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Genetic polymorphism between and within Mediterranean and Red Sea populations of the green turtle (*Chelonia mydas*) as revealed by Sequence-Related Amplified Polymorphism (SRAP)

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ABSTRACT

The present study is considered as a preliminary experiment to generate new markers that include other segments along marine turtles' genome, as a tool to investigate new areas of variation/polymorphism between, among and within populations and individuals. The PCR-based technique Sequence-Related Amplified Polymorphism (SRAP), best to our knowledge, was used for the first time to investigate the genetic polymorphism between and within Mediterranean and Red Sea green turtle (*Chelonia mydas*) populations. Based on the data obtained using twenty SRAP primer combinations, the polymorphism within the Mediterranean population was 73.8% while it reached 96% within the Red Sea population. Genotypic specific markers produced by SRAP for Mediterranean and Red Sea green turtle populations were 48 and 22 markers, respectively. The cluster analyses showed that the Mediterranean and Red Sea populations can be distinguished from each other into 2 completely different clusters. The similarity between individuals from Ras Muhammad Protected Area (PA), Hurghada, and some individuals from Zabargad showed distinguished and close relationships. This study indicated that SRAP proved to be an easy and rapid method to investigate genetic variation between and within the Mediterranean and Red Sea marine turtles' populations.

INTRODUCTION

Biodiversity covers, starting from habitats and species to populations then genes diversity at all various stages of biological system **(Abdel-Azeem, 2010; Gaston, 2010)**.

One of the oldest extant creatures on the earth are marine turtles **(Joyce, 2015; Rees *et al*., 2017)**. Due to its critical and integral role in marine and coastal environments, it is considered a crucial stone species **(Bjorndal and Jackson, 2002; Spotila, 2004;**

Casale *et al.*, 2018), in addition to its fundamental role in the culture of coastal communities **(Goatley *et al.*, 2012)**. It is also, recorded as an indication of the effect on coastal and marine ecosystem on climate change **(Hawkes *et al.*, 2009)** and marine litter pollution **(Matiddi *et al.*, 2017; UNEP, 2019)**.

In the past few decades, the molecular ecology of marine turtles has increased, almost exclusively for studies related to behavior, ecology and evolution; demanding for the ideal molecular markers for screening of genome-wide polymorphisms and large-scale, high performance genotyping. Various techniques and genetic markers such as: allozymes, microsatellites, minisatellites, mtDNA haplotypes, RAPDs, RFLPs, AFLPs and SNPs have been used for marine turtles researches **(Bowen and Karl, 2007; Carreras *et al.*, 2007; Roden *et al.*, 2009; Roden *et al.*, 2013; Tikochinski *et al.*, 2018; Tolve *et al.*, 2018)**. All these techniques were used for: a) linking foraging areas to rookery origins and to assessing population genetic structure **(Roden and Dutton, 2011; Garofalo *et al.*, 2012; Jordão *et al.*, 2015; Leroux *et al.*, 2012; Vilaça *et al.*, 2012; Dutton *et al.*, 2013; Vilaça *et al.*, 2013; Daniel and Wolfe, 2014; Tikochinski *et al.*, 2018; Tikochinski *et al.*, 2020)**, b) natal homing phenomenon **(Aves and Bowen, 1994; Lee, 2008; Kaska, 2016)**, c) hybridization phenomenon, d) multiple paternity **(Jensen *et al.*, 2013; Tedeschi *et al.*, 2014; Sari *et al.* 2017)**, e) sex ratio and sex linked markers (**Spotila, 2004; Stewart and Dutton, 2014)**, bar-coding documentation and conservation genetics **(Naro-maciel *et al.*, 2010; Jensen *et al.*, 2013)**.

PCR based technique Sequence-Related Amplified Polymorphism (SRAP) has recently been used to target the Open Reading Frame (ORF); the interval between genes and their non-coding flanking regions is amplified, and closely linked to actual genes, which would produce a fingerprint of the coding sequences and allow these bands to be easily isolated for sequencing **(Yu *et al.*, 2008; Li *et al.*, 2014).** SRAP PCR based marker has mixed co-dominant nature **(Li and Quiros, 2001; Roberts and Wolfe, 2014)**, helps to demonstrate effectiveness investigation of genetic polymorphism between species and/or populations **(Li *et al.*, 2014)** besides it can be used for further sequencing research.

Since 2001 SRAP has been used in molecular biology for plant, but it's application taking a new turn to be used with different species rather than plant to identify polymorphism in Entomophatogenic nematodes **(Abd ElAzim *et al.*, 2019)**, determine genetic variation within Arctic fox **(Zhang *et al.*, 2013)**, and polymorphism among giant prawn **(Zhou *et al.*, 2006)**. By applying SRAP marker for the first time on marine turtles new insight will be given to be used more on marine species. Thus there is need to apply SRAP technique on vertebrates to confirm its validity in a large number of animal species.

Along Mediterranean and Red Sea Egyptian coasts *Chelonia mydas* (green sea turtle) populations found for nesting and/or foraging **(Attum *et al.*, 2014; Mancini *et al.*, 2015; Casale *et al.*, 2018)**. As a threatened species according to IUCN reports and assessments more work on both ecological and molecular levels was required **(Casale *et***

***al*., 2018)**. In the current study SRAP technique was used to detect genetic variation and polymorphism between populations and individuals of sea turtles *Chelonia mydas* (green sea turtle) that nest in the Mediterranean and Red Sea Egyptian coast.

MATERIALS AND METHODS

Study area

Field work was carried out during nesting seasons of *Chelonia mydas* (green turtle) from 2016 to 2019, at different locations (Table 1, Fig. 1) on both Mediterranean and Red Sea coasts in Egypt. These locations represent most known nesting sites and sites that predicted to be suitable as nesting areas. Monitoring was conducted through day and night patrols. Night patrols allow directly observing nesting female sea turtles while day or morning patrols allow recording tracks and missing nests surveys.

Table 1. Green turtle samples' locations

Location	Coordinates	Samples	Type of samples
Ashtoum El-gamil PA	31°18'8.91"N 32°10'25.59"E	M3	Blood and skin
Alexanderia	31°12'13.24"N 29°52'7.92"E	M1, M2	Blood and skin
El-Sahel	30°51'19.13"N 29°16'1.21"E	M4	Blood and skin
Ras Muhammed PA	27°47'29.04"N 34°13'17.59"E	R1, R13, R14, R15, R16	Blood
Hurgada	27°17'0.66"N 33°46'21.89"E	R17	Blood
Zabargad Island	23°36'36.31"N 36°11'48.41"E	R2, RR3, R4, R5, R6, R7, R8, R9, R10, R11, R12	Skin

All samples of the green sea turtle (*Chelonia mydas*), M1-M4: Mediterranean population, R1-R17: the Red sea populations

Sample Collection and DNA extraction

Skin and blood samples were collected from 21 live and stranded individuals (Table 1). Skin samples obtained after cleaning the sampling area and it should not be deeper than 0.5 mm and ≈ 1*1 cm and placed in absolute ethanol. Blood samples collected from dorsal cervical sinus by 21g needle and syringe then placed in heparinised tubes (sodium heparin). Blood and tissue samples were stored at -20°C till DNA extraction.

DNA was extracted by using BioBasicEZ-10 Spin Column Genomic DNA Minipreps kit (blood) and Applied Biosystems™ MagMAX™ DNA Multi-Sample Ultra Kit for both tissue and swap samples. DNA was collected and stored at −70°C until use. One percent agarose gel electrophoresis and spectrophotometer were used to determine the quality and quantity of DNA.

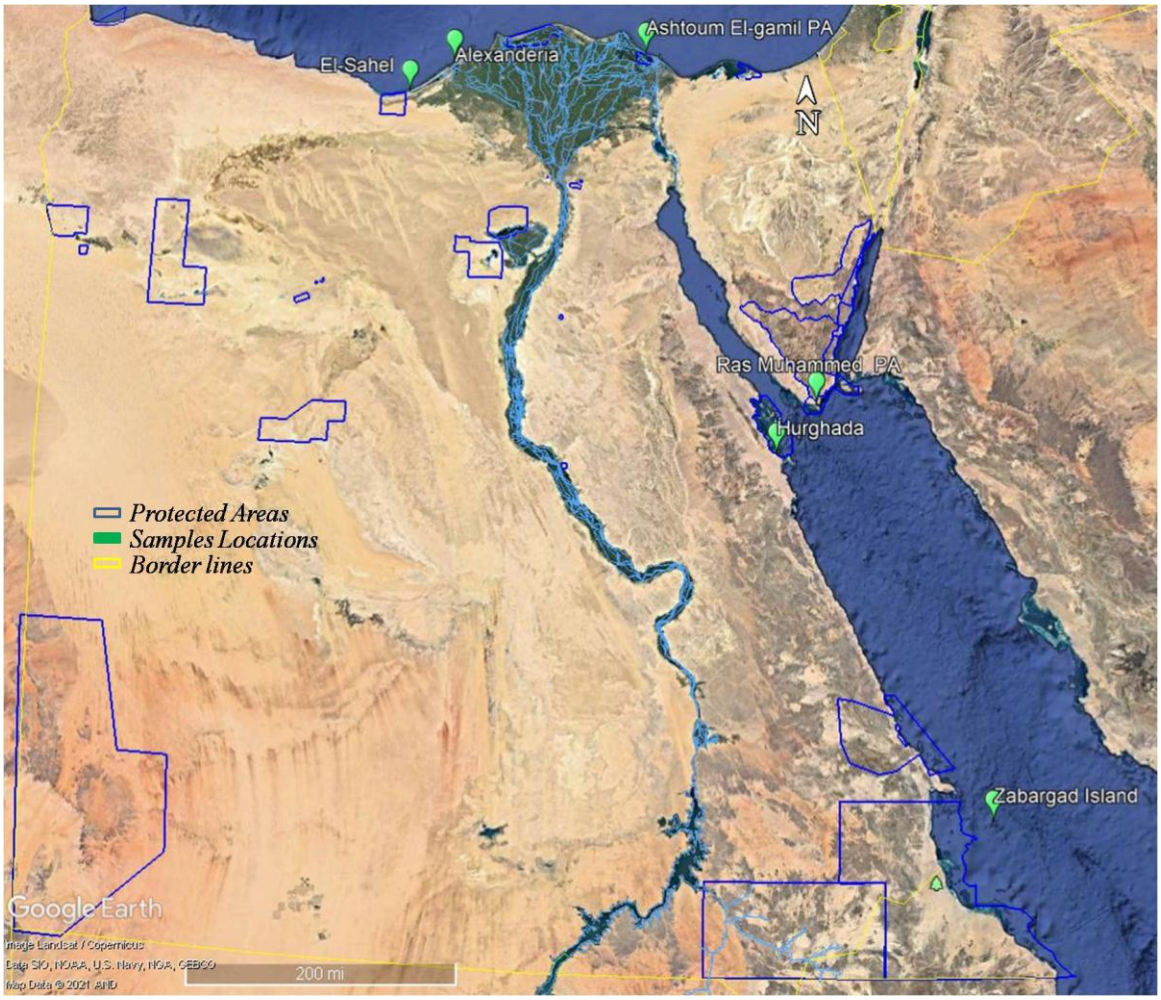

Fig. 1. A map of Egypt showing the sampling locations

Sequence-Related Amplified Polymorphism (SRAP)

Sequence-Related Amplified Polymorphism (SRAP) technique was performed according to **Li and Quiros (2001)** and **Li *et al*. (2014)** in a 20 µl reaction mixture containing: approximately 1 µl (25 ng) DNA, 1 µl of forward and reverse primers (0.5 mM of each primer) (Table 2), 10 mMTris-HCl (pH 8.4), 50 mMKCl, 200 mM of dNTPs, 4 mM of MgCl2, and 1.25 unit of *Taq* polymerase. optimized PCR reaction at a 5 min of initial denaturation at 94°C, then five cycles of three steps: 1 min of denaturation at 94°C, 1 min of annealing at 35°C, 1 min of extension at 72°C, followed by another 30 cycles of annealing temperature rising to 50°C, with a final extension step of 5 min at 72°C. The PCR reactions have been performed at least 3 times and the products were resolved by eclectorphersis in 2.5% agarose gels with TAE buffer containing ethidium bromide **(Alasaad *et al*., 2008)**.

Each primer combination undergoes blast analysis through NCBI before performing PCR reactions for validation and to explorer the predicted segments and PCR product size.

Table 2. Primer combinations sequence designed for SRAP

Primer	Forward sequence	Reverse sequence
ME1-EM1	5`-TGAGTCCAAACCGGATA-3`	5`-GACTGCGTACGAATTAAT-3`
ME1-EM4	5`-TGAGTCCAAACCGGATA-3`	5`-GACTGCGTACGAATTTGA-3`
ME1-EM5	5`-TGAGTCCAAACCGGATA-3`	5`-GACTGCGTACGAATTAAC-3`
ME1-EM6	5`-TGAGTCCAAACCGGATA-3`	5`-GACTGCGTACGAATTGCA-3`
ME2-EM1	5`-TGAGTCCAAACCGGAGC-3`	5`-GACTGCGTACGAATTAAT-3`
ME2-EM3	5`-TGAGTCCAAACCGGAGC-3`	5`-GACTGCGTACGAATTGAC-3`
ME2-EM4	5`-TGAGTCCAAACCGGAGC-3`	5`-GACTGCGTACGAATTTGA-3`
ME2-EM5	5`-TGAGTCCAAACCGGAGC-3`	5`-GACTGCGTACGAATTAAC-3`
ME2-EM6	5`-TGAGTCCAAACCGGAGC-3`	5`-GACTGCGTACGAATTGCA-3`
ME3-EM1	5`-TGAGTCCAAACCGGAAT-3`	5`-GACTGCGTACGAATTAAT-3`
ME3-EM3	5`-TGAGTCCAAACCGGAAT-3`	5`-GACTGCGTACGAATTGAC-3`
ME3-EM4	5`-TGAGTCCAAACCGGAAT-3`	5`-GACTGCGTACGAATTTGA-3`
ME3-EM6	5`-TGAGTCCAAACCGGAAT-3`	5`-GACTGCGTACGAATTGCA-3`
ME4-EM1	5`-TGAGTCCAAACCGGACC-3`	5`-GACTGCGTACGAATTAAT-3`
ME4-EM3	5`-TGAGTCCAAACCGGACC-3`	5`-GACTGCGTACGAATTGAC-3`
ME4-EM5	5`-TGAGTCCAAACCGGACC-3`	5`-GACTGCGTACGAATTAAC-3`
ME5-EM3	5`-TGAGTCCAAACCGGAAG-3`	5`-GACTGCGTACGAATTGAC-3`
ME5-EM4	5`-TGAGTCCAAACCGGAAG-3`	5`-GACTGCGTACGAATTTGA-3`
ME5-EM5	5`-TGAGTCCAAACCGGAAG-3`	5`-GACTGCGTACGAATTAAC-3`
ME5-EM6	5`-TGAGTCCAAACCGGAAG-3`	5`-GACTGCGTACGAATTGCA-3`

Band scoring and cluster analysis

LabImage 1D Version 7.1.3 Core 4.2.3 **(Kapelan Bio-Imaging, Leipzig, Germany)** software was used for band scoring pattern analyses. The Systat ver. 7 computer programs were used to measure the pairwise differences matrix and plot the dendrogram among 22 individuals. Cluster analysis was based on similarity matrices obtained using the arithmetic average for dendrogram estimation with the unweighed pair-group method **(UPGMA)**.

RESULTS

In the present study, combinations of 20 SRAP primers were used for evaluating the genetic variation between the Mediterranean and Red Sea populations of green turtles (*Chelonia mydas*). The total scorable bands that resulted in all SRAP primers combination were 187 scorable bands, of which 182 bands were polymorphic, reflecting 97.3% polymorphism among the tested samples.

Ninety polymorphic bands were found within the Mediterranean population out of 122 total scrabble bands with 73.8% polymorphism (Table 3, Fig .2). The SRAP primer combinations that recorded the highest scorable bands number in Mediterranean population was ME4-EM1 with 14 bands followed by ME2-EM4 with 13 bands, while ME1-EM5 and ME2-EM5 recorded the lowest scorable bands (2 bands each) (table 3). The primer combinations that recorded the highest polymorphic bands were ME2-EM4 (13 bands) then ME3-EM1 (12 bands). While, ME1-EM5, ME2-EM5, ME5-EM4, and ME5-EM6 recorded the lowest polymorphic bands (one band each). No polymorphic bands were detected by ME1-EM1 (Table 3, Fig. 2).

In the Red Sea population, 95 polymorphic bands were detected out of 99 scorable bands, representing 96% polymorphism within this population (Table 3, Fig .3). The SRAP indicates that, the primer combination ME3-EM3 recorded the highest number of bands (12 bands) while ME2-EM3 recorded the lowest band number (6 bands). The SRAP primer combinations which recorded the highest number of polymorphic bands were ME3-EM3 (12 bands) compared with ME1-EM1, ME2-EM3, and ME5-EM6 that recorded 6 scorable bands each (Table 3).

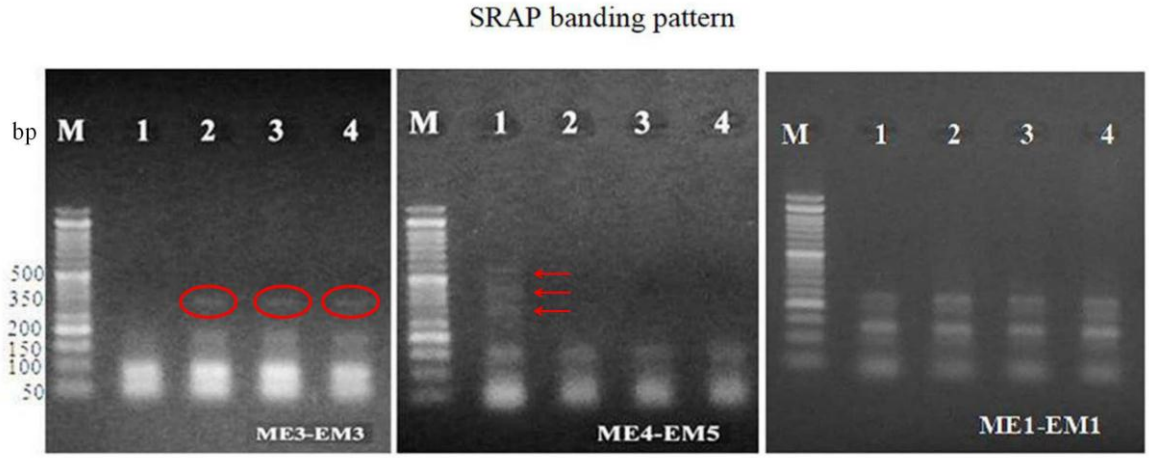

Fig. 2. SRAP banding pattern for green sea turtle (*Chelonia mydas*) in the Mediterranean population. Numbers from 1 to 4 represent individuals according to table 1. Marker used were 50bp DNA Ladder (ready to use).

Remarkably, within each studied population there was quite numbers of unique bands (Table 4). The data indicate that 48 genotypic-specific SRAP markers were detected among the Mediterranean population and 22 genotypic-specific SRAP markers were detected among the Red sea populations. The highest number of genotype-specific SRAP markers was generated by ME2-EM1 and ME3-EM1 with 6 unique bands in Mediterranean population; while within Red Sea population, the primer combination ME3-EM3 recorded the highest number of genotype-specific SRAP makers (6 unique bands).

Table 3. Total number of scorable bands, polymorphism % and band size of SRAP markers obtained by 20 SRAP primer combinations for both Mediterranean and Red Sea green turtle populations

Primers' combination	Within Mediterranean population				Within Red Sea population			
	S.	P.	P%	Amp. Size (bp)	S.	P.	P%	Amp. Size (bp)
ME1-EM1	4	0	0	150-250	7	6	86	200-550
ME1-EM4	-	-	-	-	10	10	100	100-1000
ME1-EM5	2	1	50	100-300	-	-	-	-
ME1-EM6	3	3	100	100-200	-	-	-	-
ME2-EM1	10	9	90	100-1500	11	10	91	100-1000
ME2-EM3	4	4	100	150-500	6	6	100	200-700
ME2-EM4	13	13	100	150-1250	10	10	100	100-700
ME2-EM5	2	1	50	150-350	-	-	-	-
ME2-EM6	4	3	75	125-600	-	-	-	-
ME3-EM1	12	12	100	100-1000	11	10	91	100-1500
ME3-EM3	3	1	33	100-350	12	12	100	100-1500
ME3-EM6	8	7	87.5	150-1200	-	-	-	-
ME3-EM4	5	2	40	100-800	-	-	-	-
ME4-EM1	14	10	71	100-1600	7	7	100	150-800
ME4-EM3	8	7	87.5	100-1000	-	-	-	-
ME4-EM5	4	3	75	100-600	8	8	100	100-500
ME5-EM3	12	9	75	100-1500	-	-	-	-
ME5-EM4	4	1	25	100-350	10	10	100	100-1220
ME5-EM5	5	4	80	200-1000	-	-	-	-
ME5-EM6	3	1	33	263-900	7	6	86	100-900
Total	**122**	**90**	**73.8**		**99**	**95**	**96**	

S: Scorable bands, P: Polymorphic bands, P%: Polymorphism %, Amp. Size: Amplicone size range.

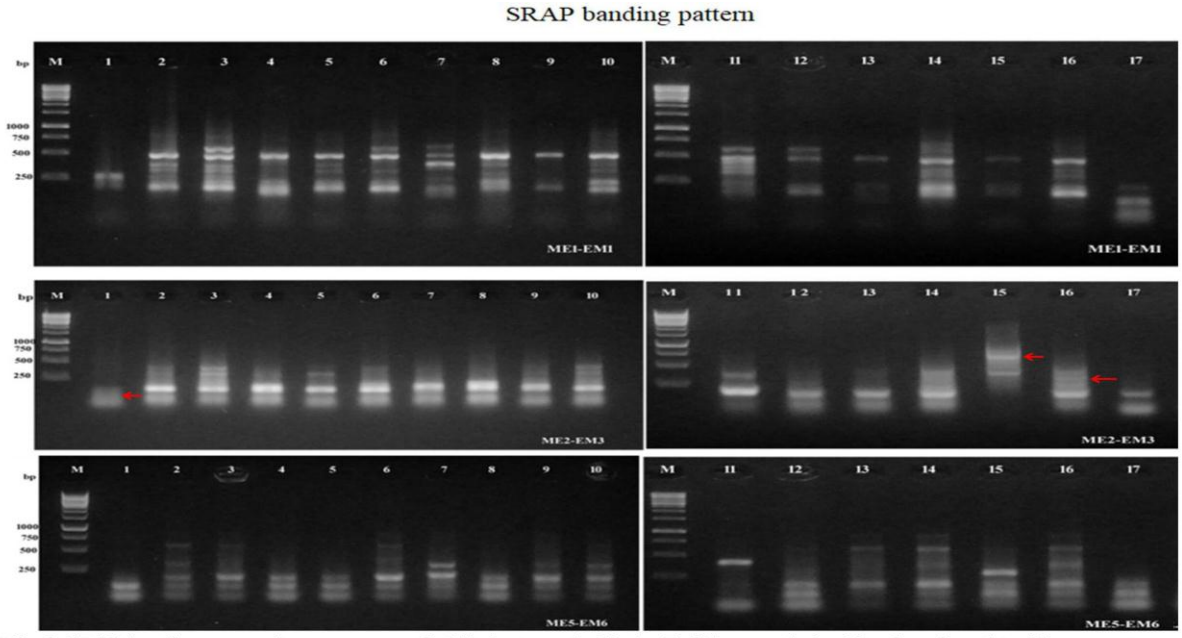

Fig. 3. SRAP banding pattern for green sea turtle (*Chelonia mydas*) in the Red Sea population. Numbers from 1 to 17 represent individuals according to table 1. Marker used were 1Kb DNA Ladder (ready to use).

Table 4. Genotypic specific SRAP markers within each population of the Mediterranean and Red sea green turtles

	SRAP markers (bp)	**Total number of unique markers**
Mediterranean	ME1-EM6 (200)	1
	ME2-EM1(200, 700, 900, 950, 1000, 1250)	6
population	ME2-EM3 (300, 500)	2
	ME2-EM4 (150, 200, 300, 400, 1250)	5
	ME2-EM5 (350)	1
	ME2-EM6 (300, 600)	2
	ME3-EM1(200, 220, 600, 700, 900, 1000)	6
	ME3-EM4 (400, 800)	2
	ME3-EM6 (500, 600, 1000)	3
	ME4-EM1(100, 250, 950, 1050, 1600)	5
	ME4-EM3 (100, 200, 1000, 800)	4
	ME4-EM5 (300, 400, 600)	3
	ME5-EM3 (300, 350, 600, 950, 1000)	5
	ME5-EM4 (250)	1
	ME5-EM5 (350, 1000)	2
Total		48
Red Sea	ME1-EM4 (750)	1
population	ME2-EM1(400)	1
	ME2-EM3 (150, 230, 500)	3
	ME2-EM4 (550, 600)	2
	ME3-EM1(125, 800, 900, 1500)	4
	ME3-EM3 (400, 600, 700, 800, 950, 1500)	6
	ME4-EM1(400, 800)	2
	ME4-EM5 (170, 400)	2
	ME5-EM4 (1220)	1
Total		22
Grand Total		**70**

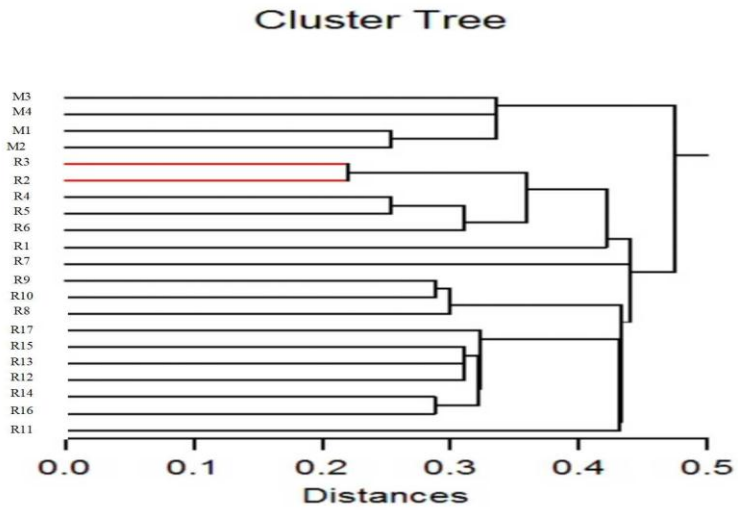

Fig. 4. Adendogram of all the examined individuals from both the editerranean (M1-M4) and Red Sea (R1-R17) populations.

Cluster analysis of the examined individuals with the 10 common primers' combinations produced a dendrogram, in which the similarity coefficient ranged from 0.22 to 0.47 (Fig .4). The examined individuals were divided into two major groups at the 0.47 similarity level; one group contained only the Mediterranean populations, while the other group was divided into three groups at similarity level 0.38. Within the Mediterranean population, M1 and M2 were clustered in one group followed M4, as they are from adjacent locations (Alexandria and El-Sahel) while M3 from Port Said. Also, there were individuals from the Red Sea populations that have showed distinguished and close relationship *i.e.* R2, R3, R4, R5, R8, R9, R10 from Zabargad island, R13, R14, R15, R16 from Ras Muhammad Protectorate and R17 from Hurghada.

DISCUSSION

Nuclear markers alongside mtDNA were always used to reveal the complete picture in the genetic variation between marine turtle populations, hence, increase the positive impacts of conservation efforts towards determined measures **(Bjorndal and Jackson, 2002; Matiddi *et al.*, 2017)**. Several types of makers such as RFLP **(Kaska, 2016)** and AFLP **(Roden *et al.*, 2009)** were used to examine variation/polymorphism in marine turtles even through mtDNA and nDNA.

The development of a set of SNP loci for marine turtles promises to be a rapid way of genotyping individuals **(Roden *et al.*, 2013)**, which can eliminate the variability of microsatellite genotypes due to differences in technology and scoring methods between laboratories **(Aitken *et al.*, 2004)**. These methods can be applied to a broad range of taxa to aid in a variety of applications including ecological, behavioural, forensic, and population structure studies.

SRAP markers have been widely used in plant for delimitation and assessment of variation within and between individuals and prove invaluable discoveries of polymorphism **(Daniel and Wolfe, 2014)**. The developed primers combinations developed by **Li and Quiros (2001)** were aimed to targeting exons in open reading frame (ORF), promoters and introns. **Abd El-Azim *et al.* (2019)** assert the success of the SRAP as molecular marker for estimating the genetic variability and relationship among Entomophatogenic nematodes. The PCR based SRAP markers revealing a high level of genetic diversity and many genetic relationships were defined between and within the populations of Arctic fox **(Zhang *et al.*, 2013)** and the giant river prawn **(Zhou *et al.*, 2006)**.

Using PCR based SRAP marker, giving opportunities to obtain good amplificons and subsequently identify genetic polymorphism. This study provides the first data of genetic variation using these markers in marine turtles that enabling identification of phylogenetic relationships among populations.

Tikochinski *et al.* (2018) showed that the population genetic analysis is polymorphism dependent, and the crucial selection of markers to identify variation between and among populations. Hence, PCR-based SRAP marker will help in identifying more polymorphism as discovered in this study. From this study, we could consider that the SRAP markers as an additive tool along with other techniques using a) mtDNA such as highly polymorphic haplotyping method **(Tikochinski *et al.*, 2018; Tolve *et al.*, 2018; Tikochinski *et al.*, 2020)**; and b) nDNA to reveal variation/polymorphism between marine turtle populations and then we would have a clear genetic structure of the green turtle population in Egypt.

SRAP marker were successfully used to assess genetic differentiation among 7 Arctic fox populations **(Zhang *et al.*, 2013)** that were found highly polymorphic and thus enabling identification of phylogenetic relationships among populations of *A. Lagopus*. SRAP successfully used as molecular markers for estimating the genetic variability and the genetic relationships among the Entomophatogenic nematodes **(Abde ElAzim *et al.*, 2019)** that can be used for identification of different Entomophatogenic nematodes species and isolates, which can be used in biological control programs. In addition, the SRAP marker was used for comparative analysis on genetic diversity of 4 populations of giant freshwater prawn (*Macrobrachium rosenbergii*) after introducing another population to overcome degenerated productivity due to inbreeding; providing an effective solution in breeding programmes **(Zhou *et al.*, 2006)**. Also, SRAP marker has been used to study genetic variation in the coding regions among *Fasciola hepatica* parasites from different host species and geographical localities in Spain with significance to human and animal health **(Alasaad *et al.*, 2008)** indicating that there seemed no genetic association between *F. hepatica* and their hosts and/or geographical locations in Spain.

The present study is considered as a preliminary experiment to generate new markers that include other segments along marine turtles' genome, as a tool to investigate new areas of variation/polymorphism between, among and within populations and individuals.

SRAP showed an easier and rapid technique to investigate genetic variation between and within Red sea and Mediterranean marine turtles populations' in Egypt; and individuals as well. Unique bands generated in this study by using SRAP combination primers could be used as an approach for genotyping individuals of the same species from different populations and to investigate their polymorphism and genetic diversity.

Results obtained from SRAP analyses indicated that there were variations between Mediterranean and Red sea populations with a high variation among individuals within the same population. There was significant polymorphism within the studied individuals/population. The diversity within the investigated individuals has been

recorded through presences of uniqueness of certain bands that referred as genotypic specific SRAP markers. The 70 recorded genotypic specific SRAP markers, showed significant diversity within investigated individuals that can be providing new information.

According to the similarity level, the cluster tree showed how much the Mediterranean and Red Sea populations are distinguished from each other into 2 completely different clusters. Besides, similarity between individuals from Ras Muhammad PA, Hurghada and some individuals from Zabargad showed distinguished and close relationship.

The present study implies that SRAP can be successfully used as molecular markers for estimating the genetic variability and the genetics relationship among and between green turtle populations.

CONCLUSION

The present study showed that SRAP can be successfully used as molecular markers for estimating the genetic variability and the genetics relationship among and between green turtle populations' in the Red sea and Mediterranean in Egypt; and to be an additive tools to detect genetic diversity within species with the most coomon and used techniques to reveal other genomic segments.

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