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SHORT COMMUNICATION

Multiple paternity in egg clutches of hawksbill turtles (*Eretmochelys imbricata*)

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Abstract We present the first data collected on the genetic mating system of the hawksbill turtle *Eretmochelys imbricata*, the only marine turtle not studied to date. We examined paternity within 12 egg clutches from ten female hawksbill turtles from Sabah Turtle Islands, Malaysia. A total of 375 hatchlings were analysed using five microsatellite markers. Results demonstrated that clutches from two out of ten females were sired by multiple males (maximum of two). Although at a low frequency, observation of multiple paternity indicates that hawksbill turtles exhibit the same genetic mating system (polyandry) as observed for other species of marine turtles. Consistent paternity across multiple clutches laid by individual females in one breeding season supports the hypothesis that sperm are stored from mating prior to nesting and are then used to fertilize all subsequent clutches of eggs that season.

Keywords *Eretmochelys imbricata* · Hawksbill turtle · Multiple paternity · Microsatellites · Marine turtles · Endangered species

Introduction

The investigation into mating systems of reptiles has advanced substantially over the past decade (e.g. Madsen et al. 1992; Davis et al. 2001; and Pearse et al. 2001). In marine turtles, a number of studies using molecular genetic methods have indicated that the frequency of multiple mating and subsequent multiple paternity varies from species to species and from location to location (e.g. FitzSimmons 1998; Kichler et al. 1999; Hoekert et al. 2002; Theissinger et al. 2009). Knowledge of the reproductive biology and mating systems of endangered species is important to understand the effect that conservation strategies may have on the preservation of genetic variation. To date, however, no studies of mating patterns have been conducted on the hawksbill turtle *Eretmochelys imbricata* (Linnaeus 1776).

The hawksbill turtle is listed as Critically Endangered in the IUCN Red List Categories (IUCN, 2010), and is listed in Appendix I of CITES (Groombridge and Luxmore 1989). The main threat faced by this animal is the intentional harvest of their meat, eggs and shell for international trade. A complete knowledge of the mating system of this species is important, but most studies have been hampered by small local population sizes.

Gulisaan, one of the three Turtle Islands in Sabah, Malaysia, provides a nesting habitat to the largest remaining hawksbill turtle population in Southeast Asia (Chan and Liew 1996). Current nestings of hawksbill turtles stand at over 600 clutches per year (Chan et al. 1999), almost double the levels recorded in the mid 1980s, a recovery attributed to conservation measures by the Sabah Government. This island was chosen as our study site as it represents one of the few sites where in-depth studies of hawksbill mating system can be made. The main objectives of this study were to: (i) conduct a first genetic test of mating patterns in hawksbill turtles; (ii) determine if multiple paternity occurs in egg clutches of hawksbill turtle breeding in the Sabah Turtle Islands, Malaysia; and

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(iii) test the hypothesis that sperm is stored during mating prior to nesting.

Materials and methods

Field sampling

Samples were collected from Gulisaan (6°09′N, 118°03′E), from March to September 2004. The incidence of multiple paternity within clutches of hawksbill turtle was assessed by sampling of known females and their clutches. Blood samples (1–2 ml) from ten nesting females, after egg laying had finished, were collected from the dorsal cervical sinus following the method described by Dutton (1996) and stored in lysis buffer. As part of the long-term conservation programme by Sabah Parks, all clutches were relocated to a beach hatchery and placed in nest chambers dug to resemble natural nests. Hatchlings emerged after 45–60 days. Between 14 and 40 hatchlings were chosen randomly from each nest. Not more than 0.1 ml blood was taken from the dorsal cervical sinus of the hatchlings using a 1 cc disposable insulin syringe. The hatchlings were released immediately after blood collection. Multiple clutches laid within a nesting season were obtained only from two females, H6 and H10, each with 2 laying events separated by 43 and 30 days respectively.

Microsatellite analysis

DNA was extracted using a CTAB protocol (Bruford et al.1992). Genotype profiles of females and their clutches were obtained for five microsatellite loci: Ei8, Cm58, Cm72 and Cc7 (FitzSimmons et al. 1995), plus nCm84 (FitzSimmons *pers. comm.*) a shorter version of Cm84 (FitzSimmons et al. 1995). Polymerase chain reactions to amplify microsatellite regions were performed using a MJ Research DNA Engine in 10 µl volumes containing 25–50 ng of genomic DNA, 0.2 U *Taq* DNA polymerase (Bioline), 1 × PCR buffer, 1–1.5 mM $MgCl_2$, 0.125 mM of each deoxynucleotide triphosphate (dNTPs mix, Bioline) and 0.2 µM of each primer (forward primer 5′ end-labelled with a Cy5 fluorescent dye group). Cycling parameters consisted of an initial denaturation at 94°C for 60 s, followed by 30–35 cycles of denaturation at 94°C for 30 s, an optimal annealing temperature for 30 s, extension at 72°C for 30 s, followed by a final elongation step at 72°C for 2 min. Following PCR, amplified products were resolved on 6% denaturing polyacrylamide gels run on an ALFexpress II^{TM} (Amersham Pharmacia Biotech) automated sequencer, product size being determined against internal standard size markers using Fragment Manager v1.2 (Amersham Pharmacia Biotech). Products from adult females were run adjacent to samples of their offspring. Any samples displaying unexpected alleles were re-amplified and re-run for confirmation.

Data analysis

Genotype data from nesting females at each locus were checked for the presence of null alleles using MICRO-CHECKER (Oosterhout et al. 2004), and tested for departure from Hardy–Weinberg equilibrium and for genotypic linkage disequilibrium using GENEPOP (Raymond and Rousset 1995). Maternal genotypes were determined directly from the sampled female, and maternally-derived alleles were identified in offspring genotypes. Paternal alleles were inferred from offspring genotypes once maternal alleles were accounted for. To assess the number of fathers in a clutch, a multi-locus approach was used to reconstruct the paternal genotypes and therefore assign individual offspring to individual males (DeWoody et al. 2000). For confirmation of paternal genotypes, maternal and offspring genotypes were also analysed using GERUD 2.0 (Jones 2005). To test for ability to detect multiple paternity, mean relatedness within clutches was calculated using MER (Wang 2004) and used to estimate effective number of mates (M_e—after Bretman and Tregenza 2005).

Results and discussion

Marker analysis

All five loci were highly polymorphic, with 3–12 alleles, and expected heterozygosity from 0.67 to 0.88 (Table 1). No loci exhibited significant departure from Hardy–Weinberg equilibrium ($P > 0.05$), and no linkage disequilibrium was detected between loci. Null alleles were not indicated at any of the five loci used.

Table 1 Summary statistics for five polymorphic microsatellite loci used for paternity analysis in hawksbill turtle. H_E = expected heterozygosity

Locus	Number of alleles	H_E	Exclusion probability (one parent known with certainty)
Ei8	10	0.78	0.68
Cm58	6	0.74	0.49
Cm72	3	0.67	0.42
nCm84	12	0.88	0.76
Cc7	9	0.87	0.6
Multi-locus	8	0.79	0.99

Paternity analysis

The identity of the nesting females, and thus their genotypes, were known which allowed for unambiguous identification of maternal alleles in all hatchlings. Reconstruction of paternal genotypes within clutches using multilocus parsimony (confirmed by outcomes in GERUD 2.0 and MER) identified that clutches from eight of the ten genotyped hawksbill families were sired by a single male, whereas only two families were sired by multiple males, in each case by two males (Table 2). Twelve different male genotypes were identified as contributing to the offspring of the ten females tested, with none of the inferred paternal genotypes common to more than one family (Table 2). With the loci and sample sizes used there is high confidence that the detection of multiple paternity is accurate: allele frequencies calculated from adult females give a 5–locus exclusion probability, with one parent known, of 0.99 (GERUD 2.0); and simulation analyses (GERUDsim 2.0—Jones 2005) indicated that the contribution of multiple males would be detected in 99.9% of clutches. Although GERUDsim simulations indicated a "worst case" situation possible amongst the 12 clutches tested (15 offspring sampled, 70:30 skewed contribution from two males) would result in 68.4% of offspring successfully assigned to the correct male genotype, for the observed clutch patterns (Table 2—single paternity, or multiple paternity with 40 offspring sampled) the predicted success rate was 94.8–100%.

Our data represent the first genetic tests of paternity in hawksbill turtles. Although the number of families tested is small and from a limited part of the species distribution, which limits the conclusions that can be drawn with regard to the species as a whole, some important initial indications on mating system of hawksbill turtles can be made. The genetic data presented here support the assumption made from behavioural observations (reviewed by Witzell 1983) that at least some female hawksbill turtles mate with and produce egg clutches sired by multiple males. A low frequency of multiple paternity was observed, but this result indicates that hawksbill turtles (at least in Gulisaan) exhibit the same polyandrous mating pattern as observed for other species of marine turtles (FitzSimmons 1998; Kichler et al. 1999; Hoekert et al. 2002; Moore and Ball 2002).

Sperm storage is considered to play an important role in turtle reproduction where male and female cycles do not coincide, so that mating at the beginning of the season allows females to ensure fertilization of all clutches throughout a protracted nesting season, even if males are a limiting resource or at low population density (Galbraith et al. 1993). Such behaviour has been described in other sea turtle species by the observation of single paternity across multiple clutches laid by individual females during

Table 2 Maternal genotypes at five microsatellite loci and number of hatchlings assigned to each inferred paternal genotype within clutches from ten female hawksbill turtles from Gulisaan

Female ID	Maternal genotype					Date of nesting	Inferred paternal genotypes					No. of hatchlings assayed	Total no. of males
	Ei8	Cm58	Cm72	nCm84	Cc7		Ei8	Cm58	Cm72	nCm84	Cc7		
H1	200/234	132/132	232/232	196/196	179/181	29/04/04	212/220	132/132	232/240	182/196	181/183	40	1
H2	210/210	122/132	232/236	194/194	177/185	03/05/04	216/234	122/126	236/240	208/208	175/181	14	1
H3	202/214	122/132	236/240	180/184	181/205	06/05/04	210/214	126/128	232/232	194/200	179/185	22	1
H4	200/210	122/132	238/238	186/190	173/181	01/05/04	210/208	122/128	234/242	182/206	173/187	21	2
							214/222	122/122	234/234	190/196	183/187	19	
H5	208/208	128/142	232/236	190/198	171/183	02/05/04	214/216	122/132	232/236	194/194	179/181	40	1
H6	200/208	122/132	236/236	194/208	181/185	24/03/04	208/228	128/132	232/232	194/214	171/183	15	1
						06/05/04	208/228	128/132	232/232	194/214	171/183	40	1
H7	208/226	122/128	236/240	182/202	179/185	06/04/04	208/210	126/128	232/236	194/214	177/203	33	1
H8	208/210	122/126	232/232	188/200	181/185	09/05/04	210/228	128/132	228/240	188/196	179/181	28	2
							198/198	116/228	232/240	188/204	181/183	12	
H9	192/236	116/122	232/240	194/196	185/187	11/05/04	214/228	126/128	236/236	190/206	173/191	37	1
H10	200/244	128/132	232/236	196/202	185/185	10/06/04	212/206	122/122	236/238	188/196	181/191	29	1

Allele designations are PCR product length (bp)

the same nesting season. In our study, we sampled two successive clutches from two females (H6 and H10); in each case, we observed a high likelihood that the same male sired both clutches. Although sample numbers are low and clutches intermediate between those collected may not have been sampled this result indicates that the same males' sperm fertilized successive clutches up to 43 days apart, consistent with the hypothesis of sperm being stored from mating(s) prior to the nesting period and being used to fertilize all subsequent clutches of eggs that season without additional inter-nesting mating with new mates. Single paternity across multiple clutches laid by individual females has been observed previously in sea turtles (e.g. FitzSimmons 1998; Kichler et al. 1999).

The low level of multiple paternity observed in the Gulisaan hawksbill population could be influenced by a variety of factors such as mating behavior, reproductive cycle, the timing of female receptiveness and the abundance and sex ratio of turtles at breeding sites (FitzSimmons 1998). As this is the first study of hawksbill genetic mating patterns it is difficult to exclude female preference for single mating, although behavioural observations (Witzell 1983) do not support such preference. High levels of multiple paternity within at least some populations of other marine turtles (e.g. Lee and Hays 2004; Theissinger et al. 2009) also do not support this explanation. It is possible that low population density is affecting polyandry rates, even though the Gulisaan hawksbill breeding population is one of the largest in Southeast Asia. The level of multiple paternity observed in the hawksbill population is much lower than that observed in the green turtle (*Chelonia mydas*) population nesting in the same area of the Turtle Islands, for which 71% ($N = 14$) of clutches were fertilized by multiple males (Joseph 2006), and it is unlikely that our reported multiple paternity rate of 20% for hawksbill misrepresents a higher true rate due to limited sample size (binomial $P = 0.0016$, for observing 2/10 cases of multiple paternity when true rate is 70%). The most recent data available (2000–2004, Sabah Parks, unpublished data) indicate that the green turtle population is much larger (6,000–15,000 nesting per year) than the hawksbill population (400–600 nesting per year).

Skewed sex ratios also may be a factor affecting low polyandry rates in the Sabah Turtle Islands hawksbill population. Like many reptiles sea turtles exhibit temperature-dependent sex determination: warmer nest temperatures produce more females and cooler nests result in more males (Standora and Spotila 1985). As a conservation measure protected hatcheries have been operational at Sabah Turtle Islands since the 1960s, where egg clutches are transferred to an open beach hatchery. Studies of sex ratio of hatchlings (Tiwol and Cabanban 2000) suggest that the open beach hatchery often produced 100% female hatchlings. This practice might not have serious implications for a larger and more widespread population such as the green turtles in this area, but it may have a serious impact on a smaller and declining population such as that of the hawksbill turtle: the skewed sex ratio in beach hatchery-produced hatchlings may skew wild sex ratios and adversely affect future reproduction. Furthermore, nesting of hawksbill turtles in this area occurs predominantly on one particular island (Gulisaan). A more balanced conservation breeding technique may be desperately needed in order to save this species from extinction. Genetic testing of further hawksbill populations, both small and large, is needed to assess whether low rates of multiple paternity are the norm in this species, and to clarify which issues (behavioural flexibility, population size, sex ratios) affect mating patterns.

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