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MULTIPLE PATERNITY IN EGG CLUTCHES OF GREEN TURTLES IN REDANG ISLAND AND SABAH TURTLE ISLANDS PARK, MALAYSIA

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Abstract: The green turtle, *Chelonia mydas*, has suffered from population declines throughout its range, mainly due to a continuous over-exploitation of eggs and adults. To better understand the mating strategy of this endangered animal, paternity in egg clutches of 36 green turtles from two major rookeries in Malaysia were investigated using microsatellite markers. A high incidence of multiple paternity for the green turtles from Sabah was discovered, with 71% of egg clutches showing evidence of being sired by at least two different males. However, for the egg clutches from Terengganu, lower incidences of multiple paternity (36%) were recorded. This study also documents the occurrence of sperm storage in the green turtles from both sites. Similar patterns of paternity were observed across successive clutches, consistent with the hypothesis of sperm being stored from mating(s) prior to nesting and being used to fertilize all subsequent clutches of eggs for that season. These data provide the first examples of multiple paternity and sperm storage in the green turtle populations in Malaysia.

Keywords: Endangered species, mating, microsatellites, marine turtles, sperm storage, sustainability.

Introduction

Previous studies have reported the frequency of multiple mating in green turtles, where it varies widely between populations (Parker *et al*., 1996; FitzSimmons, 1998; Lee & Hays, 2004; Wright *et al*., 2012; Ekanayake *et al*., 2013). Variation occurs as populations within a species can have different environmental and demographic variables that influence factors such as breeding, mate availability, mate quality and mate competitiveness (Lasala *et al*., 2013). There are many explanations for why females might mate multiple times. The two broad categories of hypothesized benefits to explain why females mate with multiple males are material and genetic benefits (Walker, 1980; Reynolds, 1996). Lee & Hays (2004) could not detect the benefits of multiple mating to female green turtles and suggested that environmental factors create substantial variation in reproductive success. They suggested that multiple paternity in sea turtles is largely a result of male coercion, where females have given in to harassment as a means of reducing their overall costs. Lasala *et al*., (2013) reported that there was no relationship between hatching success and the number of fathers per clutch, suggesting that more fathers add to the variability but not to viability of hatchlings. The mating pattern of green turtle populations in Malaysia is worth investigating as it is an important component of life-history traits and provide information on the population structure relevant for conservation plans. Except for the hawksbill turtles (Joseph & Shaw, 2011), currently there is no studies on the mating pattern of the green turtles in Malaysia.

In Malaysia, population decline of turtles are attributed to the long history of egg exploitation, commercial hunting and harvesting of sea turtles at foraging grounds by illegal fishermen, incidental captures in fishing gear and loss of breeding habitats. In this study, we investigated the multiple paternity in egg clutches of green turtles from two major rookeries in Malaysia.

The Sabah Turtle Islands situated at the Sulu Sea provide an important nesting habitat for the green turtle in Southeast Asia with nesting densities for the last five years ranging from 10,000 to 15,000 per year (Sabah Parks, unpublished data). All nests at Sabah Turtle Islands were transferred to a beach hatchery since 1966. Starting 1997, the hatcheries were partially shaded to ensure the production of balanced sex ratios in the population (Tiwol & Cabanban, 2000). On the other hand, Redang Island, Terengganu which is situated in the South China Sea is an important nesting rookery for the green turtles on the Malay Peninsula with nesting densities for the last five years ranging from 1000 to 3000 per year (Terengganu Fisheries Department, unpublished data), and the only nesting beach in Malaysia that conduct *in-situ* egg incubation. In addition, Dethmer *et al*. (2006) confirmed the spatial genetic differences between the green turtle population of Sabah Turtle Islands and Redang Island.

This study used microsatellite DNA markers to document patterns of paternity within broods of the green turtles, by genotyping females and their offspring at five highly polymorphic loci. The aims of this study were to (i) determine the multiple paternity in egg clutches of green turtles from the two major breeding sites in Malaysia and (ii) to determine if the same male(s) sired successive clutches of individual females over repeated laying periods.

Materials and Methods

Sampling from Nesting Females and Hatchlings

Samples were collected at the Sabah Turtle Islands Park (6°09'N, 118°03'E) and Redang Island, Terengganu (05°49'N, 103°00E), Malaysia (Figure 1) from March 2003 to May 2004. Blood samples were collected from 36 adult females. Of these, 14 females were from Sabah and 22 females were from Terengganu. Multiple clutches laid within a nesting season were obtained from three females from Sabah (S10, S11 and S12) and four females from Terengganu (T1, T2, T4 and T8), with 2 – 6 laying events separated by 9 to 43 days. Blood samples were collected from the dorsal cervical sinus of each female after egg laying, following Joseph and Shaw (2011). For turtles not tagged from a previous nesting season, Inconel tags (style 681; National Band and Tag Co., Newport, KY, USA) were applied in the trailing edges of both front flippers for identification.

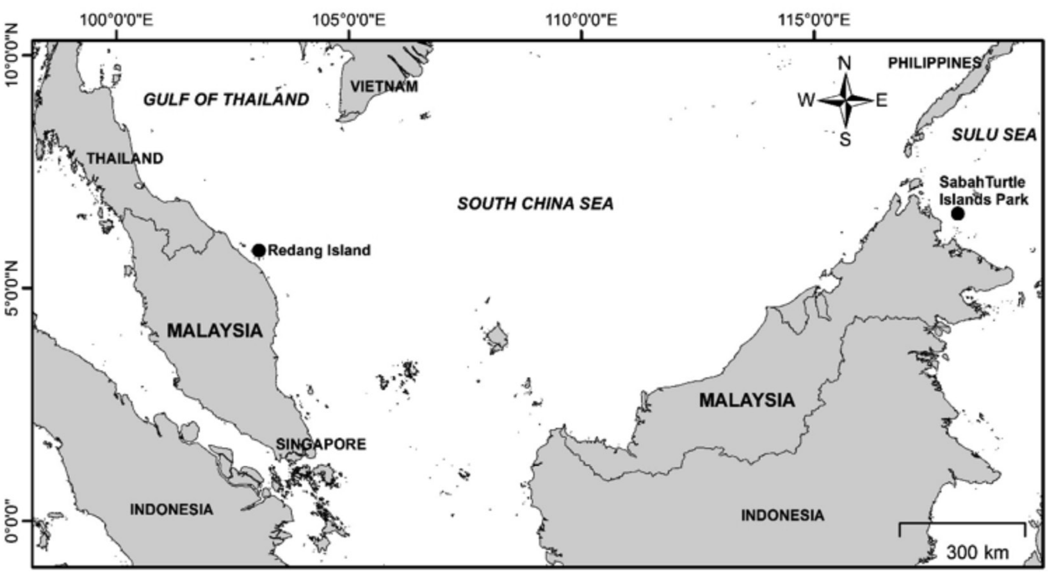

Figure 1: Map of Malaysia showing the location of sampling sites (Sabah Turtle Islands Park and Redang Island) for the paternity study

Hatchlings emerged after 45 – 60 days of incubation, and were randomly chosen from each nest. Not more than 0.1 ml blood was taken from the hatchlings' dorsal cervical sinus using 1cc disposable insulin syringe and stored in a tube containing lysis buffer (Dutton 1996). All hatchlings were released immediately after blood collection.

DNA Extraction and Amplification

Total genomic DNA was extracted using CTAB protocol (Bruford *et al*., 1992). Genotype profiles of females and their clutches were obtained for five microsatellite loci – Cm3, Cm58 and Cm72 known to be polymorphic in *C. mydas* (FitzSimmons *et al*., 1995). The fourth locus, nCm84 (FitzSimmons, *pers. comm*.) was a shorter version of the previous Cm84 (FitzSimmons *et al*., 1995), and the fifth locus, Cc7 was isolated from *Caretta caretta* (FitzSimmons, 1998). The PCR reactions to amplify microsatellites were based on the protocol by Joseph and Shaw (2011). Amplified products were resolved on 6% denaturing polyacrylamide gels run on an ALFexpress IITM (Amersham Pharmacia Biotech) automated sequencer, with the product size being determined against internal standard size markers using Fragment Manager v1.2 (Amersham Pharmacia Biotech). Products were run with samples of adult females run adjacent to samples of their offspring.

Statistical Analyses

Genotype frequencies of nesting females at each locus were tested for departure from the Hardy-Weinberg equilibrium and each pair of loci were tested for genotypic linkage disequilibrium using GENEPOP (Rousset, 2008). Null alleles were checked using Micro-Checker (Van Oosterhout *et al*., 2004). Maternal genotypes were determined directly from the sampled female and in her offsprings. 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 then analysed using GERUD 2.0 (Jones, 2005), as the software GERUD has been used for parentage analysis in many natural populations (e.g., Jensen *et al*., 2006; Yue & Chang, 2010; Joseph & Shaw, 2011; Duran *et al*., 2015). To test for the ability to detect multiple paternity, mean relatedness within clutches was calculated using MER (Wang, 2004) and used to estimate effective number of mates (Me - after Bretman & Tregenza, 2005).

Results and Discussion

All five loci were highly polymorphic, with 7 to 25 alleles, and expected heterozygosity from 0.69 to 0.91 (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 detected at any of the five loci used.

Reconstruction of paternal genotypes within clutches using multi locus parsimony (confirmed by outcomes in GERUD 2.0 and MER) had identified that 71% and 36% of all egg clutches from Sabah and Terengganu, respectively were being sired by more than one male (Table 2). The green turtle clutches from Sabah were sired by maximum of two possible fathers, whereas three possible fathers were detected in the egg clutches from Terengganu (T4 & T11). Two patterns of mating were found, either females mated with only one male or alternatively, females mated with multiple males (two or three males) to fertilize her clutches. In all cases of multiple paternity, evidence of three or more paternal alleles were found in at least four of the five loci. 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).

The results obtained support the hypothesis of multiple paternity in the Malaysian green turtle mating patterns. The results are consistent with

Table 1: Number of alleles, expected heterozygosity (H_E) and exclusion probability for the five microsatellite loci used for paternity analysis in the green turtles from Sabah Turtle Islands Park and Redang Island, Terengganu

	Sabah Turtle Islands Park			Redang Island, Terengganu		
Locus	**Number of alleles**	**H_E**	**Exclusion Probability (one parent known with certainty)**	**Number of alleles**	**H_E**	**Exclusion Probability (one parent known with certainty)**
Cm3	13	0.89	0.82	17	0.86	0.82
Cm72	15	0.87	0.84	25	0.91	0.88
nCm84	13	0.85	0.78	16	0.89	0.83
Cm58	7	0.69	0.62	11	0.73	0.70
Cc7	16	0.90	0.83	15	0.86	0.79
Multi-locus	12.8	0.84	0.99	16.8	0.85	0.99

behavioural observations of multiple matings in the green turtle (pers. observations), and in agreement with previous paternity studies in sea turtles (e.g. Parker *et al*., 1996; FitzSimmons, 1998; Kichler *et al*., 1999; Hoekert *et al*., 2002; Lee & Hays 2004; Theissinger *et al*., 2009; Joseph & Shaw, 2011; Stewart & Dutton, 2011; Ekanayake *et al*., 2013, Duran *et al*., 2015). The present study thus suggests that multiple mating by females resulting in multiple paternity might be the dominant breeding strategy in green turtles, and an important factor shaping the mating system of the green turtle populations in Malaysia.

Multiple paternity was common in Sabah, with 71% of the nests exhibiting multiple fathers. This level of multiple paternity exceeds the level reported in other studies of green turtle (Parker *et al.,* 1996; FitzSimmons, 1998; Lee & Hays, 2004; Wright *et al*., 2012; Ekanayake *et al*., 2013). As compared to Sabah, multiple paternity in Terengganu were lower, with only 36% of all clutches exhibiting multiple fathers. Despite the agreement of multiple paternity in the green turtle, all previous studies and the present study show a wide range of different incidences of multiple paternity. Several factors could have influenced the incidence of multiple paternity in individual turtle populations such as breeding sex ratio (Bollmer *et al*., 1999) and sperm competition (FitzSimmons, 1998). The present study demonstrates variation in the incidence of multiple paternity between two nesting populations in the same geographical area (Malaysia), which might be related to nesting densities (i.e. the natural population size). A lower breeding population size is likely to reduce nesting density and also reduces the chances of a female to mate with more than one male. The population in Sabah Turtle Islands Park is much bigger and more stable compared to the population in Redang Island,Terengganu. Furthermore, the Sabah population is considered to be part of a larger population with nesting grounds extending to the Philippines Turtle Islands (Moritz *et al*., 2002). In addition, in larger female breeding populations such as Sabah, male turtles show fidelity to particular courting sites, moving very little during the mating period (Limpus 1993; FitzSimmons *et al.,* 1997a; 1997b). This would also increase the opportunity of multiple matings. Besides that, a longer breeding season in Sabah (all year round) might also increase the incidences of multiple matings in the population.

Paternity Analysis within Successive Clutches from the Same Nesting Females

Multiple clutches per female were observed for seven individual green turtles (between 2 and 6 successive laying events) throughout the nesting season. In all cases tested, the same paternal alleles as observed in the first clutch at all five loci were also detected among the offspring

Table 2: Parental genotypes and number of hatchlings identified to each sire, within green turtle clutches at five microsatellite loci. Allele designations refer to the base-pair length of the alleles

a) Green turtle from Sabah Turtle Islands Park

Female ID	Maternal genotypes					Date of nesting	Inferred paternal genotypes					No. of hatchlings assayed	Total no. of males
	Cm3	Cm72	nCm84	Cm58	Cc7		Cm3	Cm72	nCm84	Cm58	Cc7		
S1	170/182	254/260	190/206	132/138	175/199	01/04/03	168/184	254/286	206/208	136/138	169/173	15	
							172/182	252/268	196/200	136/138	209/215	13	2
S2	162/182	238/274	198/206	130/138	183/193	01/05/03	168/170	250/280	206/216	130/136	169/171	32	
							172/188	250/250	206/206	138/142	175/179	8	2
S3	156/190	250/278	196/198	132/144	177/195	20/03/03	172/174	274/286	192/204	130/136	185/199	24	
							156/186	278/278	202/202	132/140	177/199	9	2
S4	168/174	260/290	208/220	138/140	205/211	01/05/03	186/188	274/290	198/212	136/140	173/181	32	
							168/168	250/268	198/216	140/140	173/175	5	2
S5	174/184	230/290	198/212	132/138	173/193	07/04/03	168/168	274/290	190/204	136/138	185/187	23	
							184/184	242/278	200/220	138/144	173/179	12	2
S6	154/188	240/254	200/210	132/138	169/221	04/05/03	168/188	248/278	216/198	138/144	173/185	39	1
S7	162/172	270/290	190/212	130/134	205/209	08/04/03	176/182	240/248	208/214	130/138	183/209	40	1
S8	168/188	250/282	194/206	132/136	175/201	04/05/03	152/176	250/266	206/210	130/136	185/199	17	
							168/168	238/244	204/204	136/136	185/189	8	2
S9	170/172	248/276	204/210	132/132	173/179	05/05/03	184/188	232/250	202/204	138/138	185/185	27	
							168/180	248/280	198/204	136/138	169/179	13	2
S10	156/176	238/244	198/208	130/138	175/187	11/04/03	168/168	268/270	184/198	138/146	169/171	40	1
S11	168/190	274/282	212/218	132/136	183/207	06/04/03	184/186	240/242	194/198	136/136	183/201	40	1
S12	160/184	240/290	188/208	134/136	169/175	29/04/03	170/184	286/290	200/222	132/134	169/169	23	
							170/170	250/286	188/222	132/136	185/197	16	2
S13	162/182	250/280	198/206	132/138	183/193	30/06/03	168/172	240/260	212/216	138/142	169/171	15	
							170/188	240/274	206/216	142/144	169/175	10	2
S14	156/156	248/280	204/208	134/144	183/209	11/05/03	168/190	238/248	204/216	134/136	171/193	22	
							162/174	242/248	208/216	136/144	177/193	17	2

b) Green turtle from Redang Island, Terengganu

Female ID	Maternal genotypes					Date of nesting	Paternal genotypes					No of hatchlings assayed	Total no. of males
	Cm3	Cm72	nCm84	Cm58	Cc7		Cm3	Cm72	nCm84	Cm58	Cc7		
T1	158/176	246/278	202/202	136/144	169/191	08/06/03	162/176	240/280	206/210	132/134	169/183	34	1
T2	160/174	252/274	182/192	130/134	185/185	06/05/03	168/184	242/286	206/218	130/136	171/181	28	
							160/170	226/302	192/200	134/136	169/183	12	2
T3	174/184	278/306	200/218	132/138	177/187	18/05/03	174/184	244/286	200/214	128/146	185/195	39	1
T4	170/194	250/284	198/202	134/142	177/181	11/05/03	158/170	258/278	194/210	134/136	165/185	18	
							184/170	284/292	206/210	136/142	185/191	12	
							158/158	278/278	202/210	134/138	181/181	10	3
T5	168/172	274/304	194/202	136/138	169/183	25/05/03	168/188	274/288	202/206	130/144	177/183	33	1
T6	156/168	270/286	200/206	134/138	189/189	02/06/03	174/180	250/254	206/206	142/144	177/183	22	
							168/184	286/286	206/210	136/142	171/181	17	2
T7	154/184	280/292	194/202	130/142	171/177	13/06/03	156/174	270/270	184/198	134/138	193/171	28	
							156/178	280/298	210/210	136/142	183/185	12	2
T8	166/196	248/300	200/206	130/138	171/185	22/05/03	180/194	278/300	192/212	130/132	179/185	32	1
T9	154/156	224/240	186/190	130/132	169/169	06/07/03	156/170	246/282	218/290	130/136	169/179	39	1
T10	158/182	244/280	200/206	130/138	181/187	16/03/04	158/170	240/280	206/214	144/144	183/191	12	
							182/190	244/250	200/214	136/144	175/181	28	2
T11	168/170	242/282	198/210	130/136	171/187	04/03/04	152/156	254/278	206/212	134/140	175/183	22	
							156/174	274/288	198/208	138/140	177/203	13	
							168/168	278/288	192/192	138/138	169/177	5	3
T12	184/190	274/276	190/218	136/138	173/201	10/03/04	154/158	252/274	198/206	134/136	169/173	20	
							180/192	274/280	198/210	132/136	187/195	18	2
T13	158/184	250/266	200/200	132/136	179/195	03/03/04	168/178	224/262	192/206	136/144	185/191	30	
							168/172	224/250	200/206	136/142	185/185	9	2
T14	156/168	258/280	184/206	136/142	169/175	11/04/04	166/188	224/252	184/214	136/144	181/219	39	1
T15	158/190	280/290	184/198	132/136	169/171	25/02/04	158/170	234/280	192/206	136/142	181/199	40	1
T16	158/168	278/278	198/204	136/138	183/205	01/03/04	182/188	288/300	212/220	130/136	163/183	40	1
T17	158/174	252/300	182/182	138/148	169/181	21/03/04	174/178	286/300	190/216	130/146	191/197	38	1
T18	176/182	274/280	196/204	136/146	181/201	22/03/04	156/160	240/304	196/214	134/136	195/203	39	1
T19	174/174	242/300	196/204	130/136	171/181	30/03/04	174/164	272/300	204/218	136/146	171/203	40	1
T20	160/174	230/246	206/220	132/138	169/191	10/04/04	174/182	224/246	198/206	138/142	175/191	38	1
T21	168/170	250/284	194/194	128/140	181/183	24/03/04	170/192	240/272	202/210	136/140	175/183	20	1
T22	172/172	234/278	198/214	134/140	181/189	05/04/04	184/192	224/294	198/202	128/134	169/181	20	1

Table 3: Parental genotypes of green turtle clutches from Sabah Turtle Islands Park (S) and Redang Island, Terengganu (T) at five microsatellite loci with their successive clutches for the nesting season. Instances of multiple paternity for a clutch are indicated in bold. Males are designated as ‘m’

Female ID	Maternal genotypes					Clutch	Paternal genotypes					No. of Hatchlings assayed
	Cm3	Cm72	nCm84	Cm58	Cc7		Cm3	Cm72	nCm84	Cm58	Cc7	
S10	156/176	238/244	198/208	130/138	175/187	11/04/03	168/168	268/270	184/198	138/146	169/171	40
						06/05/03	168/168	268/270	184/198	138/146	169/171	40
S11	168/190	274/282	212/218	132/136	183/207	06/04/03	184/186	242/240	194/198	136/136	183/201	40
						08/05/03	184/186	242/240	194/198	136/136	183/201	40
						20/06/03	184/186	242/240	194/198	136/136	183/210	31
S12	**160/184**	**240/290**	**188/208**	**134/136**	**169/175**	**29/04/03**	**170/184** **170/170**	**286/290** **250/286**	**200/222** **188/222**	**132/134** **132/236**	**169/169** **185/197**	**23** (m_1) **16** (m_2)
						10/05/03	**170/184** **170/170**	**286/290** **250/286**	**200/222** **188/222**	**132/134** **132/236**	**169/169** **185/197**	**26** (m_1) **14** (m_2)
						02/06/03	**170/184** **170/170**	**286/290** **250/286**	**200/222** **188/222**	**132/134** **132/236**	**169/169** **185/197**	**8** (m_1) **15** (m_2)
T1	158/176	246/278	202/202	136/144	169/191	08/06/03	162/176	240/280	206/210	132/134	169/183	34
						18/06/03	162/176	240/280	206/210	132/134	169/183	40
						07/07/03	162/176	240/280	206/210	132/134	169/183	40
T2	**160/174**	**252/274**	**182/192**	**130/134**	**185/195**	**06/05/03**	**184/168** **160/170**	**242/286** **226/302**	**206/218** **192/200**	**130/136** **134/136**	**171/181** **169/193**	**28** (m_1) **12** (m_2)
						28/05/03	**184/168** **160/170**	**242/286** **226/302**	**206/218** **192/200**	**130/136** **134/136**	**171/181** **169/193**	**22** (m_1) **18** (m_2)
						29/06/03	**184/168** **160/170**	**242/286** **226/302**	**206/218** **192/200**	**130/136** **134/136**	**171/181** **169/193**	**14** (m_1) **23** (m_2)

T4	**170/194**	**250/284**	**198/202**	**134/142**	**177/181**	**11/05/03**	**158/170**	**258/278**	**194/210**	**134/136**	**165/185**	**18** (m_1)
							170/184	**284/292**	**206/210**	**136/142**	**185/195**	**12** (m_2)
							158/158	**278/278**	**202/210**	**134/138**	**181/181**	**10** (m_3)
						22/05/03	**158/170**	**258/278**	**194/210**	**134/136**	**165/185**	**12** (m_1)
							170/184	**284/292**	**206/210**	**136/142**	**185/195**	**12** (m_2)
							158/158	**278/278**	**202/210**	**134/138**	**181/181**	**4** (m_3)
						31/05/03	**158/170**	**258/278**	**194/210**	**134/136**	**165/185**	**21** (m_1)
							170/184	**284/292**	**206/210**	**136/142**	**185/195**	**7** (m_2)
							158/158	**278/278**	**202/210**	**134/138**	**181/181**	**12** (m_3)
						11/06/03	**158/170**	**258/278**	**194/210**	**134/136**	**165/185**	**21** (m_1)
							170/184	**284/292**	**206/210**	**136/142**	**185/195**	**7** (m_2)
							158/158	**278/278**	**202/210**	**134/138**	**181/181**	**12** (m_3)
						20/06/03	**158/170**	**258/278**	**194/210**	**134/136**	**165/185**	**13** (m_1)
							170/184	**284/292**	**206/210**	**136/142**	**185/195**	**15** (m_2)
							158/158	**278/278**	**202/210**	**134/138**	**181/181**	**12** (m_3)
						30/06/03	**158/170**	**258/278**	**194/210**	**134/136**	**165/185**	**14** (m_1)
							170/184	**284/292**	**206/210**	**136/142**	**185/195**	**13** (m_2)
							158/158	**278/278**	**202/210**	**134/138**	**181/181**	**13** (m_3)
T8	166/196	248/300	200/206	130/138	171/185	22/05/03	180/194	278/300	192/212	130/132	179/185	32
						01/06/03	180/194	278/300	192/212	130/132	179/185	40
						04/07/03	180/194	278/300	192/212	130/132	179/185	40
						16/07/03	180/194	278/300	192/212	130/132	179/185	39

in the subsequent clutches (Table 3). Out of the seven multiple clutches, only three were multiply sired (S12, T2 and T4). FitzSimmons (1998) also reported the identical paternity for all successive clutches of nine green turtle females from the southern Great Barrier Reef. Other sea turtles also exhibit the same paternity across multiple clutches laid by individual females of Kemp's ridley (Kichler *et al.,* 1999), leatherback turtle (Crim *et al*., 2002), loggerhead (Moore & Ball, 2002) and hawksbill turtle (Joseph & Shaw, 2011). These data are consistent with the hypothesis of sperm being stored from mating(s) prior to nesting and being used to fertilize all subsequent clutches of eggs that season without additional inter-nesting mating. This also suggests that females do not mate with new (extra) males during the egg-laying season. Sperm storage is considered to play an important role in reproduction of turtles in which male and female cycles do not coincide. In sea turtles, mating only occurs at the beginning of the season and male sea turtles will migrate to the feeding areas once the mating season ends. Nesting of female sea turtles will take several months and sperm storage can increase the probability of fertilizing all clutches, particularly if males are a limiting resource or in a population of low density (Galbraith *et al*., 1993).

Conclusion

This study demonstrates multiple paternity in green turtle clutches - suggesting that multiple paternity might be a common breeding strategy of green turtle populations in Malaysia. Multiple paternity has positive implications for this endangered species because it can increase the effective population size, thus reducing the loss of genetic variability through drift (Sugg & Chesser, 1994). Furthermore, given the large energy involved in migration and egg production, it may be advantageous for female sea turtles to have multiple matings to reduce the risk of mating singly with sterile males. It is also concluded from this study that there is sperm storage in nesting female green turtles from Malaysia, and that mating probably only occurs prior to the beginning of the nesting season.

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