings (Table 1). Primers were radiolabelled with [γ^{32}P]dATP by incubation with T4 polynucleotide kinase (Promega) for 1 h at 37 °C. All PCR reactions used a volume of 20 μL containing 250 μM dNTP solution (Applied Biosystems), 1.5 mM Mg^{2+}, 10× PCR buffer, ~50 ng template DNA, 0.10 μL Ampli*Taq* polymerase (Perkin Elmer), and 40 pmol of each primer. We used a Perkin-Elmer DNA Thermal Cycler 9600 to process all PCR reactions and applied the following conditions: (i) initial denaturation of DNA at 95 °C for 3 min, (ii) a three-step cycle repeated 30 times (94.5 °C for 30 s, 56 °C or 60 °C for 30 s, and 72 °C for 30 s), (iii) final extension at 72 °C for 5 min, and (iv) cooling phase at 4 °C. We stored all PCR products at –20 °C. We analysed the PCR products on 6%

Table 1 Microsatellite loci investigated in 36 adult female leatherback DNA samples collected at Playa Grande, Costa Rica during the 1998–99 nesting season

Locus	Annealing temp. (°C)	Primer sequence (5′ → 3′)	Allele length (bp) Expected	Observed	*H**	*q**	*d**
Cc117†	56	TCTTTAACGTATCTCCTGTAGCTC CAGTAGTGTCAGTTCATTGTTTCA	224–252	227–248	0.730	0.109	0.513
Cm3†	56	AATACTACCATGAGATGGGATGTG ATTCTTTTCTCCATAAACAAGGCC	169–187	176–182	0.507	0.253	0.341
Cm84†	56	TGTTTTGACATTAGTCCAGGATTG ATTGTTATAGCCTATTGTTCAGGA	348–354	355–407	0.749	0.099	0.534
Ei8†	60	ATATGATTAGGCAAGGCTCTCAAC AATCTTGAGATTGGCTTAGAAATC	192–254	237–299	0.488	0.357	0.210
Dc99‡	56	CACCCATTTTTTCCCATTG ATTTGAGCATAACTTTTCGTGG	130–140	129–142	0.775	0.086	0.562

PCR primer sequences, annealing temperature and product sizes are indicated. Heterozygosity, probability of observing two individuals sharing the same genotype, and probability of detection of multiple paternity were calculated as described in Materials and methods.
*H = heterozygosity, q = probability that two random individuals share the same genotype at one locus, d = probability of detection of multiple paternity at one locus.
†Fitzsimmons *et al*. 1995; ‡Dutton 1995.

polyacrylamide gels. Typically, products for nestmates and their mothers were run on the same gel which also included a size standard (ΦX 174/*Hae*III DNA Marker, Promega). We ran gels at 60 W for 2.5–3.5 h, fixed them in 12% methanol–acetic acid solution for 30 min and dried them at 70 °C under vacuum. Gels were exposed to X-ray film (BioMax MR Kodak; Rochester, NY) for 1–2 days at either room temperature or –80 °C. We scored autoradiographs manually. DNA from all individuals displaying unanticipated genotypes was re-amplified and re-scored for confirmation. If an aberrant allele could not be confirmed, that genotype was eliminated from the analysis. Allele sizes were determined by comparison with the size marker using the Kodak 1D Image Analysis Software.

Statistical analysis

We assessed compliance to Hardy–Weinberg equilibrium, allele frequency distribution, and genotypic disequilibrium for 36 adult females using the software GENEPOP version 3.1d (Raymond & Rousset 1995). The Markov chain method was used to estimate probability values for Hardy–Weinberg equilibrium. Probability values greater than 0.05 indicated no significant deviations from the null hypothesis of equilibrium. For the purpose of determining the probability of detecting multiple paternity, we assumed that the allele frequency distribution in males was similar to that in females. We calculated the proportion of heterozygotes for a specific locus (heterozygosity) using the equation given by Ott (1991), $H = 1 - \Sigma(p_i^2)$, where p is the frequency of the ith allele for n alleles. To calculate the probability that two unrelated individuals shared a common genotype at a single locus, we utilized the following formula: $q = \Sigma(p_i^2)^2 + \Sigma(2p_ip_j)^2$ (Hanotte *et al*. 1991). We calculated the probability that two unrelated individuals shared a common genotype across several loci (Q) by multiplying the probabilities derived for each locus (Hanotte *et al*. 1991). We calculated the probability of detecting multiple paternity at a single locus (d) using the following formula: $d = 1 - 2a_a + a_3 + 3(a_2a_3 - a_5) - 2(a_2^2 - a_4)$, where $a_n = \Sigma p_i^n$ (Westneat *et al*. 1987). The probability of detecting multiple paternity across all loci (D) was given as $D = 1 - \Pi(1 - p_i)$. The last two equations are based on Hardy–Weinberg equilibrium predictions of allele frequencies in a given population (Westneat *et al*. 1987).

We also estimated the probability of finding two individuals in a group of N individuals with identical genotypes at all three loci by Monte Carlo simulation. Genotypes for N individuals were generated by selecting alleles randomly and independently (assuming Hardy–Weinberg equilibrium at each locus and no linkage between loci) based on the allele frequencies observed in the 36 females. In each group of N individuals, we recorded (i) whether two or more individuals shared the same genotype at all three loci, (ii) the number of individuals whose genotype matched another in the group, and (iii) the number of different genotypes in the group that match other genotypes in the group. Each simulation was based on 2×10^5 simulated groups. The probability of finding shared genotypes in a group was the number of groups in which two or more individuals shared genotypes divided by the total number of groups.

To assess whether any loci were linked on the same chromosome, we calculated the likelihood of observing a particular configuration of alleles within a family if the loci

were unlinked (the null hypothesis of free recombination) and compared it with the likelihood of observing the same configuration if the two loci were linked at a range of recombination fractions. The $\log_{10}$ of the odds for linkage, or the lod score, as originally defined by Morton (1955) is represented as $z(x)$ in the following formula:

$$z(x) = \log_{10}\{[(\theta)^{R}(1-\theta)^{NR}] + [(\theta)^{NR}(1-\theta)^{R}]\}/2(0.5)^{R+NR}$$

where θ = recombination fraction, R = number of recombinants observed and NR = number of nonrecombinants observed.

We tested for deviations from the expectation of Mendelian inheritance at one locus in each family using a χ^2 test. To account for multiple testing, we applied the Bonferroni correction, thus accepting $P < 0.001$ as statistically significant, and rejected the null hypothesis of no deviation from Mendelian inheritance.

Results

We genotyped 36 female leatherbacks at five microsatellite loci (Table 1). Allele size range agreed with that in other turtle species except for loci Ei8 which was 45 bp larger in *Dermochelys coriacea* than in *Eretmochelys imbracata*, and Cm84 which had a broader size range than in *Chelonia mydas*. The number of alleles varied from three (locus Cm3) to 12 (locus Ei8) (Fig. 1), and heterozygosity values ranged from 0.488 (locus Ei8) to 0.775 (locus Dc99, Table 1). The probability of two individuals sharing the same genotype at a single locus ranged from 0.086 to 0.357, and the probability of detecting multiple paternity at a single locus ranged from 0.21 to 0.562 (Table 1). All allele frequencies were in Hardy–Weinberg equilibrium except for Cm84 ($P = 0.010$) for which there was a deficit of heterozygotes ($P = 0.001$). We used Cc117, Ei8 and Dc99 to determine genotypes of the hatchlings because Cm3 showed only

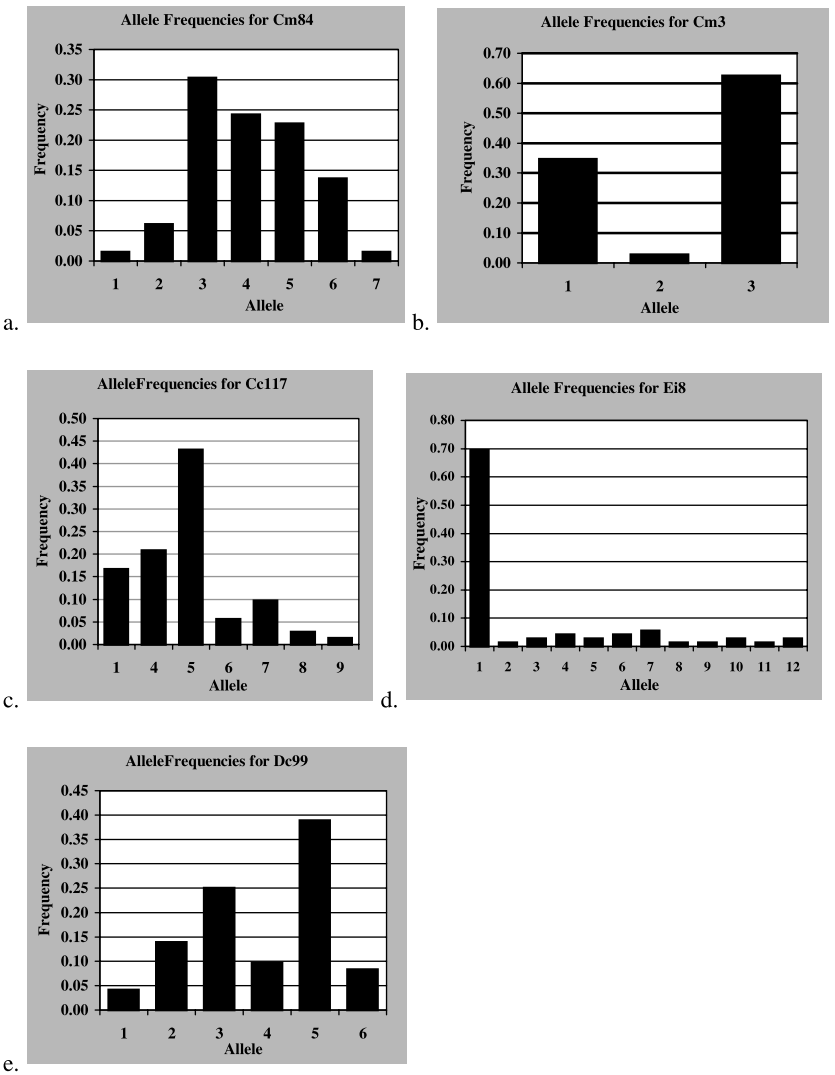

Fig. 1 Allele frequencies for five microsatellite loci observed in 36 adult female leatherbacks from Playa Grande, Costa Rica.

three alleles and Cm84 deviated from Hardy–Weinberg equilibrium.

We calculated the probabilities of individuals sharing the same genotypes at various combinations of loci and of detecting multiple paternity across combinations of loci (Table 2). The probability of detecting multiple paternity using all three loci was 0.85, and the probability that two randomly chosen individuals would share the same three-locus genotype was 0.0027. Pairwise linkage disequilibrium using Fisher's exact method indicated no significant deviation from independent assortment (Table 2). We also performed linkage analysis on pairs of loci in three clutches of 20 hatchlings in which the maternal and paternal alleles were unambiguous (Table 3). Lod scores of less than – 2.0 are significant for no linkage between loci.

We determined the genotypes of at least 20 hatchlings from both the first and last clutches of 20 females at loci Cc117 and Ei8. At least one clutch of 20 hatchlings from each female was also tested for Dc99. For families exhibiting more than two nonmaternal alleles at any locus or combination of loci, we analysed Dc99 in all clutches of that family. Because there was a possibility of internesting matings, we genotyped hatchlings from three or four clutches of seven of 20 females. In total, we obtained 973 hatchling genotypes at Cc117, 957 at Ei8, and 676 at Dc99.

Following simple autosomal Mendelian inheritance, a hatchling's genotype at any locus will consist of one allele from its mother and the other from its father. Thus, the genotype distribution among the hatchlings should approximate a frequency of 0.25 for each of the four possible combinations of alleles. Because the maternal genotypes were tested directly, we could, in most cases, unambiguously determine the maternal alleles in each hatchling. Paternal alleles were inferred from hatchling genotypes after the maternal alleles were excluded. In the event that the exact parent contributing an allele to a particular hatchling was ambiguous (i.e. when a hatchling had the same genotype as the mother), we adopted a conservative method of assigning paternal alleles based on the other genotypes observed in hatchlings of that clutch. We interpreted the presence of one paternal allele as a single homozygous father and the presence of two inferred paternal alleles as a single heterozygous father.

Twelve (60%) of the 20 genotyped families demonstrated only two paternal alleles (data available at http://www.leatherback.org). Two families (10%) demonstrated at least three paternal alleles at one or more loci, and multiple individuals with the same third unambiguous paternal allele at a locus occurred (Family CC and R, Table 4). Family CC exhibited at least three paternal alleles at all three loci, and a fourth paternal allele in three individuals at one locus (Ei8). Family R had three inferred paternal alleles at Cc117 represented in multiple hatchlings in all tested clutches, and two hatchlings that did not have the observed maternal allele at this locus. One of these hatchlings had a unique third paternal allele at locus Dc99 as well. In both families CC and R, there was significant deviation from the expectations of Mendelian inheritance.

Six families (N, S, T, W, Z and EE; Table 4) deviated from the expectations of Mendelian inheritance because a third paternal allele occurred with low frequency. Two hatchlings from clutches 1 and 5 of family T each had a different extra nonmaternal allele at the Ei8 locus. (All individual genotype data by clutch are available http://www.leatherback.org) There were two hatchlings in clutch 5 of family Z who each had a different extra allele at the Ei8 locus. Families W and EE each had a single hatchling with an extra nonmaternal allele at Ei8. The same hatchling in family EE also had a unique genotype at locus Dc99. A single hatchling in family S did not have either of the expected maternal alleles at Cc117, and family N had two hatchlings that did not have an expected maternal allele at Ei8. Hatchling DNA that demonstrated these inconsistencies

Table 2 Probability of two individual leatherback turtles at Playa Grande, Costa Rica sharing genotypes (*Q*) and of finding multiple paternity (*D*) using various combinations of loci

Locus groupings	*Q*	*D*	Linkage disequilibrium (*P* value)‡
Cc117 & Ei8	0.028	0.680	0.845
Cc117 & Dc99	0.011	0.773	0.931
Ei8 & Dc99	0.025	0.693	0.371
Cc117, Ei8, & Dc99	0.0027	0.851	

*Q = Probability that two random individuals share the same genotype across a combination of loci.
†D = probability of detecting multiple paternity across a combination of loci.
‡Significant linkage disequilibrium indicated by $P < 0.05$.

Table 3 Linkage results between pairs of loci for leatherback turtles at Playa Grande, Costa Rica

θ*	Cc117 vs. Dc99 Lod†	Cc117 vs. Ei8 Lod	Ei8 vs. Dc99 Lod
0.001	–21.3	–18.3	–15.6
0.01	–12.3	–10.3	–8.6
0.02	–9.6	–7.9	–6.6
0.05	–6.3	–4.9	–3.9
0.10	–3.8	–2.8	–2.1
0.20	–1.3	–1.0	–0.6

*θ = recombination distance between two loci. 0.5 = free recombination.
†The lod score is defined as the $\log_{10}$ of the likelihood ratio of observing the results if two loci are linked vs. not linked.

Table 4 Genotypes* for females and hatchlings and inferred alleles for males at three microsatellite loci; data from families that demonstrated deviation from the expectations of Mendelian inheritance are presented

	Maternal genotypes			Hatchling genotypes (*n*)			Inferred paternal alleles		
Family†	Cc117	Ei8	Dc99	Cc117	Ei8	Dc99	Cc117	Ei8	Dc99
H (3,60)	4/7	1/3	3/4	4/4 (17) 4/5 (16) 4/7 (15) 5/7 (12)	1/10 (7) 1/12 (14) 3/10 (27) 3/12 (9)	1/3 (12) 1/4 (18) 3/6 (17) 4/6 (13)	4,5	10,12	1,6
I (2,40)	5/5	1/1	4/5	4/5 (20) 5/5 (20)	1/1 (33) 1/5 (7)	3/4 (7) 3/5 (3) 4/5 (4) 5/5 (6)	4,5	1,5	3,5
N (2,40)	5/6	1/10	5/6	1/5 (11) 1/6 (12) 5/6 (12) 6/6 (5)	1/1 (6) 1/7 (9) 1/10 (15) 7/10 (8) 7/11 (2)	2/5 (4) 2/6 (6) 5/6 (3) 6/6 (7)	1,6	1,7	2,6
R (4,80)	1/1	1/4	2/5	1/4 (20) 1/5 (17) 1/7 (40) 2/5 (1) 4/5 (1)	1/1 (34) 1/4 (46)	2/3 (7) 2/5 (29) 3/5 (10) 5/5 (33) 5/6 (1)	4,5,7	1	3,5,6
S (2,40)	5/7	1/1	3/5	4/5 (5) 4/7 (9) 5/5 (10) 5/7 (15) 4/6 (1)	1/1 (39)	3/3 (5) 3/5 (5)	4,5	1	3 or 5
T (3,52)	4/5	1/11	4/5	4/4 (13) 4/5 (30) 5/5 (8)	1/1 (33) 1/11 (17) 1/12 (1) 1/9 (1)	3/4 (8) 3/5 (14) 5/5 (13) 4/5 (10)	4,5	1,9,12	3,5
W (2,40)	4/4	5/6	3/5	4/5 (40)	1/5 (10) 1/6 (13) 5/11 (9) 6/11 (7) 6/13 (1)	2/3 (6) 2/5 (16) 5/6 (8) 3/6 (6)	5	1,11,13	2,6
Z (4,80)	4/7	1/10	2/5	4/5 (25) 4/6 (20) 5/7 (23) 6/7 (9)	1/1 (45) 1/10 (33) 1/5 (1) 1/7 (1)	2/2 (23) 2/3 (20) 2/5 (18) 3/5 (19)	5,6	1,5,7	2,3
CC (4,88)	1/1	1/1	3/5	1/4 (24) 1/5 (39) 1/8 (22)	1/1 (38) 1/6 (31) 1/12 (16) 1/7 (3)	2/3 (7) 2/5 (14) 3/5 (26) 3/6 (8) 5/5 (23) 5/6 (10)	4,5,8	1,6,7,12	2,5,6
EE (2,40)	4/4	1/1	2/3	4/4 (13) 4/5 (27)	1/1 (24) 1/3 (13) 1/9 (1)	2/5 (17) 3/5 (20) 2/3 (1)	4,5	1,3,9	2 or 3,5

*Alleles are designated by consecutive numbers beginning with the smallest allele as 1.
†Each family is designated by a letter(s); the number of clutches and the number of hatchlings are given in parentheses, respectively.

was re-isolated and the PCR was repeated, but the same results were observed.

Two families (H and I) displayed only two paternal alleles, but the observed frequencies of hatchling genotypes deviated significantly from the expectation of Mendelian inheritance ($P < 0.001$). Both families had skewed genotype frequency distributions at locus Ei8 (Table 4).

From the hatchling genotypes we could unambiguously infer the genotypes of 17 males. The paternal three-locus genotypes of clutches C and E and of clutches H and K were identical (data not shown). There were no females ($n = 20$) who shared genotypes at all three loci. Simulations of genotypes at three loci based on allele frequencies observed in the group of 36 females indicated that the probability of any two individuals sharing a three-locus genotype in a group of 17 individuals was 0.294, and in a group of 20 individuals was 0.381. The probability of there being two distinct sets of matched genomes in a group of 17 was 0.034.

We sampled a total of 25 dead stage-three embryos from clutches CC4 ($n = 8$) and EE4 ($n = 17$). Genotypes of dead embryos were consistent with the genotypes of hatchlings, and are included in Table 4.

Table 5 Summary of inferred paternal haplotypes at three microsatellite loci in four clutches of leatherback turtle family CC

Paternal three-locus genotype* Cc117–Ei8–Dc99	Clutch 1	2	3	4
Father A				
5–12–2	4	3	2	2
5–12–6	0	0	4	1
5–1–2	4	0	3	3
5–1–6	0	2	4	5
Father B				
4–6–5	2	3	3	1
4–1–5	0	0	1	0
4–1–3 or 5	2	3	0	1
4–6–3 or 5	1	1	0	4
8–1–5	1	0	0	3
8–6–5	1	3	1	2
8–1–3 or 5	1	0	0	2
8–6–3 or 5	1	3	0	3
Father C, D				
5–6–5	0	1	0	0
5–1–5	1	0	0	0
4–7–3 or 5	1	0	0	1
8–7–3 or 5	1	0	0	0

*Paternal haplotypes were inferred from the hatchling genotypes after the maternal haplotypes were excluded.

Discussion

In this study, we determined the genotypes of nearly 1000 leatherback sea turtle hatchlings at three informative microsatellite loci. These hatchlings represented 50 clutches laid by 20 females at Playa Grande on the Pacific Coast of Costa Rica during the 1998–99 nesting season. This is the largest single study of paternity in leatherbacks to date (Rieder 1996; Curtis 1998; Dutton *et al*. 1998).

Heterozygosity values for the three loci examined in the nesting leatherback population ($n = 36$) were lower at locus Ei8 (0.507 vs. 0.847), similar at locus Cc117 (0.730 vs. 0.754) and higher at locus Dc99 (0.749 vs. 0.639) than for the stable leatherback population on the Caribbean coast of Costa Rica (Curtis 1998). Global values of heterozygosity for leatherbacks (FitzSimmons *et al*. 1995) were considerably lower than those estimated here, possibly because of our larger sample size. Dutton (1995) estimated heterozygosity values for Dc99 to be 0.594 for leatherback populations globally and 0.647 for the Pacific Costa Rican leatherbacks.

Paternity

The null hypothesis of single paternity could not be rejected in 12 of the 20 families**.** In the remaining eight families, six (CC, R, T, W, Z, EE) demonstrated more than two paternal alleles and two families (N and S) had single hatchlings that lacked an observed maternal allele.

There are two potential sources of observing more than two nonmaternal alleles: multiple paternity and mutation. We accepted the hypothesis of multiple paternity in families CC and R that demonstrated three or more paternal alleles at one or more loci. There was a contribution of both fathers in each of these two families in successive clutches suggesting that two successful matings occurred prior to the beginning of the nesting season, and that sperm from both fathers was stored.

Inspection of the paternal genotype contribution to hatchlings from family CC suggests that there was a third male contributing to this family (Table 5). From the number of hatchlings with each paternal allele combination, we can infer that male A had the following three-locus genotype: 5/5, 1/12, 2/6. Similarly, male B had a three locus genotype of 4/8, 1/6, 5/3, or 5. However, there were five hatchlings with four paternally contributed genotypes that suggest a third and possibly a fourth male (bottom group in Table 5). Three of these five hatchlings were from the first clutch laid by female CC. Since this turtle was a remigrant, it is possible that there was sperm remaining from the previous mating season. The minor representation of the putative third male is consistent with a time-dependent reduction in sperm viability (Ewing 1943), sperm competition (Martin *et al*. 1974; Thornhill & Alcock 1983), or with a second mating during the season.

If we are less conservative in acceptance of multiple paternity, we could include families T, W, Z and EE that each have a low number of hatchlings with extra paternal alleles. However, the pattern of multiple paternity in these families would be highly skewed in favour of one male. Pearse *et al*. (2001) reported that the rate of multiple paternity in the painted turtle (*Chrysemys picta*) was 13.2% and that the contribution of the second father ranged from nearly equal to highly skewed. Since we have sampled many more hatchlings than previous studies of the leatherback, this may be the first report of a second pattern of multiple paternity. To investigate this further, genotyping at additional loci would be necessary.

On the other hand, these rare hatchling genotypes, together with those genotypes that fail to include an observed maternal allele, may represent mutations. The range of mutation rate would be from 0 (if rare events do in fact represent multiple paternity) to 4.2×10^{-3} (if all rare events are mutations). The range of mutation for microsatellites in general reported by Edwards *et al*. (1992) and by Fitzsimmons (1998) for these loci in the green turtle is 5.7×10^{-4}–9.6×10^{-3}. Thus, the maximum mutation rate calculated here is within that range.

Seventy per cent of the rare hatchling genotypes occurred at locus Ei8, suggesting that, although this locus has the lowest heterozygosity value, the larger alleles may be hypervariable. The sequence of the Ei8 PCR product (GenBank accession #L42236) from *Eretmochelys imbricata* indicates several stretches of different simple sequence repeats, thus having more than one block potentially capable of variation. Types of mutations that might be observed include those that prevent amplification of an allele (i.e. a null mutation, Pemberton *et al*. 1995) and those that alter the number of repeats by a discrete number. Our methodology did not identify null alleles, and there did not appear to be a heterozygote deficiency for the three loci in the population of 36 females (Table 1) (Gullberg *et al*. 1997). In general, microsatellite repeats mutate by gaining or losing a single or a few repeats/mutation event (Schlötterer & Pemberton 1994), and the probability of mutating to an allele differing by one repeat is greater than the probability of mutating to an allele differing by multiple repeats (Kichler *et al*. 1999). Of the 13 putative mutations we observed, six differed by one repeat, one differed by two repeats and five involved more than two repeats.

Two observations suggest that two females may mate with the same male (polygyny). First, the frequency of matched genotypes among 36 females was 0 whereas among the 17 males for which a three-locus haplotype could be unambiguously inferred it was 11.7%. Secondly, simulations indicated that among the females we should expect a match 38% of the time in a single group of 20 genotypes. The probability of observing two distinct matches in the set of 17 inferred male genotypes is only 3.4%. Families C and E shared paternal alleles 4 and 7 at locus Cc117 which have a frequency of 0.208 and 0.097, respectively, in the population. Families H and K shared paternal alleles 10 and 12 at locus Ei8 which have a population frequency of 0.028 and alleles 1 and 6 at locus Dc99 which have population allele frequencies of 0.042 and 0.083, respectively. Given the rarity of these specific alleles in the population, it is probable that these familial groups were fathered by the same male. Rieder (1996) also found that the clutches of two different females at Playa Grande exhibited the same paternal genotypes at two loci. We suggest that this indicates a moderate rate of polygyny in this population of leatherbacks.

Polygyny may be a result of a female-biased sex ratio. Leatherbacks experience temperature-dependent sex determination and at Playa Grande there is a female-biased sex ratio among hatchlings (Binckley *et al*. 1998). This bias has been in place for 50 years (Binckley, unpublished), and the current breeding sex ratio may also be female-biased. There was no indication of polygyny in Caribbean leatherbacks (Curtis 1998). This may be because of the stability of the Caribbean leatherback population and the more balanced sex ratio of its hatchlings (Leslie *et al*. 1996).

Families H and I did not demonstrate contribution from a second father, but did deviate from the expected genotype frequencies of Mendelian inheritance, and thus may illustrate the potential for differential fitness of sperm, oocyte or zygote. Such a phenomenon would result if a deleterious allele for a coding gene was transmitted in conjunction with the assayed marker loci (Ei8 in both cases).

Sperm storage

Since we tested multiple clutches, and in some cases four successive clutches, from each of 20 nesting females, we could evaluate whether there was sperm storage. The sampling effort in this study represented the majority of the reproductive output of these females because Playa Grande leatherbacks average 4.3–7.9 clutches per season (Reina *et al*. in press). In every family, the same paternal alleles were present in every clutch examined, with the exception of minor alleles seen only once or twice. Therefore, our data indicated that paternity did not change over the entire nesting season for any female sampled, although the quantitative contribution from sperm of multiple fathers may alter from clutch to clutch. For example, clutch 3 of family CC showed greater contribution from the father with three-locus genotype 5/5–1/12–2/6, whereas clutch 2 had greater contribution from the father with genotype 4/8–1/6–3(or 5)/5 (Table 5). Rieder (1996) reported the same paternity for two clutches of a female leatherback at Playa Grande during the 1995/96 nesting season. Caribbean leatherbacks also exhibit the same paternity across multiple clutches laid by individual

females (Curtis 1998). These data are consistent with the hypothesis that mating did not occur between nesting events. The alternative possibility of multiple matings with the same male is unlikely. This would require stringent pair bonds to explain the general pattern of single paternity as well as mating with the same two males for the cases of multiple paternity. A second possibility to explain these data is that a female mated with two males of the same genotype. We consider this unlikely because the probability that two individuals would share the same genotype is 0.0027. The data are consistent with sperm storage, as has been demonstrated in other turtles (Ewing 1943; Galbraith 1993). This adaptation may provide reproductive assurance that clutches will be fertilized in the event that females are unable to locate a mate between clutches (Galbraith & Brooks 1987).

We conclude from this study that there is sperm storage in nesting female leatherbacks and that mating occurs prior to the beginning of the nesting season. There is a low rate of multiple paternity (10%) in which both males contribute equally to the gene pool. There may be a low rate of multiple paternity in which a second male contributes a minor portion of the hatchling gene pool. However, this pattern is not distinguishable from anomalies or mutations. Thus, we conclude that only 10% of the females in this study exhibited polyandrous behaviour. The greater frequency of matched genomes among the males is consistent with a small amount of polygyny in this population.

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References

Binckley CA, Spotila JR, Wilson KS, Paladino FV (1998) Sex determination and sex ratios of Pacific leatherback turtles, *Dermochelys coriacea*. *Copeia*, **1998**, 291–300.

Bollmer JL, Irwin ME, Rieder JP, Parker PG (1999) Multiple paternity in loggerhead turtle clutches. *Copeia*, **1999**, 475–478.

Carr A, Carr N (1986) *Dermochelys coriacea* (leatherback sea turtle) copulation. *Herpetological Review*, **17**, 24–25.

Curtis C (1998) Genotyping with polymorphic markers reveals multiple paternity in leatherback turtle clutches. Master's Thesis, Drexel University, Philadelphia, PA, USA.

Dutton PH (1995) Molecular evolution of sea turtles with special reference to the leatherback *Dermochelys coriacea*. PhD Dissertation, Texas A & M University.

Dutton PH, Bixby E, Davis SK (1998) Tendency towards single paternity in leatherbacks detected with microsatellites. In: *Proceedings of the 18th International Symposium on Sea Turtle Biology and Conservation* (eds Abreu-Grobois FA, Briseno-Duenas R, Marquez-Millian R, Sarti-Martinez L), p. 39. NOAA Technical Memorandum NMFS-SEFSC-436. National Marine Fisheries Service, Miami, FL, USA.

Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R (1992) Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. *Genomics*, **12**, 241–253.

Ewing HE (1943) Continued fertility in female box turtles following mating. *Copeia*, **1943**, 112–114.

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

FitzSimmons NN, Moritz C, Moore SS (1995) Conservation and dynamics of microsatellite loci over 300 million years of marine turtle evolution. *Molecular Biology and Evolution*, **12**, 432–440.

Galbraith DA (1991) Studies of mating systems in wood turtles (*Clemmys insculpta*) and snapping turtles (*Chelydra serpentina*) using DNA fingerprinting. PhD Dissertation, Queen's University, Kingston, Ontario, Canada.

Galbraith DA (1993) Review: Multiple paternity and sperm storage in turtles. *Herpetological Journal*, **3**, 117–123.

Galbraith DA, Brooks RJ (1987) Survivorship of adult females in a northern population of common snapping turtles, *Chelydra serpentina*. *Canadian Journal of Zoology*, **65**, 1581–1586.

Galbraith DA, White BN, Brooks RJ, Boag PT (1993) Multiple paternity in clutches of snapping turtles (*Chelydra serpetina*) detected using DNA fingerprints. *Canadian Journal of Zoology*, **71**, 318–324.

Gibson AR, Falls JB (1975) Evidence for multiple insemination in the common garter snake, *Thamnophis sirtalis*. *Canadian Journal of Zoology*, **53**, 1362–1368.

Gullberg A, Olsson M, Tegelström H (1997) Male mating success, reproductive success, and multiple paternity in a natural population of sand lizards: behavioural and molecular genetics data. *Molecular Ecology*, **6**, 105–112.

Hanotte O, Burke T, Armour J, Jeffreys AJ (1991) Hypervariable minisatellite DNA sequences in the Indian pea fowl *Pavo cristatus*. *Genomics*, **9**, 587–597.

Harry JL, Briscoe DA (1988) Multiple paternity in the loggerhead turtle (*Caretta caretta*). *Journal of Heredity*, **79**, 96–99.

Kichler K, Holder MT, Davis SK, Marquez R, Owens DW (1999) Detection of multiple paternity in the Kemp's ridley sea turtle with limited sampling. *Molecular Ecology*, **8**, 819–830.

Leslie AJ, Penick DN, Spotila JR, Paladino FV (1996) Leatherback turtle, *Dermochelys coriacea*, nesting and nest success at Tortuguero, Costa Rica, in 1990–91. *Chelonian Conservation and Biology*, **2**, 159–168.

Martin PA, Reimers TJ, Lodge JR, Dziuk PK (1974) The effect of ratios and numbers of spermatozoa mixed from two males on the proportion of offspring. *Journal of Reproductive Fertility*, **39**, 251–258.

Morton NE (1955) Sequential tests for the detection of linkage. *American Journal of Human Genetics*, **7**, 277–318.

Ott J (1991) *Analysis of Human Genetic Linkage*. The Johns Hopkins University Press, Baltimore.

Pearse DE, Janzen FJ, Avise JC (2001) Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles. *Heredity*, **86**, 378–384.

Pemberton JM, Slate J, Bancroft DR, Barrett JA (1995) Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, **4**, 249–252.

Raymond M, Rousset F (1995) GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Reina RD, Mayor PA, Spotila JR, Piedra R, Paladino FV (2002) Nesting ecology of the leatherback turtle, *Dermochelys coriaeca*, at Parque Nacional Marino Las Baulas, Costa Rica: 1988–89–1999–2000. *Copeia*, **2002**, 653–664.

Rieder JP (1996) Mating systems and conservation biology: microsatellite analysis of paternity in leatherback sea turtle clutches. Master's Thesis, Ohio State University, Columbus, OH, USA.

Schlötterer C, Pemberton J (1994) *The Use of Microsatellites for Genetic Analysis of Natural Populations*. In: *Molecular Ecology and Evolution: Approaches and Applications* (eds Schierwater B, Streit B, Wagner GP, DeSalle R), pp. 203–214. Birkhäuser. Basel.

Spotila JR, Dunham AC, Leslie AJ *et al*. (1996) Worldwide population decline of *Dermochelys coriacea*: are leatherback turtles going extinct? *Chelonian Conservation and Biology*, **2**, 209–222.

Spotila JR, Reina R, Steyermark AC, Plotkin PT, Paladino FV (2000) Pacific leatherback turtles face extinction. *Nature*, **405**, 529–530.

Steyermark AC, Williams K, Spotila JR *et al*. (1996) Nesting leatherback turtles at Las Baulas National Park, Costa Rica. *Chelonian Conservation and Biology*, **2**, 173–183.

Tegelström H, Höggren M (1994) Paternity determination in the adder (*Vipera berus*)—DNA fingerprinting or random amplified polymorphic DNA? *Biochemical Genetics*, **32**, 249–256.

Thornhill R, Alcock J (1983) *The Evolution of Insect Mating Systems*. Harvard University Press, Cambridge, MA.

Westneat DF, Frederick PC, Wiley RH (1987) The use of genetic markers to estimate the frequency of successful alternative reproductive tactics. *Behavioral Ecology and Sociobiology*, **21**, 35–45.

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