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Genetic variation of *Diplodus sargus* and *Diplodus vulgaris* in four Mediterranean coastal regions of Egypt based on microsatellites

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ABSTRACT

The use of microsatellite is important for determination of regional patterns of genetic connectivity between marine populations which is necessary for proper geographical scale setting.The objective of this study is to employ cross-species primers from *Sparus aurata* in *D. sargus* and *D. vulgaris* in four Mediterranean coastal regions of Egypt for studying their genetic diversity, population differentiation in nearby regions.

Four microsatellite markers (SaI10, SaI12, SaI19 and SaI21) were used among 112 *Diplodus sargus* and *Diplodus vulgaris* species. The samples represented four Mediterranean coastal regions located from 31.22° N to 31.62° N and from 29.88° E to 30.85° E. Variable levels of genetic diversity were observed in the four studied regions, SaI19 locus had no heterozygosity in all populations, and better heterozygosity was observed in all loci with SaI10 and SaI21 in *D. sargus* and *D. vulgaris* respectively. A low level of variation in both fishes among the four regions was observed amounting to 5% in *D. sargus* and 8% in *D. vulgaris*. Meanwhile, higher levels of variation between individuals within populations were observed amounting to 56% in *D. sargus* and 71 % in *D. vulgaris*. In this study the levels of genetic variability observed in *D. sargus* (0.048) and in *D. vulgaris* (0.078) were lower than those observed in other Sparidae fishes assessed with microsatellites.In conclusion, three microsatellite loci (SaI10, SaI12, and SaI21) can be applicable in *D. sargus* and *D. vulgaris* and could be used in other species of family Sparidae.

INTRODUCTION

Diplodus sargus (White seabream) and *Diplodus vulgaris* (the common two-banded seabream) are commercially important fish species, belonging to Sparidae family. The *Diplodus* genus is the largest of family Sparidae, containing 23 species (e.g., *D. sargus*, *D. vulgaris*, *D. annularis*, *D. cervinus*, *D. noct*, *D. puntazzo*) **(Fishbase, 2016)**. They are distributed in the Eastern Atlantic Ocean and along the Mediterranean Sea **(Whitehead *et***

***al.*, 1986; Fischer *et al.*, 1987)**. Many authors reported different features of their ecology and biology **(Rosecchi, 1987; Garcia and Macpherson, 1995; Macpherson *et al.*, 1997; Macpherson, 1998; Vigliola and Harmelin-Vivien, 2001)**, reproduction **(Morato *et al.*, 2003; Pajuelo and Lorenzo, 2004)** and feeding habits **(Zander and Sötje, 2002)**. Environmental conditions of Sparidae have also been studied by **Planes *et al.*, (1997)** and **Lloret and Planes (2003)**, where they showed that they are omnivorous fish, prefer small molluscs and crustaceans, and would also consume algae and small corals. Marine environment at the Mediterranean tends not to have major distinguished boundary lines with homogeneous marine species, nevertheless, it still shows varying levels of population differentiation **(Bianchi and Morri, 2000)**.

Several techniques were used for the identification of fish species of family Sparidae, including morphological traits **(Whitehead *et al.*, 1986)**, amylase activity present in the gut of different fish species **(Fernández *et al.*, 2001)**, allozymes variation **(Alarcón and Alvarez, 1999)**. DNA sequence analysis, isoelectric focusing (IEF) of water-soluble proteins and Single strand conformation polymorphism (SSCP) were also used **(Schiefenhövel and Rehbein, 2013)**. DNA Inter-Simple Sequence Repeat (ISSR) markers were also used for the identification of *Diplodus* spp. **(Casu *et al.*, 2009)**. Moreover, random amplified polymorphic DNA (RAPD) method, was used in this area **(Ali *et al.*, 2004)**. The application of restriction fragment length polymorphism (RFLP) was applied to distinguish between many fish species **(Cocolin *et al.*, 2000)**. DNA barcoding was applied to Family Sparidae **(Armani *et al.*, 2015; Abbas *et al.*, 2017, 2018)**. Phylogeography and genetic structure have been genetically studied in several species of genus *Diplodus* **(Planes and Lenfant, 1996; González-Wangüemert *et al.*, 2007; Kaouèche *et al.*, 2011; Mercedes González-wangüemert *et al.*, 2011; El-deeb *et al.*, 2014; Abbas *et al.*, 2017, 2018)**.

Microsatellites, is another important genetic marker related to the evolution of ecology and natural systems. Microsatellites are simple sequence repeats (SSRs) that consist of 2-6 nucleotides long **(Field and wills, 1996)**. These markers are important for the recognition of individuals, parentage and also for detecting mating success in multifarious breeding systems, as in Sparidae family **(Brown *et al.*, 2005)**. He used six new microsatellite markers in *Sparus aurata* and cross-species amplification within the Sparidae family in Greece. Also, **Roques *et al.*, (2007)** characterized nine polymorphic microsatellite markers in *Diplodus vulgaris* and *Diplodus sargus* in western Mediterranean Sea. Additionally, **Pérez *et al.*, (2008)** identified another eight microsatellite markers and used them in wild population of *Diplodus sargus* from Spain.

Cooke *et al.*, (2016) estimated 810–11,692 km broad confidence intervals for the spatial scale gene flow at marine fish communities needed for a meaningful evolution, nevertheless, they also added that short distances could also be matter for some particular taxa. Additionally, **Planes and Lenfant (1996); González-Wangüemert *et al.*, (2004, 2007); Pérez-Ruzafa *et al.*, (2006)** detected a significant differentiation between white sea bream population at different spatial ranged from 101–103 km which is similar to values found between populations separated by 10^2 km. However, **Roques *et al.*, (2007)** suggested that, analysis of genetic populations' by using microsatellite is important for determination of regional patterns of genetic connectivity between marine populations which is necessary for proper geographical scale setting.

Therefore, the goal of the present work was to employ cross-species primers from *Sparus aurata* in *D. sargus* and *D. vulgaris* in four Mediterranean coastal regions of Egypt for studying their genetic diversity, population differentiation in nearby regions.

MATERIALS AND METHODS

Samples collection and identification

Diplodus sargus and *Diplodus vulgaris* were collected from four different locations in Egypt. These locations are Lake Burullus (31.62° N, 30.85° E) Bahari (31.22° N, 29.88° E), Abo Qir (31.31° N, 30.09° E) and Rashid (31.51° N, 30.34° E). The map of the study area was shown in Figure1.

Figure 1. The map of the study area was accessed from http://www.gmapgis.com

Amplification of microsatellites

Genomic DNA was extracted from muscles of 112 fish (56 fish samples from *D.sargus* and 56 fish samples from *D. vulgaris*) by Phenol-Chloroform technique **(Fan and Gulley, 2001; Cseke *et al.*, 2011; Zhou *et al.*, 2015)**. DNA was used for the amplification of four microsatellite markers by using SaI10, SaI12, SaI19 and SaI21 primers **(Brown *et al.*, 2005)**.

The primer sequences of these four microsatellite markers with their annealing temperature, GenBank accession numbers and their length of repeats were shown in Table 1. Total PCR volume was 10 µl, consisting of: 20 ng genomic DNA template, 0.75 µmole (0.4 µl) forward and reverse primers, 5 µl Bioline MyTaq™ Red master mix. PCR amplification was performed at a temperature ranged from 60 °C to 62 °C, and the exact annealing temperature (T_a) for each locus were listed in Table 1. The reactions were done in Applied Biosystems verity 96 wells thermal cycler using the following programme: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 50 s at 95 °C, 50 s at the corresponding primer T_a and 1 min at 72 °C; a final extension for 7 min at 60 °C. Gel electrophoresis was performed according to **(Surzycki, 2000)**. The gel was visualized and photographed by the Gel Documentation system (GelStudio Digital Compact, Biometra, UK).

Table 1. Primer sequences of four microsatellite markers, annealing temperature, GenBank accession number and their repeat structure.

Locus name	Repeat structure	Primer sequence (5′–3′)	Annealing Temp.	GenBank accession no.
SaI10	$(GT)^{37}$	F: TCACGGGGGACCAAGACTG R:CTCACACTGCCTAATTAGCACAGA	62 °C	AY322107
SaI12	$(GT)^{30}$	F: ACGGTATGGAGTCAACTGC R: CCCCTTTTGGTACATCATAG	60 °C	AY322108
SaI19	$(GT)^{25}$	F: ATTCTTCACAGGCCCAACACAAA R: GAAAACACCGGCCCAGTACGA	60 °C	AY322111
SaI21	$(GT)^{41}$	F: GGACGCCACACCATGTTCA R: ACCGAAGCTGATTGTTAGTGTGA	60 °C	AY322112

Data analysis

Each microsatellite locus was scored in all *D. sargus* and *D. vulgaris* individuals using BioDocAnalyze 2.2 software (Biometra). Genetic diversity within sampling locations was measured by calculating the allele frequencies using GenAlEx 6.5 **(Peakall and Smouse, 2012)**. Hardy-Weinberg values of the four examined microsatellite markers were tested by Chi square method, and other AMOVA tests was performed by ARLEQUIN v3.5 software **(Excoffier and Lischer, 2010)**.

RESULTS

Amplification of microsatellite loci in *Diplodus sargus*

For all 56 samples of *D. sargus* isolated from Lake Burullus, Bahari, Abo Qir and Rashid, the presence of the microsatellite loci (SaI10, SaI12, SaI19 and SaI21) was determined by PCR reaction. The Genetic diversity within sampling locations was summarized in Table 2. The number of alleles per locus ranged from 3 to 19. The observed heterozygosity ranged from 0 (no heterozygosity) to 1 (for a system have a large number of equally frequent alleles). SaI19 locus had no heterozygosity in all populations which means that SaI19 locus had no genetic variability between populations. SaI10 locus had high heterozygosity in all populations that equals 1 in Lake Burullus. SaI12 locus had moderate to high genetic variability in Bahari, Abo Qir and Rashid but low genetic variability in Lake Burullus. SaI21 locus had low genetic variability in Abo Qir and Rashid but it had moderate genetic variability in Lake Burullus and Bahari.

Table 2. **Results of the amplification of four microsatellite loci (SaI10, SaI12, SaI19 and SaI21) in *D. sargus* from 4 different locations.**

Sample location*	Locus	*N*	Allele size range	No. of alleles	H_O	H_E
Lake Burullus	SaI10	12	169-243	19	1.000	0.941
	SaI12	3	112-162	3	0.333	0.500
	SaI19	12	223-249	7	0.000	0.847
	SaI21	8	177-264	10	0.500	0.875
Bahari	SaI10	13	164-286	19	0.846	0.938
	SaI12	5	112-207	7	0.800	0.840
	SaI19	14	223-271	10	0.000	0.878
	SaI21	10	282-186	15	0.600	0.925
Abo Qir	SaI10	13	185-230	11	0.846	0.840
	SaI12	7	94-195	10	0.571	0.888
	SaI19	14	241-265	4	0.000	0.704
	SaI21	8	72-226	10	0.250	0.891
Rashid	SaI10	14	186-237	10	0.786	0.870
	SaI12	12	153-240	19	0.833	0.941
	SaI19	14	238-282	9	0.000	0.878
	SaI21	10	130-336	12	0.200	0.910

* = 14 individuals were used from four different locations (Lake Burullus, Bahari, Abo Qir and Rashid). *N* = the number of successful samples, H_O = observed heterozygosity, H_E = Expected heterozygosity.

The analysis of molecular variance (AMOVA) showed little genetic differentiation among locations (F_{st} = 0.048; *P* = 0.001). Genetic variation in the studied populations was mainly due to individual variation within populations with a percentage of variance of 56%. Individual-level variation among the population represented 39% of the variation while variation between populations was 5% (Table 3).

Table 3. Analysis of Molecular Variance (AMOVA) within and among *D. sargus* populations.

Source of variation	Variance component	Percentage of variation	Fixation indices	Probability (*P*)
Among populations	0.087	5%	F_{st} = 0.048	0.001
Among individuals within populations	1.003	56%	F_{is} = 0.590	0.001
Within individuals among all populations	0.696	39%	F_{it} = 0.610	0.001

Hardy-Weinberg values of the four examined microsatellite markers were shown in Table 4. Genotype frequencies at all four loci, except SaI12 locus, indicate an overall departure from Hardy-Weinberg expectations in two or more loci per location, which was attributed to the heterozygosity of the SaI19 and SaI21 loci. However, a significant HW values in all the populations were observed with the exception of the locus SaI21 in the Lake Burullus population. Hardy-Weinberg value of SaI12 locus was not significant in all populations, this means that SaI12 locus is in Hardy-Weinberg equilibrium.

Table 4. Hardy-Weinberg values (HW) of four microsatellite markers used in *D. sargus* isolated from 4 different locations.

Locus	Lake Burullus	Bahari	Abo Qir	Rashid
SaI10	Ns	ns	0.000	0.013
SaI12	Ns	ns	ns	ns
SaI19	0.000	0.000	0.000	0.000
SaI21	Ns	0.049	0.006	0.001

ns = not significant, significant value when $p < 0.05$.

Amplification of microsatellite loci in *Diplodus vulgaris*

The presence of the microsatellite loci (SaI10, SaI12, SaI19 and SaI21) were detected in 56 individuals of *D. vulgaris* from different locations including Lake Burullus, Bahari, Abo Qir and Rashid**.**

The Genetic diversity within sampling locations were summarized in Table 5. The number of alleles per locus ranged from 1 to 14. The observed heterozygosity ranged from 0 to 0.857.

SaI10 had moderate genetic variability in *Lake Burullus* population, low genetic variability in Bahari and no heterozygosity in both Abo Qir and Rashid. SaI12 had high genetic variability that reached 0.857 in Lake Burullus, moderate genetic variability in Bahari, low genetic variability in Abo Qir and no heterozygosity in Rashid. SaI19 locus had no heterozygosity in all populations which means that SaI19 locus had no genetic variability between populations. SaI21 had moderate genetic variability in Lake Burullus, low genetic variability in both Bahari and Abo Qir and High genetic variability in Rashid.

The results of molecular variance (AMOVA), was summarized in Table 6. Little genetic differentiation among locations were observed (F_{st} = 0.048; P = 0.001). Genetic variation in the studied fish was mainly due to the individual's variation within populations with a percentage of variance of 71%. Individual-level variation (individual relative to all individuals) represented 21% of the variation while variation between populations (a population relative to other populations) was 8%.

Table 5. Results of the amplification of four microsatellite loci (SaI10, SaI12, SaI19 and SaI21) in *D. Vulgaris*.

Sample location*	Locus	*N*	Allele size range	No. of alleles	H_O	H_E
Lake Burullus	SaI10	14	153-199	7.000	0.500	0.753
	SaI12	7	100-163	12.000	0.857	0.908
	SaI19	14	233-248	8.000	0.000	0.837
	SaI21	7	154-246	10.000	0.571	0.888
Bahari	SaI10	14	168-223	4.000	0.286	0.589
	SaI12	5	102-191	7.000	0.600	0.840
	SaI19	14	238-260	10.000	0.000	0.888
	SaI21	7	174-323	7.000	0.143	0.847
Abo Qir	SaI10	14	239-265	6.000	0.000	0.755
	SaI12	6	104-194	8.000	0.333	0.861
	SaI19	14	244-268	8.000	0.000	0.806
	SaI21	11	152-268	14.000	0.364	0.921
Rashid	SaI10	14	225-284	6.000	0.000	0.806
	SaI12	1	182	1.000	0.000	0.000
	SaI19	14	223-282	11.000	0.000	0.898
	SaI21	11	143-316	17.000	0.727	0.930

* = 14 individuals were used from four different locations (Lake Burullus, Bahari, Abo Qir and Rashid). *N* = the number of successful samples, H_O = observed heterozygosity, H_E = Expected heterozygosity.

Table 6. Analysis of Molecular Variance (AMOVA) within and among *D. vulgaris* populations.

Source of variation	Variance component	Percentage of variation	Fixation indices	Probability (*P*)
Among populations	0.133	8%	F_{st} = 0.078	0.001
Among individuals within populations	1.207	71%	F_{is} = 0.776	0.001
Within individuals among all populations	0.348	21%	F_{it} = 0.793	0.001

Hardy-Weinberg values of the four examined microsatellite markers are shown in Table 7. An overall departure from Hardy-Weinberg expectations in two or more loci per locations was observed in the genotype frequencies at all four locations. This departure is due to a heterozygosity at SaI10, SaI19 loci that showed significant HW values in all populations. The HW expectations showed non-significant values in SaI12 locus of fish samples from Lake Burullus and Bahari. Meanwhile, SaI12 locus was monomorphic in fish species taken from Rashid (only one successfully amplified sample with one allele). SaI21 in Lake Burullus and Rashid populations showed non-significant values.

Table 7. Hardy-Weinberg values (HW) of four microsatellite markers used in *D. vulgaris* isolated from 4 different locations.

Locus	Lake Burullus	Bahari	Abo Qir	Rashid
SaI10	0.045	0.002	0	0
SaI12	ns	ns	0.043	Monomorphic
SaI19	0	0	0	0
SaI21	ns	0.023	0.003	ns

ns = not significant, significant value means $p < 0.05$.

DISCUSSION

The genotype data collected on *D. sargus* and *D. vulgaris* from the four geographical sites (Lake Burullus, Bahari, Abo Qir and Rashid) indicate sharing of the genetic material among populations as the F_{st} value = 0.048 in *D. sargus* and 0.078 in *D. vulgaris*. **Roques *et al.* (2007)** used nine microsatellite markers for *D. vulgaris* to test the cross-species amplification in *D. sargus* and *Oblada melanura.* Their results indicated that there were close phylogenetic relationships between the three species which agreed with our results.

In the present study, all loci were polymorphic in two species of the four populations except SaI12 locus in Rashid populations when examined in *D. vulgaris*. Greater variation was seen within *D. sargus* than that of *D. vulgaris*. In other studies a high levels of polymorphism was observed in microsatellite loci of *D. sargus* and *D. vulgaris* species which is persistent with the mean allele number observed in Sparidae fish species **(Brown *et al.*, 2005; Liu *et al.*, 2007; González-wangüemert *et al.*, 2010)**.

The large observed divergence of the observed (H_O) from the expected (H_E) heterozygosity, and also low number of successful amplified samples at some loci may indicate the need for further PCR optimization in some cases. However, locus SaI10 (in all locations) in *D. sargus*, SaI19 in *D. sargus* and *D. vulgaris* and SaI21 in *D. vulgaris* showed encouraging results for the likely use of these loci in the microsatellite examinations.

SaI10 locus showed low heterozygosity in *D. sargus* and high heterozygosity in *D. vulgaris* which agreed with the results of **Brown *et al.* (2005)** who found that SaI10 locus showed high heterozygosity in *Pagrus pagrus*, *Dentex dentex* and *Spondyliosoma cantharus*. These results also agreed with **Liu *et al.* (2007)** who found that SaI10 locus showed high heterozygosity in *Acanthopagrus schlegeli*.

SaI12 locus showed low heterozygosity in Lake Burullus population of *D. sargus* (0.333), and in *D. vulgaris* populations of both Abo Qir and Rashid populations reaching 0 (no heterozygosity) These results are in harmony with the results of **Brown *et al.* (2005)** who found that SaI12 locus showed no heterozygosity in *D. sargus*.

SaI19 locus showed no heterozygosity in all locations in both *D. sargus* and *D. vulgaris*. These results contradicts the findings of **Brown *et al.* (2005)** who found that this locus showed high heterozygosity in *D. sargus*, *Pagrus pagrus* and *Dentex dentex*. Similarly, Liu et al. (2007) found that SaI19 locus exhibited high heterozygosity in *Acanthopagrus schlegeli*.

In our results, SaI21 locus showed successful amplification in both *D. sargus* and *D. vulgaris*. These results disagree with those of **Brown *et al.* (2005)** who reported a failure

of amplification of SaI21 microsatellite locus in some species of family Sparidae including *D. sargus* and *D. vulgaris*.

In the present study, the analysed populations of both fishes showed some variability in the values of the observed heterozygosity of the microsatellite loci (ranged from 0 to 1 in *D. sargus* and from 0 to 0.857 in *D. vulgaris*). Meanwhile the expected heterozygosity showed high values in both *D. sargus* (0.5 to 0.95) and *D. vulgaris* (0.75 to 0.93), similar to or slightly lower than that observed for marine fish that varied between 0.84 and 0.92 as reviewed by **Carvalho and Hauser (1998)**.

The levels of genetic variability observed in *D. sargus* and *D. vulgaris* were lower than those in other Sparidae fishes assessed with microsatellites markers (0.048 in *D. sargus* and 0.078 in *D. vulgaris* in this work). Meanwhile, levels in *Pagrus major* was 0.69, in *Acanthopagrus schlegeli* was 0.55–0.95 and in *Pagellus bogaraveo* was 0.72 **(Perez-Enriquez and Taniguchi, 1999; Liu *et al.*, 2007; Piñera *et al.*, 2007)**. Similarly, **González-Wangüemert *et al.* (2010)** found that similar genetic diversity and non-significant genetic differences of white seabream (*Diplodus sargus*) were recorded at Mediterranean islands in the vicinity of continental samples. He also suggested that proximity of the coasts and the current system could contribute to an optimal fish larval dispersion among Mediterranean coasts with high gene flow.

CONCLUSION

Three microsatellite loci (SaI10, SaI12, and SaI21) isolated from *S. aurata* can be applicable in *D. sargus* and *D. vulgaris*. SaI10 and SaI21 in *D. sargus and D. vulgaris* respectively and could also be used for the examination of their presence in other species of family Sparidae. These cross-amplified markers showed a statistical power that can be used in the analysis performed in this study as much as it has been used in *S. aurata.*

In terms of population subdivision in the 4 geographical regions in the Mediterranean Sea in Egypt, the results showed no significant genetic differentiation throughout the four studied locations. The cause of the lower observed heterozygosity compared to the expected heterozygosity in both *D. sargus* and *D. vulgaris* populations may be due to inbreeding. The shared genetic material in the studied region of the Mediterranean Sea could be explained by the free movement of the fish along the area.

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ARABIC SUMMARY

التنوع الوراثي لسمكتى الشرغوش الحر والرشيدي *Diplodus sargus* و *Diplodus vulgaris* في البحر المتوسط بمصر مستخدما محددات المايكروساتلايت

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تم تجميع ١١٢ عينة من الشرغوش الحر والرشيدي على ان يكون عدد العينات ٥٦ عينة من كل نوع، حيث تم تجميع ١٤ سمكة من أربعة مواقع مختلفة في مصر وهما رشيد وبحيرة البرلس وبحري وأبوقير. ثم تم عزل الـ د ن ا من لحم الاسماك، تم عمل تفاعل انزيم البلمرة المتسلسل لأربعة من التوابع الدقيقة (المايكروستالايت) وهما SaI10 و SaI12 و SaI19 و SaI21 في كل من الشرغوش الحر والرشيدي.

تشير بيانات التنوع الجيني التي تم جمعها من أسماك الشرغوش الحر والرشيدي من أربعة مواقع جغرافية وهي رشيد وبحيرة البرلس وبحري وأبو قير أن قيمة F_{st} = ٠.٠٤٨ في الشرغوش الحر، بينما تساوي ٠.٠٧٨ .في الشرغوش الرشيدي. جميع المواقع المستخدمة في كلا النوعين في العشائر الاربعة متعددة الأشكال فيما عدا موقع SaI12 في عشيرة رشيد حيث أنها وحيدة الشكل (monomorphic). أسماك الشرغوش الحر كانت أكثر تباينا وتنوعا من أسماك الشرغوش الرشيدي. كشفت ترددات النمط الجيني في جميع المواقع الأربعة انحرافا شاملا عن توقعات هاردي-واينبرغ في موقعين أو أكثر في كل عشيرة في كل من الشرغوش الحر والرشيدي ويرجع ذلك الانحراف إلى العجز في heterozygosity والتي تتمثل بشدة في موقع SaI19 الذي يظهر التماثل homozygosity في جميع العشائر في أسماك الشرغوش الحر والرشيدي.

الاستنتاجات والتوصيات:

- أن الاربعة توابع دقيقة المستخدمة في هذه الدراسة قادرة على تعريف اسماك الشرغوش الحر والرشيدي لذلك فيمكن استخدامها في تعريف أي سمكة من أسماك العائلة المرجانية.
- أنه لا يوجد انقسامات بين العشائر المختلفة وذلك بسبب تماثل المادة الوراثية في العشائر المختلفة وذلك يرجع إلى غياب الحدود البحرية للبحر الأبيض المتوسط كما أنه يرجع إلى سرعة حركة الأسماك خلال البحر.
- أن سبب انخفاض قيمة الـ heterozygosity المرئية عن الـ heterozygosity المتوقعة يمكن أن يرجع إلى التزاوج الداخلي للأسماك بين العشائر المختلفة.