Detection of multiple paternity in green turtle clutches during a reproductive season at Khram Island, Thailand.

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ABSTRACT
Although direct observations have been suggesting that multiple mating in green turtle (*Chelonia mydas*) may be quite common at Khram Island, Chonburi, Thailand, the number of successful males is still in question. Genetic analysis technique is a tool for investigation. We preliminary evaluated the proportions of successful breeding males and females of green turtle in a natural population at Khram Island, Gulf of Thailand, using two microsatellite markers. We analyzed genotypes of 77 tissue samples collected from 3 nesting females, and at least seven offsprings per clutch in three successive clutches within a reproductive season (total of nine clutches). We were able to detect multiple paternity of the hatchlings. For most clutches, at least two males successfully sired hatchlings within each clutch. Throughout a nesting season, at least three different males could mate with the same female and there were at least seven males successfully mating with this set of females. This study confirmed the effectiveness of microsatellite DNA markers in detecting multiple paternity within natural populations of green turtle. However, to reduce the confounding effects of mutations on allele assignment and to increase power to monitor individual's genetic contribution, we need additional variable genetic markers.

KEYWORDS: green sea turtle, paternity, microsatellite, DNA marker

INTRODUCTION

Reproductive biology in sea turtles received attention worldwide from evolutionary biology perspectives to sea turtle management. One of the central questions has been the determination of mating pattern in a natural population. For green turtle (*Chelonia mydas*), direct observation and genetic analyses have indicated that the frequencies and the degrees of polyandry (a female mates with multiple males) or polygyny (a male mates with multiple female) are population specific (Crim et al., 2002; Fitzsimmons, 1998; Ireland et al., 2003; Lee and Hays, 2004). Our study attempts to preliminary evaluate mating pattern in a green turtle from Khram Island, Chonburi, one of the most important nesting sites of green turtle in Thailand.

The nesting areas at Khram Island have been under protection of the Royal Thai Navy. This green turtle population is one of the largest populations in Thailand with the number of nesting females up to almost 1,000 nests in 1988 (Chantrapornsyl, 2003). Recent trend indicates population decline, however. During 1995-1999, the estimates for the number of nesting females were lower than 300 nests a year (Chantrapornsyl, 2003). Conservation efforts were put forth by various agencies in Thailand, including the Thai government, Department of Marine and Coastal Resources, Department of Fisheries and the Royal Thai Navy (Monanunsap and Charuchinda, 2003). Conservation planning still needs much knowledge on the basic biology of green turtle.

Previous studies on the Khram Island population showed that within a reproductive season, nesting intervals of each female ranged from 8-51 days and each female lays eggs 2-7 times in the season during April-September (Monanunsap and Charuchinda, 2000). Direct observation of mating patterns has been inadequate in determining the number of successful mates. Many studies used genetic analyses to evaluate mating patterns based on the exclusion of known maternal genetic contribution to offspring (Fitzsimmons, 1998; Ireland et al., 2003; Lee and Hays, 2004; Parker et al., 1994). In this study, we used hypervariable microstellite DNA markers. Major benefits of this technique are that microsatellite genetic markers are highly variable, which is appropriate for genetic identification at family levels, and that only small amount of preserved tissue samples is required the analyses which allows for non-lethal sampling.

Two major questions addressed in our study include (1) how many successful breeding males mate with a female and (2) how many times a

female mate with additional males within a reproductive season in a natural population of green turtle at Khram Island, Gulf of Thailand. We analyzed two microsatellite loci, shown to be highly variable in other studies (Fitzsimmons, 1998; Crim et al., 2002). We, first, evaluated polymorphism of microsatellite DNA markers for this green turtle population. We, then, identified paternal alleles by excluding maternal alleles from genotypes of hatchlings. More than two paternal alleles at each locus indicate multiple paternity. Our study is the first in Thailand attempting to address the question of multiple paternity of green turtle in a Thai natural population.

MATERIALS AND METHODS

Sample collection

During May to August 2001, we collected muscle tissue samples of three nesting female turtles and their hatchlings (sampling 10 offsprings/clutch) from Khram Island, Chonburi, Thailand. For each female, we collected three successive clutches during the nesting season (Table 1). Collected tissues were preserved in 95% EtOH. All genetic analyses were carried out at the Aquatic Genetic Laboratory at Burapha University.

Table 1. Number of offspring/clutch of nesting females, sampled during the 2001 reproductive season at Khram Island, Thailand.

Nesting Female	Clutch 1	Clutch 2	Clutch 3	Clutch 4
1	10	-	10	10
2	10	10	-	10
3	10	10	10	-

DNA extraction

DNA was extracted from turtle tissue using a salt extraction protocol (Fitzsimmons, 1998). A small piece of tissue was added to 500 μL extraction buffer (100mM Tris HCl, 100mM EDTA, 0.5% SDS, 0.2M NaCl) with an additional 10 μL proteinase K (20 mg/mL) in a 1.5 mL microcentrifuge tube. The solution was incubated at 55°C overnight. We precipitated protein using 7.5 M Ammonium Acetate. Then DNA was precipitated from the supernatant using cold 100% ethanol. DNA pellet was washed in 75% ethanol and resuspended in TE. The DNA solution was then added to polymerase chain reaction (PCR) mixtures.

Resolving microsatellite polymorphisms

We examined two microsatellite loci using PCR primers previously developed for *C. mydas* (Fitzsimmons, 1998; Crim et al., 2002). PCR was performed in a Hybaid thermal cycler using a 15 μL reaction mixture. Each reaction mixture contained 1.5 μL template DNA solution or 50 mg purified template DNA, 25 pmol of each primer, 2.0 to 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 X reaction buffer (Appendorf), and 1 unit of *Taq* polymerase (Appendorf). Temperature profiles for the PCR consisted of denaturing at 95°C for 1 minute, annealing at primer specific temperatures for 1 minute, and elongating at 72° C for 1 minute. The reaction went through 30 to 35 cycles, then the final elongation was extended for 10 minute. Two μL of loading dye were added to each tube directly, and the mixture was loaded on 8% sequencing gel at 1000 V for 2 to 2.5 hr. They were stained using Silver Staining techniques (Promega). Alleles were designated according to the sizes of the PCR product.

Data Analysis

We first estimated microsatellite variation of the two loci studied. We, then, determined parental alleles by eliminating maternal alleles from the genotypes of offspring in a clutch (Ireland et al., 2003). Within each clutch, paternal alleles were ones different from the maternal alleles and ones found in homozygous hatchlings. More than two paternal alleles within a clutch indicate multiple paternity. Because of limited information on population genetic diversity and relatively small sample sizes, we chose to estimate the minimum number of males per clutch assuming heterozygous genotypes for each male. For example, if we detected three paternal alleles, the minimum number of males would be two. Mismatching of maternal genotypes was excluded from the analysis.

RESULTS

Microsatellite variation

The two microsatellite loci used in this study revealed considerable genetic variation (Table 2), with 13 and 14 alleles per locus for Cm72 and Cm84 respectively. Observed heterozygosities index were 0.88 and 0.84. These variations allowed for the detection of multiple paternity of green turtle (*C. mydas*) hatchlings at Khram Island, Chonburi.

Table 2. Allelic diversity and observed heterozygosities at two microsatellite loci used in the paternity analysis of green turtle hatchlings

Locus	N	No.of allele	Allele size (bp)	Observed Heterozygosity
Cm72	50	13	197-289	0.88 (44/50)
Cm84	62	14	336-374	0.84 (52/62)

Paternity analysis

We determined parental alleles by excluding maternal alleles from offspring genotypes (Tables 3 and 4). We, then determined the minimum number of male turtles assuming heterozygous genotypes of each male. For most clutches, at least two males were successfully siring offspring of each nesting female (Table 4). Throughout the nesting season, each female could store sperm of at least 3-4 males. There were overlapping paternal alleles at both loci between consecutive clutches of each female. At least seven different males contributed to this set of hatchlings for the entire 2001 reproductive season.

Information inferred from these two microsatellite markers was helpful to detect multiple paternity of hatchlings in a natural population. Although two markers may not be adequate to track individual male's genetic contribution, it indicated possible polygamy (a male mates with multiple females) in a few instances. A breeding male who probably contributes genomes to a couple of nesting female no.2 and 3 might have genotype as 254/262 bp for the marker of Cm72 (see Table 3-4).

Table 3. Genotypes of three females *C. mydas* and their offsprings from three successive clutches within the 2001 nesting season. Alleles are assigned to fragment length in base pair (bp).

Maternal genotypes (bp)			N	Offspring genotypes		Freq.
Clutch	Cm72	Cm84		Cm72	Cm84	
1.1	246/246	350/358	7	246/283	338/350	1
				246/268	338/350	2
				240/246	-	1
				233/246	350/374	1
				-	338/358	1
				-	358/374	1
1.2			10	246/268	350/358	1
				-	350/358	3
				-	338/350	5
				-	350/350	1
1.3			8	246/262	350/358	1
				242/246	358/358	1
				246/246	350/350	1
				246/268	-	1
				-	350/350	1
				-	358/358	1
				-	358/362	1
				-	350/358	1
2.1	244/283	350/358	10	233/244	346/350	1
				254/283	350/358	1
				233/244	-	2
				197/244	350/358	1
				244/244	350/350	1
				233/244	350/358	1
				-	350/358	3
2.2			9	233/283	350/354	1
				233/283	-	1
				244/244	-	1
				244/262	350/354	1
				244/262	-	1
				262/283	350/358	1
				262/283	350/350	1
				262/283	-	1
				-	350/366	1
2.3			7	244/252	-	1
				244/254	-	1
				258/283	348/358	1
				-	336/350	3
				-	350/358	1
3.1	244/304	350/350	9	244/254	336/350	1
				244/254	342/350	1
				244/254	-	2
				244/262	-	1
				244/289	344/350	1
				254/304	350/358	2
				254/304	350/362	1
3.2			8	244/244	350/362	1
				244/244	342/350	1
				244/244	350/372	1
				254/304	350/362	2
				262/304	350/358	1
				262/304	-	1
				-	350/372	1
3.3			9	244/254	350/350	3
				244/262	350/358	1
				244/262	-	1
				262/304	350/362	1
				-	350/358	1
				-	340/350	1
				-	350/362	1
Total			77			

Table 4. Number of least possible successful males based on number of paternal alleles of variable markers. Alleles shown in italic indicate share alleles between successive clutches within a nesting female.

Possible male alleles (bp)			Minimum number of males
Clutch	Cm72	Cm84	
1.1	**240**	**338**	2
	233	**374**	
	268		
	283		
1.2	*268*	*338*	1
		350	
1.3	**242**	*350*	2
	246	**358**	
	262	**362**	
	268		
Throughout the reproductive season			3
2.1	**197**	**346**	2
	233*	**350***	
	244	**358***	
	254		
2.2	*233*	**354**	2
	244	*350*	
	262*	**366**	
2.3	*254*	**336**	2
	252	**348**	
	258		
Throughout the reproductive season			4
3.1	**254***	**336**	3

	262*	**342**	
	289	**344**	
		358*	
		362*	
3.2	**244***	*342*	2
	254	*358*	
	262	*362*	
		372*	
3.3	*254*	**340**	2
	262	**350***	
		358	
		362	
Throughout the reproductive season			4
All breeding males			**7**

DISCUSSION

Microsatellite genetic markers proved to be an effective tool to analyze paternity of sea turtle hatchlings in a natural population compared to direct observation. Microsatellite variation provided insights about reproductive biology of green turtle. Multiple paternity seems to be common in examined samples, similar to findings in many marine turtle species, including loggerhead (*Caretta caretta*) (Moore and Ball Jr, 2002), Kemp's ridley *(Lepidochelys kempi)* (Kichler et al., 1999) and leatherback turtles *(Dermochelys coriacea)* (Crim et al., 2002). The levels of multiple paternity in other sea turtles ranged from 16% to 57.7% of total clutches examined. In addition, our results were similar to the findings in another green turtle population in Ascension Island (Ireland et al., 2003; Lee and Hays, 2004). For the Ascension Island population, multiple paternity was relatively common (10 out of 17 clutches) based on five microsatellite loci (Lee and Hays, 2004).

Our findings, however, seem to contradict those found in a southern Great Barrier Reef (sGBR) population of green turtle (Fitzsimmons, 1998), where multiple paternity is rare based on microsatellite analysis at five loci. Only two out of 22 clutches examined were multiply sired and of those multiply sired very few hatchlings were offspring of a secondary male. Fitzsimmons (1998) speculated that mating system/sperm storage strategies and sperm competition might play a major role in Australian turtle population. We believed that reproductive behaviors of green turtle may depend on geographical specification and weather in nesting sites. Levels of multiple paternity detected in our study may reflect differences in effective proportion of males and females or mating strategies between the sGBR and Khram Island populations.

Fitzsimmons (1998), Ireland et al. (2003), Lee and Hays (2004) and our study confirmed that levels of multiple paternity in green turtle are population specific. However, our study might have overestimated the frequency of multiple paternity in the studied population due to limited sample sizes and numbers of examined loci.

We only examined limited number of females. We collected three nesting females out of approximately 40-50 females which might not be a good representation of the Khram Island population. The limited number of hatchlings per clutch (7-10) prevents us from analyzing proportion of males' genetic contribution within each clutch and also to speculate on mutations or null alleles.

We relied heavily on limited information of two microsatellite loci. In some cases, information was only available at one locus. Although two microsatellite loci were informative, we could not distinguish paternal alleles from potential mutation events. Fitzsimmons (1998) excluded 20 mutation events, indicated by mismatch genotypes at one locus out of five. Highly variable microsatellite loci (e.g., Cm72) resulted in a high number of mutation events. For our study, the numbers of mismatch genotypes (excluded from the analysis) indicated that mutation or non-amplifying fragments (null alleles) might influence our analysis.

An interesting aspect of this study was the ability to track multiple paternity in successive clutches for each nesting female for an entire nesting season. Our results suggested that females might have mated at least twice within a nesting season (new paternal alleles detected in each consecutive clutch). This contradicts the observations that a female only mate once during one to two months prior to the nesting season (Miller 1992). The portions of share alleles indicate some degrees of overlap, however. This might imply that females utilize sperms from prior mating. Due to limited ability to distinguish mutations from paternal alleles, this observation of multiple mating during a nesting season needs to be confirmed with additional variable loci.

Although some turtle species are polygamous (Crim et al. 2002), we cannot conclude that this is the case for the green turtle population at the Khram Island. To identify whether the number of breeding males are limited for this female population, we need additional variable loci and some information about allele frequencies in this population. Genetic information inferred from adequate numbers of genetic markers may shed light on the number of successful breeding males and their genetic contribution to hatchlings as well as levels of polygamy in this population.

CONCLUSION

Microsatellite proved to be an effective tool to assess multiple paternity in the Khram Island population of green turtle. Despite limited genetic information, we detected multiple paternity in this population (at least 2-3 males in each clutch and 3 - 4 males among successive clutches). Within a nesting season, females might mate more than once, implying from new paternal alleles among successive clutches. To increase the precision of our estimates and to monitor individual males' genetic contribution within this population, we need additional variable genetic markers.

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