ISSN 2347-6893

Population genetic structure of Epinephelus marginatus in the Central Mediterranean Sea (Gulf of Gabès and the coast of Libya)

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ABSTRACT

In the present study, mitochondrial DNA sequences from the cytochrome b (cytb) gene and seven nuclear microsatellites were examined to assess the genetic diversity of *Epinephelus marginatus* inhabiting Tunisian and Libyan coastal waters. Based on 940 base pairs of the cytochrome b segment, we found low level of genetic variability for the two samples analysed (h = 0.294 ± 0.097and h = 0.274± 0.142 respectively in Tunisia and Libya). An analysis of molecular variance (AMOVA) showed significant pattern of genetic structure based on nuclear data (7 microsatellite loci) (ΦST= 0.28487; P < 0.001). Conversely, no genetic structuring was found for mtDNA (ΦST = -0.0121).

In summary, this study provides preliminary assessment of geographical patterns of differentiation of E. marginatus in this region for conservation, management and stock identification.

Indexing terms/Keywords

Genetic structure; Mitochondrial DNA; Cytochrome b gene; Microssatellite; Central Mediterranean Sea; Epinephelus marginatus

ISSN 2347-6893

1. INTRODUCTION

The genus *Epinephelus* of the Serranidae family contains 129 congeneric species inhabiting marine habitats around the world [1]. In the Mediterranean Sea, the dusky grouper *Epinephelus marginatus* (*E. marginatus*) is one of the seven species belonging to the genus *Epinephelus*. This species is absent from the Black sea. In the Atlantic, it is reported as far as the British Isles in the north, South Africa in the south and the Brazilian coast in the west [2] . The highest densities of the dusky grouper occur on the north and north western coasts of Africa, from Tunisia to Senegal [3]. These groupers have a great economic importance in the fishing industry and aquaculture. However, these bony fishes are most at risk, probably due to their large body size, long lifespan, late sexual maturity[4], overfishing, pollution and lack of ecosystem protection.

The dusky grouper *E. marginatus* (Lowe. 1834) is one of iconic species in the Mediterranean Sea. It is the only Serranidae species considered as endangered (Annex 3 of both Bern and Barcelona Conventions). As all groupers (sub-family Epinephelinae), the dusky grouper is a protogynous hermaphrodite with a complex and socially structured reproductive behaviour[5]; [6]. First sexual maturity is reached when females are 5 years old and 40–50 cm total length [3,6,7] to 6-7 years old and 36 cm total length [8]. While sex inversion occurs when individuals are 9 to 16 years old and 70–90cm in total length [3]. The species is reputed to be sedentary and territorial[9]; [10]; [11]; [12]; [13]. Many studies based on partially overlapping samples of dusky grouper have also found evidence of population differences[14]; [15]. Variation in mtDNA cytb sequences suggested differentiation among Algerian and French dusky groupers[16]. [14], and [17] analysed two different classes of molecular markers, allozymes and microsatellites, rejected the null hypothesis that Mediterranean dusky groupers are a single panmictic unit.

Identification guides based on morphological characteristics are available for the identification of almost all grouper species in the world [1]. Moreover misidentification between species is still common.

Molecular conservation genetics seeks to manage biological threats by protecting, maintaining and restoring unique species and their genetic diversity. The integration of population distribution mapping, identification of extrinsic environmental factor(s) and population genetic theory play a significant role in the qualitative and quantitative assessment of species status and determination of sustainable conservation strategies. Candidate organisms for molecular conservation genetic analysis typically have small fragmented populations and suffer from loss of genetic diversity due to inbreeding. This results in a decreased ability to evolve in response to stochastic events and thus a decline in population. For this reason, minimizing the loss of genetic diversity from inbreeding and isolation is a major objective in genetic conservation and management [18]. The purpose of this research is to describe and define the status of the Tunisian population of dusky groupers in order to best develop a comprehensive conservation management and monitoring strategy.

2. MATERIALS AND METHODS

2-1 Sampling and DNA extraction

Samples of *E. marginatus* were collected from two sites in the Central Mediterranean Sea (south of Tunisia (Zarzis) and north of Libya) (Figure 1). A total of fifty three specimens were analyzed for the mitochondrial cytb sequence and forty one of which have been also genotyped for seven microsatellites (Table 1).

A small piece of the dorsal fin or the tail (20-50 mg) from each specimen was excised with surgical scissors and preserved in absolute ethanol at -20°C until DNA extraction. Genomic DNA was extracted using the QIAGEN DNeasy tissue kit following the manufacturer's recommendations. Purity and concentration of DNA recovered were determined with a NanoDrop spectrophotometer.

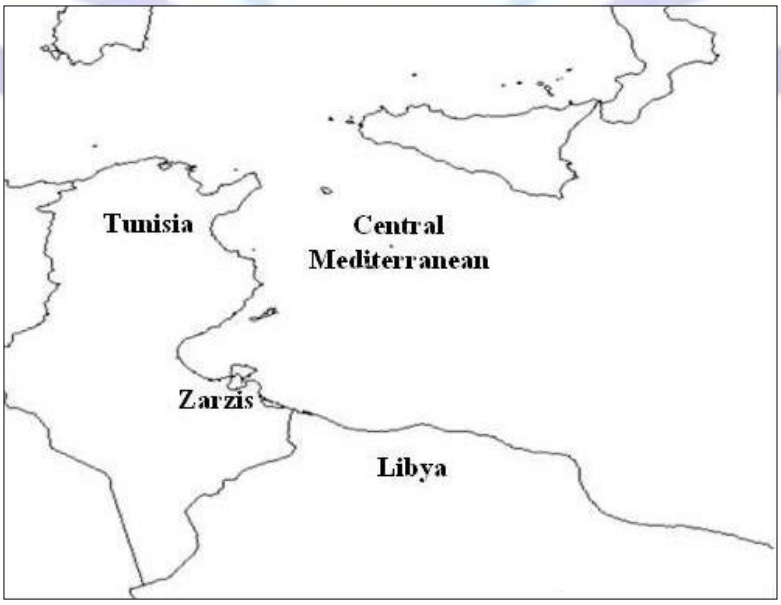


Figure 1: Location of sampling sit

ISSN 2347-6893

Table1: E. marginatus mitochondrial cytb haplotype frequencies for each site individually.

Haplotype	Variable sites	Tunisia (N=45)	Libya (N=15)	Total (N=60)
Hap_1	TCAAGCACTCT	1	–	1
Hap_2	TTAAGTACTCT	38	13	51
Hap_3	TTGAGCCCTCC	1	–	1
Hap_4	TTAAATACTCT	1	–	1
Hap_5	TTGAGCCCCCC	1	–	1
Hap_6	CTAAGCACTCT	1	1	2
Hap_7	TTAAGCACTCT	2	–	2
Hap_8	TTAGGCATTGT	–	1	1

2-2 Mitochondrial DNA amplification and sequencing

Polymerase chain reaction (PCR) was used to amplify a 940 bp fragment of the mtDNA cytb gene. A set of primer used was: 28For 5'- CGCCTGTTTATCAAAAACAT-3' described by [19] and EpiR 5'- CGCCTGTTTATCAAAAACAT-3' developed in this study. PCR amplification was carried out in 25 µL reaction mixtures containing 5 µL 5X GoTaq buffer, 2 µl 25 mM MgCl2, 2 µL dNTP (10 mM), 0.5 µL of each primer (10 µM), 0.2 µL (5U) GoTaq® DNA Polymerase (Promega), 2 µL DNA template and 13.3 µL ddH2O (Invitrogen). Cycling parameters were an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation (95°C for 1 min), annealing (52°C for 45 sec), and extension (72°C for 1 min) with the final extension step at 72°C for 10 min.

To control for contamination due to handling, a PCR negative control was run in all PCRs.

The PCR products were separated on a 1% agarose gel to confirm the amplification. All the positive PCR products were purified with the QIAquick PCR purification Kit (Qiagen), following manufacturer's instructions and sequenced on an ABI Prism 310 genetic analyser (Applied Biosystems) in both directions with the forward and reverse primers used for amplification.

2-3 Mitochondrial DNA sequence analysis

Sequences were aligned with CLUSTAL W algorithm [20] as implemented in the software BioEdit v. 7 [21].

Estimates of the number of polymorphic sites (S), number of haplotypes (K), nucleotide diversity (π), and haplotype diversity (h) were obtained using the software DNASP, ver. 5.10 [22] and Arlequin ver. 3.5 [23].

The genetic relationships amongst haplotypes were investigated by a median-joining network using the softwares NETWORK Ver. 4.6 .1.1[24] and Network Publisher ver.1.2.0.0 (www.fluxusengineering.com) software.

The genetic differentiation among the two selected populations (Tunisia - Libya) was analyzed through pairwise estimates of ΦST, the significance of which was tested in 1000 permutations.

2-4 Microsatellite genotyping

Seven microsatellite loci were amplified using primer pairs originally developed for *Mycteroperca microlepis* (GAG007, GAG010, GAG038, GAG045, GA049; [25] and for *Epinephelus merra* (Em-03, Em-08; [26]. One primer of each pair was 5'- labelled with FAM or HEX. Polymerase chain reactions (PCRs) were carried out in 12 µL volumes comprising 3.8 µL ddH2O, 6 µL Multiplex Mix Qiagen 2X, 10 µM forward and reverse primer, and 25 ng genomic DNA. Reaction profiles consisted of an initial 15 min denaturation step at 95°C followed by 35 cycles at 95°C for 30 sec, primer-specific annealing temperature for 90 sec at 60 °C and at 72°C for 1 min extension, with a final extension step at 60°C for 30 min. Following PCR, three or four amplified loci (differing in fluorochrome labelling or allelic range) for each individual were mixed to be co-scored (multiplexed), for this 0.5 µL of each product, 3.5 µL formamide, 0.7 µL of 50 mm EDTA, 0.05 µL dextran blue and 0.3 µL Tamra 500 internal size standard. Each sample was heated to 92°C for 10 min, snap-cooled in ice water, electrophoresed on a 6% denaturing polyacrylamide gel using an ABI Prism 3130 genetic analyser (Applied biosystems).

ISSN 2347-6893

2-5 Analysis of microsatellite variation

Microsatellite alleles were scored using GeneMapper version 3.7 (Applied Biosystems). Genetic diversity was evaluated using allele frequencies, observed (Ho) and unbiased expected heterozygosity (He) calculated in GENEPOP'007 [27]. The software Micro-Checker[28] was used to test for technical artefacts such as null alleles. Deviations from the Hardy–Weinberg Equilibrium (HWE) were tested using the inbreeding coefficient Fis [29] implemented in Genetix 4.05 software

[30].

3. RESULTS

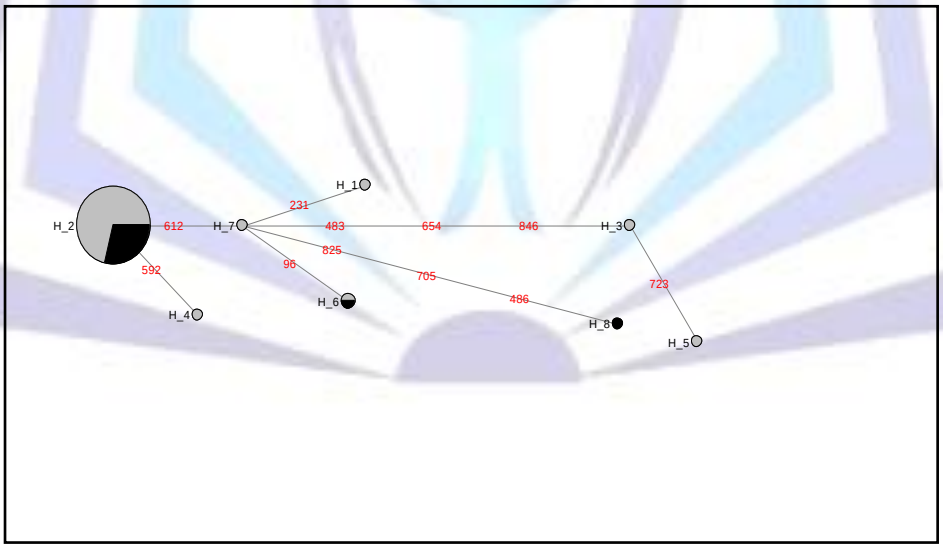
3-1 Mitochondrial DNA genetic variation

Of the 849 pb of the mitochondrial cytb gene from 53 sequenced *E. marginatus*, 11 nucleotide positions were polymorphic, 6 of which were parsimony informative sites. A total 8 distinct haplotypes were found. The species exhibit low to moderate haplotype diversity (0.294 ±0.097 and 0.257 ±0.142, respectively the Tunisian and Libyan sample).

A median joining (MJ) network was used to depict the evolutionary relationships among the 8 unique haplotypes identified here using NETWORK as shown in Figure 2. In Figure 2, circles represent individual haplotypes (different colors) and sizes of circles indicate relative frequencies of each haplotype in the sampled populations (Table 1 contains actual frequencies). Single mutational changes are presented as lines among haplotypes. The resolved network of phylogenetic relationships among the 8 cytb haplotypes revealed a star-like pattern, with many haplotypes originating from the most abundant haplotype (H2) (Figure 2). Among the eight distinct haplotypes found here (H1– H8), two of them (H2 and H6) were shared by the two sampling sites. The most common haplotype (H2) was represented in 85% of specimens, whilst the other shared haplotype (H6) was observed in a smaller proportion (4%). The remaining haplotypes (11%) were location private (Figure 2).

The overall estimate of genetic divergence was not significant (ΦST = -0.01216) (Table 2). Results indicate that no obvious genetic structure was apparent among the sampled *E. marginatus* populations based on the mtDNA cytb region sequence data and most genetic variation was present within sampled populations. *E. marginatus* population pairwise ΦSTestimates were calculated based on 10,000 permutations (Table 2), and suggested that gene flow was ongoing among sites.

Figure 2: Median-joining network of haplotypes of *E. marginatus*. Size of circles is proportional to the frequency of each haplotype



ISSN 2347-6893

Source of Variation	d.f.	Var	var (%)
mtDNA			
Among sites	1	-0.00421	-1.22
Within sites	58	0.35019	101.22
Total	59	0.34599	
Fixation Index FST :	-0.01216 (P ; N.S)		
Microsatellites			
Among sites	1	0.06518	28.49
Within sites	80	0.16363	71.51
Total	81	0.22881	
Fixation Index FST :	0.28487 (P < 0.001)		

Table 2: Results of analysis of molecular variance (AMOVA) for mitochondrial DNA and microsatellite

Degrees of freedom (d.f.), variance components (var), percent variation (var %) and F-statistics to test for evidence of genetic differentiation among *E. marginatus* populations using mitochondrial DNA and microsatellites. N.S: Not significant

3-2 Microsatellites genetic variation

Allele frequencies of the polymorphic loci (Am) are listed in Table 3. Among the seven microsatellite loci, the number of alleles per locus across all populations ranged from two (Em-03) to fifteen (GAG045). Alleles fixation (gene frequency = 1) was observed in Tunisia at Em-08*199 locus and in Libya at Em-03*152 locus. The average number of alleles per locus was 6.28 and 3.28 for the Tunisian and Libyan population respectively. At a 95% level, the percentage of polymorphism was 71 % and 85 % respectively for the Tunisian and Libyan population.

The observed (Ho) and expected (He) heterozygosity for each locus and each sample are shown in Table 3. The two populations analyzed (Tunisian and Libyan) have the same rate of heterozygosity and for the global sample expected heterozygosity is equal to 0.5 ± 0.35.

An applied test on the two samples showed significant departure from Hardy-Weinberg equilibrium (FIS= 0.177; P< 0.001), FST value (0.024; p<0.05) calculated according to Weir and Cockerham (1984). The Libyan sample appears to be in equilibrium for all loci; however, the Tunisian sample reveals heterozygote deficiency. The contribution of each locus to the deviation from the panmixia was tested by the Jackknife test (Table 4). Comparing the total value of FIS and FST subtracting each time one of seven loci, the FIS values for each locus show heterozygote deficiciency for loci GAG045, and the FST values for each locus show heterozygote deficiency for loci GAG007. The computation of Nei's distance samples gave a low value (0.035).

ΦST analysis showed significant genetic structure at the level of the whole study (ΦST = 0.28487; P < 0.001).

Table 3: Allelic variability at seven microsatellite loci in two E. marginatus populations from the Mediterranean Sea (Tunisia and Libya). Number of individuals (Nb), expected (He) and observed (Ho) heterozygosity, inbreeding coefficient (Fis).

Locus	Samples	Tunisia	Libya	All samples
Nb		32	9	41
Em-03	152	0.9643	1.0000	
	153	0.0357	0.0000	
	He	0.0689	0.0000	
	Ho	0.0000	0.0000	

	Fis	1		1
Em-08	198	0.0000	0.0556	
	199	1.000	0.0944	
	He	0.0000	0.1049	
	Ho	0.0000	0.1111	
	Fis		0	-0.03
GAG010	107	0.1481	0.1111	
	117	0.0556	0.2222	
	119	0.3148	0.4444	
	121	0.2963	0.2222	
	126	0.0556	0.0000	
	130	0.0556	0.0000	
	134	0.0741	0.0000	
	He	0.7764	0.6914	
	Ho	0.5926	0.7778	
	Fis	0. 254	-0.067	0.181
GAG049	81	0.0185	0.0000	
	85	0.0556	0.0000	
	87	0.3519	0.3889	
	89	0.0741	0.1111	
	91	0.1852	0.2778	
	94	0.3148	0.2222	
	He	0.7339	0.7099	
	Ho	0.8889	0.8889	
	Fis	-0.193	-0.196	-0.194
GAG007	141	0.0625	0.1250	
	146	0.8438	0.4375	
	151	0.0938	0.4375	
	He	0.2754	0.6016	
	Ho	0.2500	0.8750	
	Fis	0.108	-0.311	-0.07
GAG038	68	0.0161	0.0000	
	69	0.0323	0.0000	
	71	0.1290	0.2143	
	73	0.3710	0.3571	
	76	0.0484	0.0000	
	77	0.1129	0.0714	
	80	0.0484	0.0000	
	82	0.0484	0.1429	
	84	0.0645	0.0714	
	86	0.1290	0.1429	
	He	0.8039	0.7755	
	Ho	0.6129	0.5714	

	Fis	0.253	0.333	0.267
GAG045	71	0.0333	0.0000	
	77	0.2167	0.2500	
	80	0.0333	0.0000	
	82	0.1833	0.2500	
	86	0.0333	0.0000	
	89	0.0667	0.0000	
	97	0.0333	0.0000	
	100	0.2000	0.5000	
	102	0.0167	0.0000	
	104	0.0333	0.0000	
	106	0.0167	0.0000	
	108	0.0833	0.0000	
	116	0.0167	0.0000	
	120	0.0167	0.0000	
	127	0.0167	0.0000	
	He	0.8611	0.6250	
	Ho	0.4667	0.3750	
	Fis	0.471	0.455	0.469
Total loci	Am	6.28	3.28	
	P (95%)	0.71	0.85	
	He	0.5±0.37	0.5±0.31	
	Ho	0.40±0.33	0.51±0.36	0.177
	Fis	0.218*	0.040 NS	

Table 4: values of FST and FIS after the Jackknife test on each locus

Loci	FIS	FST	Probability
Without Em-03	0.16466	0.02566	P <0.001
Without Em-08	0.17891	0.02417	P <0.001
Without GAG010	0.17615	0.03194	P <0.001
Without GAG049	0.27326	0.03413	P <0.001
Without GAG007	0.20425	-0.01226	P <0.001
Without GAG038	0.15078	0.04272	P <0.001
Without GAG045	0.08858	0.02912	P <0.001
mean	0.18153	0.02145	P <0.001
Ecart-type	0.12650	0.03984	

ISSN 2347-6893

4-DISCUSSION

Species identification of grouper is problematic, since morphological traits overlap among species[1]. Molecular genetic markers have been used to resolve taxonomic ambiguity in many taxa [31] including fishes[32].

The purpose of this preliminary study was to obtain a general view of *E. marginatus* genetic structure of the south Tunisian and eastern Libyan coasts and especially on both sides of a boundary area betwen eastern and western mediterranean basins. In this context, analysing populations of marine organisms like dusky grouper *E. marginatus* is of particular interest, as it allows us to investigate the consequences of divergent biotic and abiotic conditions on population's differentiation.

The pattern of genetic diversity can be attributed to a recent population expansion after a low effective population size which has been caused by bottlenecks or founder events[33]. In the case of *E. marginatus*, a star-like network structure based on cytb haplotype showed recent démographic expansion. When analyzing patterns of genetic differentiation at mtDNA data, a low level of genetic variability was showed for the two *E. marginatus* populations. As expected, levels of genetic variability revealed by microsatellite loci were much higher. Observed and expected heterozygosities for microsatellites are shown in Table 2. The expected heterozygosities (He=0.5), for microsatellites that we observed in *E. marginatus* were comparable to those in other marine teleosts analysed with the same techniques. For example, average value of He for microsatellites is 0.48–0.66 in five other species of *Epinephelus* [26], 0.5-0.6 in *Epinephelus coioides* [34], and Ho = 0.54 for *Epinephelus* Quernus, [35]. But despite the dispersal ability of their pelagic larvae, which enhanced substantial gene flow, level of heterozygosity of epinephelin fishes, which are rather sedentary, was relatively low compared to the migratory fishes, such as cod (He = 0.898) [36], red sea bream (Ho = 0.808) [37], and king fish (Ho = 0.729) [38]. Although we did not observe particularly low levels of diversity, temporal replicates would be needed to assess the trend in genetic variability over time and to determine if Mediterranean dusky groupers are experiencing a genetic decline associated with their observed reduction in numbers. Moreover, due to the length of the generation time, any such decline would be likely to be delayed in time before becoming detectable [39]).

Our microsatellite FST values were similar or slightly higher than those obtained using microsatellites in some other marine species that show significant differentiation, such as *E. marginatus* (FST = 0.018; [17]), Atlantic cod (FST = 0.015; [36]), European hake (FST = 0.013; [40]). Two other studies based on partially overlapping samples of dusky grouper have also found evidence of population differences. Variation in mtDNA cytochrome b sequences suggested differentiation among Algerian and French dusky groupers [16;14].

Many hypotheses can be advanced to explain such heterozygotes deficiency: selective forces against heterozygote genotypes, crossing system, presence of null alleles and populations substructure (Wahlund effect). The latter can be accentuated with gene flow restricted by presence of geographic, ecological or biological barriers. The positive FIS observed in our samples might be interpreted as the result of inbreeding and thus of reduced Ne. The FIS values observed tended to be higher in those populations collected over a wide geographical area, we could interpret this variance within samples as a Wahlund effect, thus reinforcing the hypothesis of a spatial structure.

Although our data for microsatellites support the conclusion that the Central Mediterranean dusky groupers are not panmictic. Our study is preliminary, because we used only two samples of population

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