ISSN: 2458-8989

Natural and Engineering Sciences

NESciences, 2019, 4(1): 42-54

-RESEARCH ARTICLE-

Sex Determination in Green Turtle Hatchlings: Geometric Morphometry and Molecular Sex Markers

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Abstract

Determination of the sex ratio in sea turtle population is one of the most important parameters for the conservation and management of the species. We aimed to the test usability of the more than one approaches that identify the sex of hatchlings with alternative non-invasive methods. Geometric morphometry and molecular sex marker were used to indicate difference between the sexes in the green sea turtle (*Chelonia mydas*) hatchlings which sexes were previously determined by gonad histology. A total of 60 landmarks were identified from three body parts (carapace, plastron and tail) for geometric morphometry. No sex specific difference was found in these three body parts. Furthermore, we analyzed a total of 55 different oligonucleotide combinations using sex-based pool strategy but found no difference. These two techniques are insufficient for sex determination in the green turtle hatchlings. A relatively small number of the primer combinations and male hatchlings used to identify sex of *C. mydas* appears to be a disadvantage. In order to obtain clearer results with geometric morphometric and molecular sex markers, it is recommended to compare sexes in laboratory conditions with constant temperature.

Keywords:

Geometric morphometry, AFLP, Sex determination, Green Turtle, Samandağ, Sugözü Beaches.

Article history:

Received 14 November 2018, Accepted 21 January 2019, Available online 31 January 2019

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Introduction

Sex determining mechanisms in reptiles are grouped under two main branches: entirely under genetic control (genotypic sex determination, GSD) and highly dependent on temperature change during the embryonic development process (temperature-dependent sex determination, TSD) (Mittwoch, 2000; Warner, 2011). In reptiles, the effect of temperature on embryonic development is more pronounced than other environmental factors (Bull, 1980). For this reason, TSD is the most common environmental sex determination (ESD) in reptiles. Comparative analyses have indicated that the TSD expresses ancestral status for turtles (Janzen & Krenz, 2004; Organ & Janes, 2008) and the GSD have emerged independently at least 6 times in evolutionary history (Janzen & Krenz, 2004). There are some species of turtles in which there is no temperature effect in sex formation, even if there are no sex chromosomes defined (Warner, 2011). However, empirical evidence has indicated that TSD is the primary mechanism in the majority (81%) of tested species.

Knowledge of the sex ratio in sea turtles is useful to examine population dynamics. The sex ratio at hatching may be dissimilar to adult sex ratio, and thus, comparing them can provide information about differential mortality, migration, and dispersal between sexes (Bulmer, 1994). The sex ratio is a demographic variable that is affected by environmental conditions because the sex of sea turtles is determined by their incubation temperature. The relationship between sex and incubation temperature is characterized by a critical temperature which is an equal proportion of male and female individuals (Bull, 1980). In most sea turtles, for instance, embryos incubating at high temperatures (>29 °C) become mostly female whereas in cooler nests (<29 °C) a greater proportion of embryos develop into males (Mrosovsky, 1994). However; it is known that transitional range of temperature (TRT) between male and female producing temperatures is required to characterize a population (Mrosovsky & Pieau 1991). Maxwell et al. (1988) showed that the difference in mean temperature during the critical period between totally male and female clutches was only 1.1 °C in loggerhead turtles during the critical period. Both current and future populations may be affected by global climate change, and therefore, acquiring the sex ratio of hatchlings on a nesting beach is vital for the future of the species and conservation because long-term survival depends on both female and male ratios (Janzen, 1994). While morphological differences are observed between the sexes in adult turtles (Berry & Shine, 1980), few or no external sex differences are evident in hatchlings (Valenzuela et al., 2004). Therefore, researchers have been employing different techniques to assess the sex of hatchlings. For example, gonadal histology (Merchant-Larios et al., 1989; Godfrey & Mrosovsky, 2006), radioimmunoassay to measure testosterone levels in blood or chorioallantoic fluid (Gross et al., 1995), laparoscopy on live post-hatchlings (Wyneken et al., 2007), direct observations of the gonads in situ (McCoy et al. 1983), and clearing of gonads in toto (van der Heiden et al., 1985), quantitative sex identification based on the histological characteristics of the gonads and paramesonephric ducts (Ikonomopoulou et al., 2012) have been used to determine the sex. Some of these techniques require the sacrificing of animals and a complicated long laboratory processes. However, the sea turtle species are endangered, and under protection globally. Therefore, non-invasive alternative techniques for the sex determination need to be developed.

There may be morphological differences between female and male hatchlings that are not visible to the naked eye (Valenzuela et al., 2004). Valenzuela et al. (2004) suggested a two-step method of sex determination based on the morphology of *Chrysemys picta* and *Podocnemis expansa*. The first step is selecting the best method for quantifying morphology, followed by

identifying the most suitable statistical approach. Differences between male and female hatchlings may be revealed by linear measurements (Valenzuela et al., 2004). Michel-Morfin et al. (2001) found significant differences between female and male *Lepidochelys olivacea* hatchlings in terms of 9 morphological characters in discriminant function analysis (DFA). Sönmez et al. (2016) specified that the tail length was important character in establishing differences between female and male green turtle hatchling in a linear measurement. However, some authors do not find morphological differences between the sexes using this approach (Lubiana & Ferreira-Junior, 2009). Another approach is a landmark-based geometric morphometric analysis. The geometric morphometry determines the similarities and differences in the landmark using computer simulations by determining the landmark on examined individuals (Bookstein, 1992). In hatchlings of two non-sea turtle species (*C. picta* and *P. expansa*) carapace shape was significantly differentiated between sexes using landmark-based geometric morphometric analysis (Valenzuela et al., 2004).

As well as different techniques that mentioned above, molecular techniques are also used to sex determination in sea turtles and non-sea turtle species (Demas et al., 1990; Hernández-Echeagaray et al., 2012; Rovatsos et al., 2017). Long and complicated laboratory processes of existing techniques and expensive practices encourage the development of alternative methods. DNA fingerprinting technique may be an ideal approach to the question by Vos et al. (1995). Amplified fragment length polymorphism (AFLP) is relatively cheap, easy, fast and reliable method to generate hundreds of informative genetic markers (Vos et al., 1995). AFLP method does not require pre-knowledge about DNA sequence. AFLP markers are useful tools for identification of close relationships such as sibling and hybrid species, structure and diversity of the population at the levels of species and subspecies (Innan et al., 1999; Li et al., 2002). AFLP markers have been used to uncover cryptic genetic variation of strains or closely related species which were impossible to resolve with morphological or other molecular systematic characters (Li et al., 2002). AFLP has also been reported to be useful as a sex-specific marker in a large number of different groups (Griffiths & Orr, 1999; Felip et al., 2005; Quinn et al. 2007). In reptiles, it was reported that a female-specific DNA marker was successfully isolated by screening AFLP in the bearded dragon lizard (*Pogona vitticeps*) (Quinn et al., 2007).

AFLP method allows simultaneous scanning of many different DNA regions randomly distributed in the genome (Mueller & Wolfenbarger, 1999). Even the smallest changes in the genome of each organism (using primers in different combinations) can be observed because almost unlimited markers can be produced by AFLP-PCR (Mueller & Wolfenbarger, 1999). Single nucleotide changes can be detected on polyacrylamide gels or automated genotyping. AFLP provides a reliable alternative to the identification of possible gene regions that are not previously identified and possibly responsible for sex formation (Vos et al., 1995). Different regions scattered throughout the genome are a convenient path because of simultaneous scanning. Screening of sex-specific markers with AFLP is performed as follows: AFLP fragments are amplified using different primer combinations from two different DNA pools of male and female individuals. The fragment profiles displayed on the counter-rotating gel are expected in 3 different ways: (1) in both sexes, (2) only in male, and (3) only in the female. Fragments amplified in only one sex are identified as a marker of that sex, and DNA sequencing data are generated to examine sex characteristics. In this sense, the sex differences can be expected between male and female green turtles with both geometric morphometry and molecular sex markers. We, therefore, aimed to test differences

between female and male green turtle (*Chelonia mydas*) hatchlings in terms of geometric morphometry and molecular sex markers. Thus, if we can obtained a morphological difference and sex marker, we can determined exactly the number of male and female hatchlings in a nest in real time.

Materials and Methods

A total of 158 dead hatchlings of *C. mydas* were collected on Samandağ Beach (36° 7.500′ N, 35° 55.100′ E) and Sugözü Beaches (36°48.677′ N, 35°51.068′ E) located along the north eastern Mediterranean in Turkey during 2014, 2015 and 2016 nesting seasons. All dead hatchlings were found on the way to the sea. Fresh carcasses of hatchlings without any decomposition were chosen in the field and then transferred to a laboratory for gonad sampling and morphological data collection. First, we collected geometric morphometry data. Three body regions (plastron, carapace, and tail) were used for geometric morphometry. The digital images of the body region in each specimen were acquired using a high-resolution digital camera. Attention has been paid to the light quality of the environment during photographing. The camera was parallel to the image and photographed at the same distance. Later, dead hatchlings were dissected, and their gonads were preserved in 4% buffered para-formaldehyde for gonadal histology. During dissection of dead hatchlings, one forelimb was stored in 96 % ethanol for molecular analysis. The alcohol of the samples in the laboratory was periodically changed and kept in room conditions. The sex of hatchlings was identified by gonadal histology. The gonads were cut in half transversely with one-half embedded in paraffin wax, sectioned at 6–10mm from the mid, and stained with Eosin and Harris hematoxylin. The sex of a hatchling was identified using a microscopic examination of gonad sections by checking the differentiation in gonadal medulla and cortex or the absence of seminiferous tubules (Yntema & Mrosovsky, 1980).

After sex determination with gonad histology, the photographs of male and female hatchlings were transferred to the 'tpsDIG2' software (Rohlf, 2015). From each image, x, y coordinates of 60 anatomical landmarks (36, 17, and 7 are on the carapace, plastron, tail, respectively) (Figure 1) were recorded. The landmarks are positions of biologically repeatable (operationally homologous) anatomical points (Valenzuela et al., 2004). The landmarks were then transferred to the MS Office Text document, and the numerical coordinates in the text were transferred to 'MorphoJ' package software (Klingenberg, 2011) for statistical analyses. Superimposition, translation, rotation and scale changes were removed with a full Procrustes fit (Rohlf & Slice, 1990), and a covariance matrix was generated for each data set transferred to the packet program. After this process, the data-sets were prepared for statistical analysis. In the analysis of geometric morphometry, general Procrustes analysis (GPA) was applied. Procrustes ANOVA test was used to compare the sex differences in shape, and discriminant function anlysis (DFA) was used to test whether these differences were statistically significant. Moreover, principal component analysis (PCA) was used to obtain the formal appearance, and the first two principal components were used for this purpose. The geometric morphometry analysis was completed in 'MorphoJ' version 1.02d. Ten female and 10 male hatchlings were compared in terms of 3 body regions in the geometric morphometry.

Fore-limb tissues from five female (sample numbers: S26, S29, S2, S7, S10) and four male (sample numbers: S5, S23, S1, S34) were used for the molecular analysis. Total genomic DNA isolation was performed with some improvements in the standard proteinase K, phenol-chloroform

protein precipitation protocol (Hillis & Moritz, 1990). Approximately 50 mg fore-limb tissue was digested in 500 µL STE buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA, pH 8.0), 25 µL proteinase K (10 mg/mL) and 50 µL SDS (10 %). DNA was extraction by a standard phenol-chloroform procedure and precipitated with absolute ethanol. Precipitated DNA was dissolved in 100 µL 1xTE (10 mM Tris-HCI, 1mM EDTA, pH 8.0) and quantified at a wavelength of 260–280 nm using a spectrophotometer.

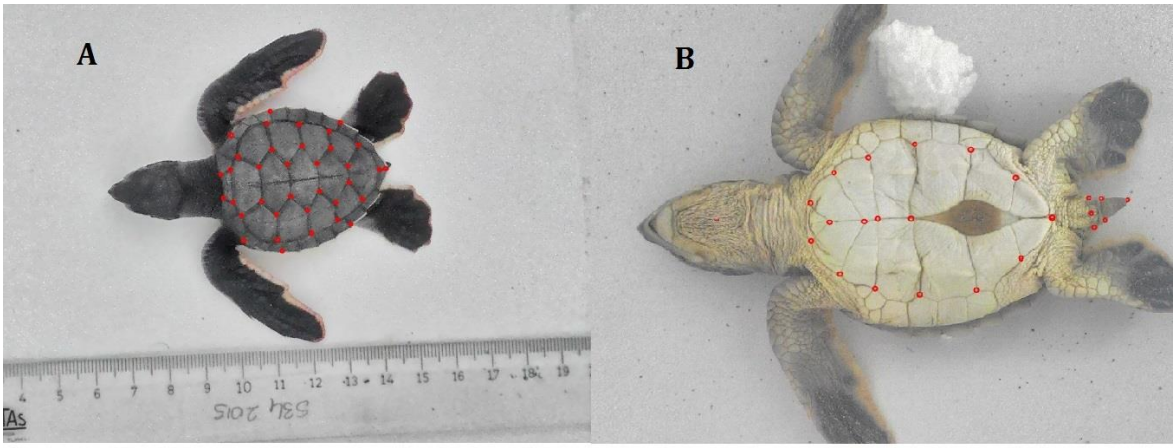

Figure 1. The location of the 60 landmarks (A= carapace, B= plastron, and tail) in green turtle hatchlings.

DNA quality was visualized by running on 0.8 % agarose gel electrophoresis. The quantitation was estimated by comparing it with a known DNA marker. DNA solutions were diluted to 50 ng/µL for PCR applications. Pooled DNA samples from each sex were prepared using 50 ng/µL of genomic DNA. Pooled DNA was initially analyzed using a total of 55 different primer combinations.

AFLP analysis was performed using a method described by Vos et al. (1995) with some modifications. DNA samples were cut with EcoRI and MseI (Fermentas, MBI, Massachusetts, USA) restriction enzymes. The oligonucleotide adaptors were ligated to cut to the DNA fragment ends to generate a template DNA for amplification. In pre-amplification step, the genomic DNAs were amplified using two AFLP primers (EcoRI-A and MseI-C) both having a single selective nucleotide. PCR (Stuart, UK) amplifications were performed 26 cycles with the following cycle profile: a 1min DNA denaturation step at 94 °C, a 1min annealing step at 56 °C, and a 1 min extension step at 72 °C. Five times diluted PCR products of DNA were used as templates for selective amplification. PCR amplifications were performed with the following cycle profile: a 30s DNA denaturation step at 94 °C, a 30s annealing step (see below) and a 2 min extension step at 72 °C. The annealing temperature in the first cycle was 60 °C, subsequently reduced at each cycle by 0.7 °C for the next 12 cycles and was continued with a 30s DNA denaturation step at 94 °C, a 30s annealing step at 56 °C and a 2 min extension step at 72 °C for the next 23 cycle (Table 1).

Polyacrylamide gel electrophoresis (PAGE) analysis of AFLP fragments was performed in two different ways using combinations of fluorescence-labeled (15) and unlabeled (40) oligonucleotides. AFLP-PCR products were denatured at 90 °C for 4min and separated on 6 % denaturing polyacrylamide gels and visualized by silver staining. AFLP markers were analyzed

manually based on the presence/absence of homolog DNA fragments. Analysis of the AFLP fragments amplified using fluorescence-labeled oligonucleotides in Macrogen Europa (South Korea) company through service procurement. The results (.fsa files) were opened using the 'Geneious' version 7.0 software.

Table 1. Primers used for the selective amplification and their sequences (*fluorescent dye-labelled).

EcoRI*-P1	5'-GAC TGC CTA CCA ATT CA CC-3'	**EcoRI-P9**	5'-GAC TGC CTA CCA ATT CA TA-3'
EcoRI*-P2	5'-GAC TGC CTA CCA ATT CA CG-3'	**EcoRI-P10**	5'-GAC TGC CTA CCA ATT CA GC-3'
EcoRI*-P3	5'-GAC TGC CTA CCA ATT CA TT-3'	**EcoRI-P11**	5'-GAC TGC CTA CCA ATT CA GT-3'
EcoRI-P4	5'-GAC TGC CTA CCA ATT CA AA-3'	**MseI-P1**	5'-GAT GAG TCC TGA GTA AC AC-3'
EcoRI-P5	5'-GAC TGC CTA CCA ATT CA AT-3'	**MseI-P2**	5'-GAT GAG TCC TGA GTA AC TG-3'
EcoRI-P6	5'-GAC TGC CTA CCA ATT CA AG-3'	**MseI-P3**	5'-GAT GAG TCC TGA GTA AC TA-3'
EcoRI-P7	5'-GAC TGC CTA CCA ATT CA CA-3'	**MseI-P4**	5'-GAT GAG TCC TGA GTA AC GC-3'
EcoRI-P8	5'-GAC TGC CTA CCA ATT CA TC-3'	**MseI-P5**	5'-GAT GAG TCC TGA GTA AC GA-3'

Results

The sex of 158 dead hatchlings was determined by histological examination, and 12 (7.6%) of them were identified as male. Two male hatchlings were excluded from the data due to their anomalies such as carapace scute deviation. The carapace shape analyses indicated no differences in shape ($F=0.89$, $df=68$, $P=0.72$) between males and females. This was further supported by DFA ($p>0.05$) with each sex had a mean of 40.3 % similarity to their group. A total of 9 principal components were produced in PCA. The first and second principal components described 50 and 14 % of the total variance, respectively. Although the total variance was high, sexes were not separated and clustered over the elliptical figure with a 95 % confidence interval (Figure 2b). The same result was also found in the canonical variance analysis (CVA), and the sexes were not distinguished in terms of the carapace shape. The distribution of landmarks on the carapace is presented Figure 2a.

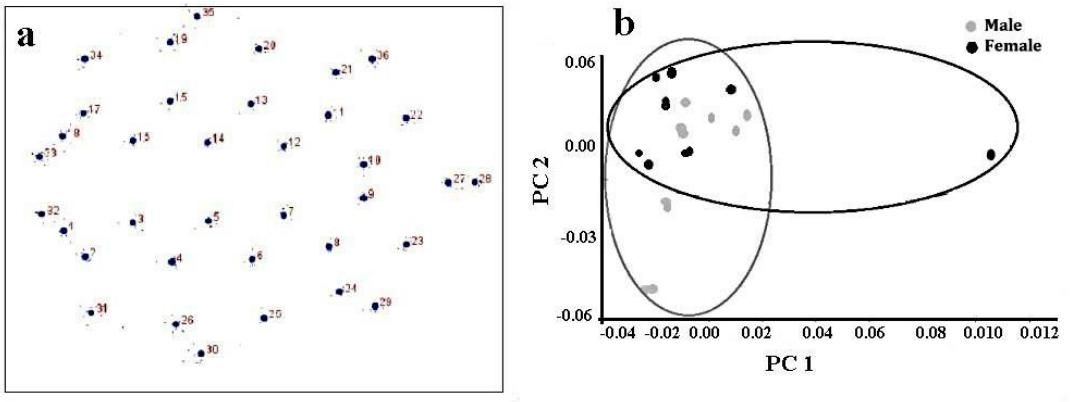

Figure 2. The distributions of landmarks on carapace (a) and PCA plot with 95% confidence ellipses for carapace (b)

The plastron shape analyses indicated no significant differences in Procrustes shape ANOVA (F=0.29, df =30, P=0.60). This was further supported by DFA (p>0.05) with each sex had a mean of 45 % similarity to their group. A total of 19 principal components were produced in PCA. The first and second principal components described 22.5 and 21.7 % of the total variance the total variance, respectively. However, although the total variance was high, sexes were not separated and clustered over the elliptical figure with a 95 % confidence interval (Figure 3b). The same result was also observed in CVA, and the sexes were not distinguished in terms of the plastron. The distribution of landmarks on the plastron is presented Figure 3a.

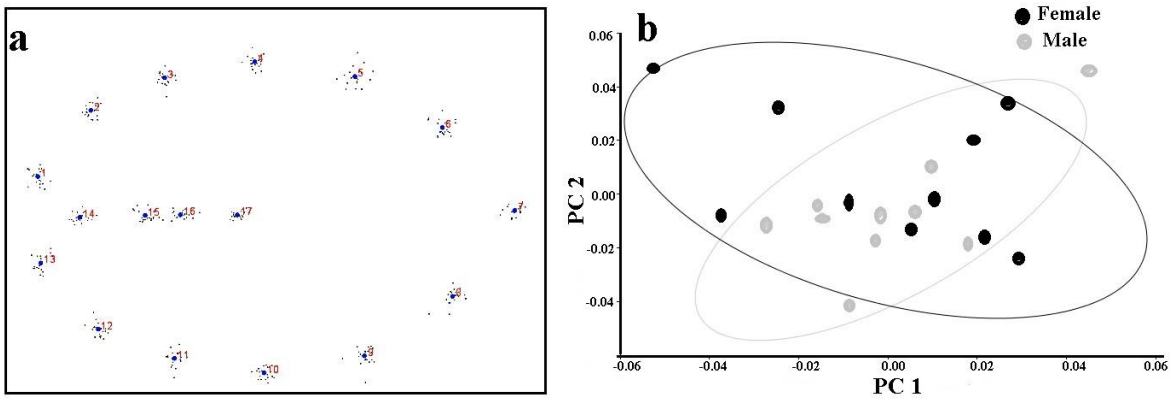

Figure 3. The distributions of landmarks on plastron (a) and PCA plot with 95% confidence ellipses for plastron (b)

The tail shape analyses found no significant differences in Procrustes shape ANOVA (F=1.72, df=10, P=0.08). This was further supported by DFA (p>0.05) with each sex had a mean of 55 % similarity to their group. A total of 10 principal components were produced in PCA. The first and second principal components described 51.7 and 22.5 % of the total variance, respectively. However, although the total variance was high, sexes were not separated and clustered over the

elliptical figure with a 95 % confidence interval (Figure 4b). The same result was also found in CVA, and the sexes were not distinguished in terms of the tail. The distribution of landmarks on the tail is indicated Figure 4a.

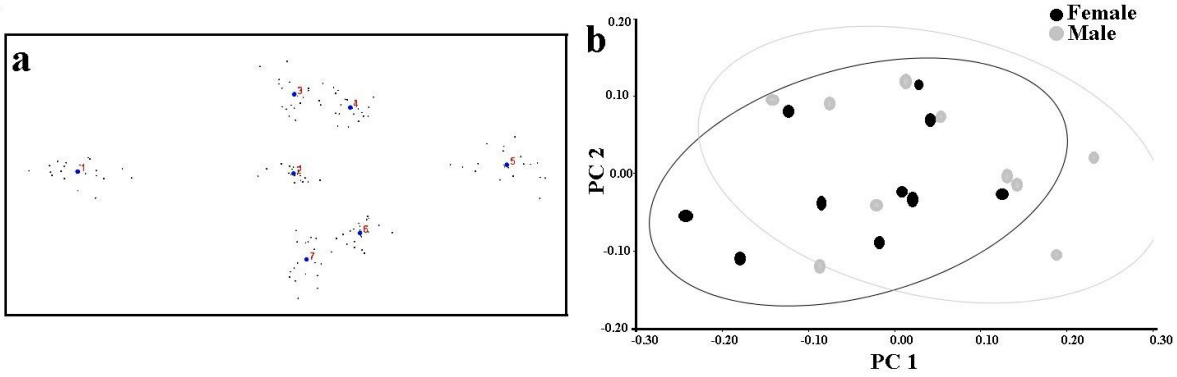

Figure 4. The distributions of landmarks on tail (a) and PCA plot with 95% confidence ellipses for tail (b)

AFLP analysis using a total of 55 different primer pairs, showed no band differences between female and male hatchlings. AFLP fragments amplified with fluorescent dye-labeled EcoRI selective primers are indicated in Figure 5. Thus, gel images (Fig. 5a) and peaks (Figure 5b) were compared to detect differences between female and male band profiles. As a result, both AFLP analysis and gel images with peaks showed no difference between female and male *C. mydas* hatchlings.

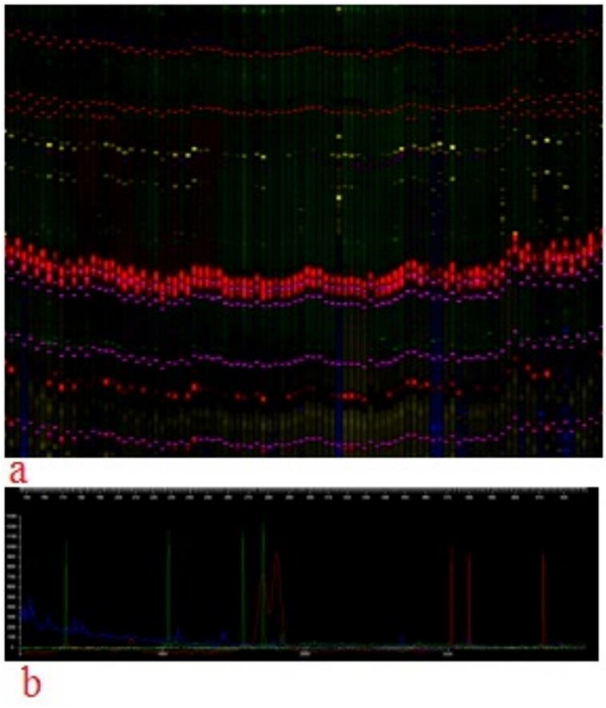

Figure 5. AFLP fragments amplified with fluorescent dye-labelled *Eco*RI selective primers (a= gel images, b= peaks)

Discussion

Comparative classical morphology is used as an alternative method for sex discrimination in sea turtle hatchlings (Michel-Morfin et al., 2001; Sönmez et al., 2016; Delgado et al., 2010). While some of these studies not found differences between male and female hatchlings, such as in our study (Delgado et al. 2010), some morphological differences were found in some studies (Michel-Morfin et al. 2001, Sönmez et al. 2016). A different approach to the determination of differences between sexes is geometric morphometry, which is more accurate results than traditional classical morphology (Valenzuela et al., 2004; Lubiana & Ferreira-Junior, 2009; Türkecan, 2010; Ceballos & Valenzuela, 2011; Ferreira Junior et al., 2011). For example, geometric morphometry has provided successful results in sex determination among non-sea turtle species (Valenzuela et al., 2004; Lubiana & Ferreira-Junior, 2009; Ceballos & Valenzuela, 2011). However; geometric morphometry studies on sea turtles are limited, and these studies were mostly done on *Caretta caretta* (Türkecan, 2010; Ferreira Junior et al., 2011). In these studies, authors indicated that there are significant shape differences between the sexes. However, the present study did not detect differences between sexes. The contradicting results in studies based on geometric morphology may be due to the development of eggs under different micro-ecological conditions or small sample size. It is known that different micro-ecological conditions cause variations on morphology of sea turtle hatchling (Reece et. al., 2002; Glen et. al., 2003). As well as micro-ecological conditions, it is important how to identified sex in the methodology. For example, Türkecan (2010) and Ferreira Junior et al. (2011) indirectly identified sex by using incubation duration and distance to the sea of sample nests for distinguishing between female and male hatchlings in their geometric morphometry studies.

A relatively small number (n=10) of the male hatchlings that used in this study to identify sex of *C. mydas* using geometric morphometric technique appears may be disadvantage. Cardini & Elton (2007) stated that the samples size in the geometric morphometric studies have frequently small sample, due to the scarcity of material available for analysis. The studies that have small samples showed fairly accurate results in mean size, standard deviation of size and variance of shape (Cardini & Elton, 2007). Whereas, the mean shapes and angles was found strongly affected by sampling error (Cardini & Elton, 2007). It was reported that the sex ratio of the present study beaches is female bias (Kılıç & Candan, 2014; Yalçın Özdilek et al., 2016). It is inevitable that the proportion of male hatchlings is low in the present study. Because we collected to the samples with a high degree of selectivity such as dead and fresh carcasses of hatchlings without any decomposition.

The success of identifying sex-specific markers mainly depends on the presence of a sex chromosome (Demas et al., 1990; Sarre et al., 2004). However, target species are sometimes found in non-chromosomal genetic determinants (Griffiths et al., 2000). Therefore, the identification of sex-linked markers within the scope of the study may be due to the absence of sex chromosomes for *C. mydas*. According to Wuertz et al. (2006), another reason why sex identifiers cannot be identified is that the sexting system in the investigated organism is processed by a gene dosage effect. There is a report on the sex-specific segregation of banded krait minor (Bkm) satellite DNA in mature Kemp's ridley and green turtle (Demas et al., 1990). However, this result has not been replicated and attempts to identify other sex-specific markers in temperature-dependent sex determination (TSD) turtles have been unsuccessful (Hernández-Echeagaray et al., 2012). The sex-specific segregation of Bkm satellite DNA in sea turtles (Kemp's ridley and green turtle) was

explained in three theories by Demas et al. (1990). Firstly, TSD may not play a key role in this species. Secondly, TSD may include structural changes in the methylation or chromatin altering the sensitivity of DNA to the restriction endonucleases. Finally, TSD may include DNA modification in sex determination sequences or contiguous regions. Sarre et al. (2004) stated that the authors did not consider in these two species can show GSD when they are incubated under natural conditions. They claimed that TSD and GSD may have an effect on each other for produce to the less-viable sex-reversed hatchlings which are selected against under natural conditions. In a different approach on molecular sex technique, it is stated that the determining of sex-linkages of protein coding genes may be a trustworthy molecular sex technique in the non-sea turtle species (Testudines: Trionychidae) (Rovatsos et al., 2017).

A relatively small number (n=55) of the primer combinations used in this study to identify sex of *C. mydas* using AFLP fingerprint technique appears to be a disadvantage. Based on the results obtained, we recommend that more AFLP primer combinations should be examined to make a conclusive decision about the status of sex markers in *C. mydas*. The use of more AFLP primer combinations allows a more exhaustive investigation of the *C. mydas* genome. Moreover, it is suggested to examine the expression of genes responsible for sex formation in reptiles, gene dosage effect and Bkm satellite DNA methylation in future studies (Hernández-Echeagaray et al., 2012; Sarre et al. 2004). If these three studies are made from sexes obtained with constant temperature in laboratory conditions, then clearer results are likely to be obtained.

In conclusion, geometric morphometry and molecular sex markers in the present study are found as unsuitable techniques that can be used for sex discrimination. The reasons for this may be effects of small sample size and gene dosage effect in the green turtles hatchlings.

Acknowledgements

This work was supported by the Scientific Research Project Fund of Cumhuriyet University under the project number KMYO-004. We thank Scientific Research Project Fund of Cumhuriyet University, volunteer students, the Ministry of Forestry and Water Affairs, Regional Directorate of Nature Conservation and National Parks, Samandağ District Governor, Samandağ Environmental Protection and Tourism Association's managers and members, Ecological Research Society and Cumhuriyet University's Advanced Technology Research Center, and Oğuz Türkozan.

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