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Microsatellite DNA Analysis on the Polyandry of Green Sea Turtle *Chelonia mydas*

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Green turtle (*Chelonia mydas*; Testudines) is included in the group of polyandry animals, which is single female mated with many male. DNA polymorphism method generally considered to have a high degree of accuracy as compared to other methods to elucidate polyandry phenomena on many animals. In this research, three microsatellite loci were used to identify the number and frequency of genotypes per locus, the number and frequency alleles per locus, and genotypes and number of alleles in the nest. The purpose of this research was to study the reproductive pattern of *Chelonia mydas* and compensation eggs of males from hatchling's population in turtle conservation area of Pangumbahan Coastal Park, West Java. The result showed that from 10 nests, we could find 37 genotypes with 11 alleles for D108 locus, 21 genotypes with 9 alleles for B103 locus, and 27 genotypes with 9 alleles for C102 locus. The alleles number of each nest was more than 5 alleles for 5 nests, and more than 4 alleles for the remaining nests. Based on the probabilities of alleles contribution of each parent, the green turtle is a polyandry animal.

Keywords: *Chelonia mydas*, polyandry, microsatellite loci, genotype, allele

INTRODUCTION

Sea turtle is the common name for Testudines that live in the sea. Out of seven species of sea turtle in the world, Indonesia has six species, i.e. green sea turtle *Chelonia mydas*, hawksbill turtle *Eretmochelys imbricata*, leatherback sea turtle *Dermochelys coriacea*, loggerhead sea turtle *Caretta caretta*, flatback turtle *Chelonia depressa*, and olive ridley sea turtle *Lepidochelys olivacea* (www.wwf.or.id). As in other Testudines, sea turtles eggshell consist of carapace and plastron. Except for leatherback sea turtle, carapace of sea turtles is protected by hard scales.

Sea turtle live in tropical and subtropical area with spawning ground almost in slightly slope sand beach which can be found in many Indonesian beaches. The breeding process of green sea turtle takes place in an area close to the spawning ground or food spot. It occurs in the morning before the sun rises (Bustar 1972; Limpus & McLachlan 1979), although green sea turtle can lay their eggs throughout the year (Nuitja 1992). The female of sea turtle reaches maturity and spawn for the first time at the age of 20-50 years with 2-8 spawning intervals every year.

Sea turtles are able to preserve sperms, even the old sperms from the last spawning season can be used to fertilize egg in the next season (Galbrait 1993). It means that mounting or sperm deposition of male sea turtle does not always followed by fertilization. After mounting at breeding ground, male sea turtle move toward to playing or feeding ground, while the female hunt for nesting sites. To reach the breeding and spawning ground, sea turtles often travel as far as 3000 km, thus there is a possibility one female mate with more than one male. It is common to find more than one male in every nest, in both case of green (Fitzsimmons 1998; Lee & Graeme 2004) and loggerhead sea turtle (Moore & Ball 2002).

DNA microsatellite or short DNA segment recurrent can be used to analyze the breeding pattern of organisms. Alleles in every microsatellite locus are mostly polymorphic and codominant and the pattern of inheritance of microsatellite DNA alleles follows the Mendel inheritance law (Levinson & Gutman 1987; Moxon & Wills 1999). Therefore, analysis of microsatellite DNA alleles can be used to study the breeding phenomenon in sea turtle.

This research aim was to investigate the reproduction pattern of *C. mydas* and eggs compensation

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(male) of the hatchling or its population that hatch at Sea Turtle Conservation Area of Taman Pesisir Pangumbahan Sukabumi, West Java. Furthermore, this research was expected to provide basic information of polyandry and microsatellite locus data of sea turtle.

MATERIALS AND METHODS

Sampling Location and Tissue Preservation. Samples of *C. mydas* were collected at Sea Turtle Conservation Area of Taman Pesisir Pangumbahan Sukabumi, West Java (Figure 1). Tissues of *C. mydas* carapace were collected from the fresh hatchling. Twelve samples were collected from each of ten nests (N = 120) and preserved in absolute alcohol.

Isolation of Total DNA. DNA was extracted from the carapace tissue of the hatchling which had been preserved in absolute alcohol. Therefore, the tissue must be cleaned from ethanol before further process. The clean tissue was lysed with proteinase-K (0.125 mg/mL) and 1% sodium dodecyl sulfate. DNA was then purified by phenol method followed Sambrook *et al*. (1989) standard method.

Cells were slowly stirred together with 40 µL 5 M NaCl, 400 µL phenol and 400 µL CIAA (chloroform isoamilalcohol = 24:1) for 1 hours at room temperature. DNA phase was precipitated by an addition of 800 µL 70% ethanol, and then suspended with TE buffer.

Amplification and Genotyping of the Microsatellite DNA. Alleles from three microsatellite DNA locus were amplified with primers for each locus as presented in Table 1 (Dutton & Frey 2009). PCR reaction was performed at 20 µL total volume consisted of 2 µL DNA template (10-100 ng), 6 µL sterile water, 1 µL each of forward and reverse primer, and 10 µL ampli Tag Kappa Ready Mix. PCR condition for all primers: pre-denaturation 94 ºC for 5 min; 30 cycles of denaturation 94 ºC for 30 seconds, primary annealing 60 ºC for 90 seconds, and elongation 68 ºC for 120 seconds; and last elongation 72 ºC for 5 min.

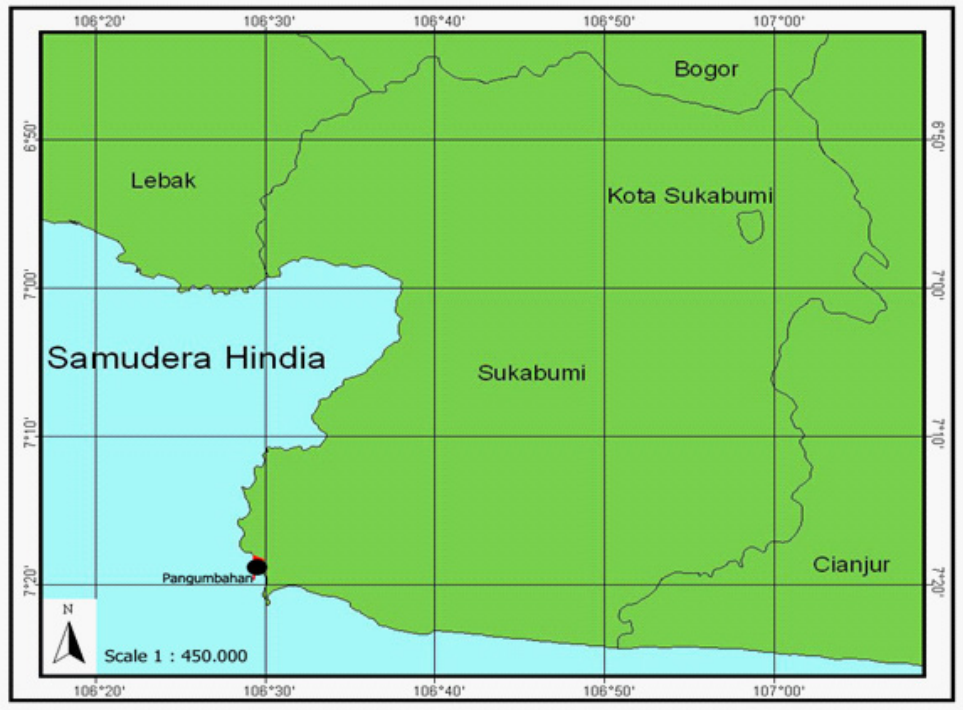

Figure 1. Location of sample collection at Pangumbahan Coastal Park, West Java.

Table 1. Names and orders of three primary microsatellite used in the research

Locus	Primary sequence (5'-3')	T (ºC)	Number of sample	Alleles size	Number of alleles
D108	Foward: TCTCCCTCACTGGCTTATGA Reverse: GCTGGCATCTTTTCTGTCAG	60	87 (72.5%)	192 - 254	11
B103	Foward: CAGTCCTTGTTGTGGTTAGAGT Reverse: GTTTCTTTTTCCCTTTCATCTTCTGTC	60	88 (73.3%)	151 - 168	9
C102	Foward: TAAAAAGGCAGCCAAGTAAG Reverse: GTTGCAGAACCAACAGAATAG	60	107 (87.5%)	212 – 266	9

Microsatellite DNA alleles detection or PCR product performed by 8% polyacrylamide gel electrophoresis (PAGE) and followed by silver stained techniques (Tegelstrom 1986). The DNA bands were scored as alleles; allele a, b, and c were named based on the migrating rate, where allele a as the fastest. If there were two alleles, then it was considered as heterozygote. Likewise, if there was only one allele then the sample was considered as homozygote.

Since the parents genotype data was absent and to determine the multiple paternity, we limited the assumption that if in one nest consist only one or two alleles, then the parent and the male alleles were homozygote. Conversely, when there were three parent alleles in one nest and the male allele was heterozygote then there were four alleles in one nest. Therefore, it would be decided as one male if there were ≤ 4 alleles in one nest, and at least two males if there were ≥ 5 alleles in one nest (Table 2).

Table 2. Alleles combination probability based on parent and male genotype

Main genotype		Number of minimum inherent alleles
Male (♂)	Female (♀)	
AA	AA	1 Allele : a
AA	AB	2 Alleles : a & b
AA	BB	2 Alleles : a & b
AB	BC	3 Alleles : a, b & c
AB	CD	4 Alleles : a, b, c, d

RESULTS

DNA Microsatellite Amplification. The percentages of successful amplified samples for locus D108, B103, C102 were 72.5, 73.33, and 87.5%, respectively (Table 1). Locus D108 has 37 genotypes, which was the highest genotype variety among others, while 21 and 27 genotype for B103 and C102 locus respectively. All successfully amplified samples were polymorphic, and there were 11 alleles in D108 locus, as well as 9 alleles each in B103 and C102 locus.

D108 locus was consisted of 24.14% homozygote and 75.86% heterozygote samples. The highest homozygote samples were found at nest number 6, while the highest heterozygote was in the nest number 9. In B103 locus, the highest homozygote samples was occurred at nest number 1 (52.27% homozygote), while the highest heterozygote were in nest 9 (47.73% heterozygote). The C102 locus had 59.05% homozygote samples and 40.95% heterozygote samples. Several highest homozygote samples were found at nest number 8, 9, and 10, while the highest heterozygote were in nest 1 and 2.

Table 3. Number of alleles for each nest at three Microsatellite DNA loci

Nest	Locus			Male minimum
	D108	B103	C102	
1	5	2	6	2
2	3	4	4	1
3	6	3	3	2
4	5	4	3	2
5	8	3	5	2
6	5	1	4	2
7	4	4	4	1
8	4	4	3	1
9	4	3	2	1
10	4	2	3	1

Alleles compositions between nests showed the existence of shared alleles. In locus D108 and C102, 100% of the alleles were shared between ten nests. In locus B103, 77.77% of the alleles were shared. In locus D108, allele e and f were the most shared alleles among nests 2, 3, 4, 5, 6, 7, and 8. Meanwhile in locus C102, allele b and c were dominantly shared among nest 3, 4, 5, 6, 7, 9, and 10; followed by allele f and g that were shared among nests 1, 2, 4, 5, 7, and 8. The most dominantly shared allele for locus B103 were allele d which was shared among nests 1, 2, 5, 6, 7, 8, and 9. Other alleles were commonly found between 2-5 different nests.

Multiple Paternity. From the male probability assumption by microsatellite DNA analysis of the hatchlings, it is noted that nest 1, 3, 4, 5, and 6 of the three observed locus had ≥ 5 alleles, therefore has at least 2 males in one nest. The others had ≤ 4 alleles which indicated that at least there was one male in each nests (Table 3). These results proved the existence of multiple paternities in green sea turtle.

DISCUSSION

The coastal of Pangumbahan has high diversity of microsatellite allele in each nest of green sea turtle. This might be due to the green sea turtle migration pattern in Indonesia which crosses the Hindia Ocean, Pacific Ocean and South China Sea (Cahyani *et al*. 2007; www.wwf.or.id.). However, other researcher mentioned that the feeding or spawning area of green sea turtle in Berau (East Kalimantan) is from Australia, Malaysia, Micronesia and New Guinea (Cahyani *et al*. 2007). Based on sea turtle conservation map (www.wwf.or.id.), both feeding and spawning area of sea turtle in Indonesia were come from Australia, Malaysia,

Thailand, Philippine, Vietnam, Brunei Darussalam, and New Guinea.

Microsatellite allele variation in every nest showed the genotype combination of the parents. Genotype or microsatellite allele combination in green sea hatchlings can be used to determine its origin. The existence of shared alleles among ten nests were found in this study, which reach 100%. Fitzsimmons (1998) found that male A was the father for two different nests. In other case, genotype combination from three male alleles was found at four different nests (Crim *et al*. 2000).

Microsatellite markers are inheritance biparental and codominant, hence can be used to measure the diversity of microsatellite alleles. These markers have been used in some of population studies for the Leatherback turtle (*Dermochelys coriacea*) (Ciofi *et al*. 2002); ornate box turtles (*Terrapene ornate*) (Kuo & Janzen 2004); Sea turtle (Bowen & Karl 2007; Lee 2008); genetic conservation programs (Fitzsimmons *et al*. 1995; Pearse *et al*. 2006), and to count the number off athersin turtle mating system (Kemp's ridleys (*Lepidochelys kempi*) (Kichler *et al*. 1999); loggerhead turtle (*Caretta caretta*) (Moore & Ball 2002; Zbinden *et al*. 2007); green turtles (*Chelonia mydas*) (Ireland *et al.* 2003); olive ridley (*Lepidochelys olivacea*) (Aggarwal *et al*. 2004; Jensen *et al*. 2006); The spur-thighed tortoise (*Testudo graeca*) (Roques *et al*. 2004); marine and freshwater turtles (Verly 2006); hawksbill turtles (*Eretmochelys imbricata*) (Joseph & Shaw 2011), and lemon sharks (*Negaprion brevirostris*) (Joseph *et al*. 2008). Furthermore, Engtrom *et al*. 2007 have made a summary of the primers that can be used in mtDNA, microsatellite and other nuclear loci of either turtles or tortoises.

Based on alleles combination in each locus, it is known that paternity system of female (parent) sea turtle is polyandry, or described as mating with more than one male. This multiple paternity is possible due to the capability of sea turtle to preserve sperms (Galbrait 1993) that remain in good condition for four years (Pearse *et al*. 2001). A total of 61% of all green turtle nests (*C. mydas*) observed in Ascension Island in Atlantic had two or more males (Lee & Graeme 2004). Moore and Ball (2002) suggested that it is common to find several males in one nest. In Melbourne Beach, Florida, around 31% of all loggerhead sea turtle (*C. caretta*) nests had two males, and almost 10% has three or more males.

DNA microsatellite of green turtle hatchlings (*C. mydas*) in ten observed nests have high variability. Based on 3 results of the analysis of DNA microsatellite loci, green turtles are performing polyandry. Microsatellite markers can be used to determine lineage and existence of multiple paternity in turtles.

Further research is expected to provide the availability of the parent genotype analysis, proximity parking study nest, nest physical parameter measurements, and satellite tagging. This data will later facilitate the analysis of multiple paternity in turtles. In addition, mtDNA analysis combined with metal tags, and satellite telemetry can be used to produce maps of migration routes and network identification on turtle conservation management.

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