BABEŞ-BOLYAI UNIVERSITY

FACULTY OF BIOLOGY AND GEOLOGY

DEPARTMENT OF EXPERIMENTAL BIOLOGY

PHD THESIS

RELEVANCE OF MITOCHONDRIAL MOLECULAR MARKERS IN THE
CHARACTERIZATION OF THE POPULATION GENETIC STRUCTURE
OF ROMANIAN BLACK SEA *Cerastoderma glaucum*, POIRET 1789
- Summary -

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Keywords :

- Romanian littoral
- population genetics
- mitochondrial DNA
- polymerase chain reaction
- bivalves
- *Cerastoderma glaucum*

I. INTRODUCTION

The main idea of the present study was to investigate through molecular means the diversity and genetic structure of the bivalve *Cerastoderma glaucum*, Poiret 1789 previously known from the literature as *Cerastoderma (Cardium) edule*, Linnaeus 1758. The main aims of the study are:

- Strengthening the identity and nomenclature of *Cerastoderma glaucum*, previously known as *Cerastoderma (Cardium) edule.*

- Small-scale genetic structure of the *Cerastoderma glaucum* populations from the Romanian Black Sea

- Does the Constanta Sud-Agigea harbor influence the structure of the populations?

- What could be the factors influencing the genetic structure of the species?

1. The Black Sea – general description

The Black Sea (fig.1) is one of the most isolated inland sea in the world. It is a typical example of an enclosed, intercontinental, Eurasian sea. Enclosed seas may have the following definition: "a sea that is surrounded by land with a relatively narrow entrance, which is at least 4 times smaller in dimension than the longest axis within the enclosed basin" (Healy and Harada, 1991). The Romanian Sector of the Black Sea lies to the west and south of the Danube Delta and has an area of over 35 000 km^2.

Fig. 1 General map of the Black Sea region

2. General considerations on Cerastoderma glaucum

C. glaucum (Poiret, 1789) is a common bivalve (Bivalvia: Cardiidae) that inhabits the Romanian infralittoral of the Black Sea (Grossu, 1962). Its distribution area reaches also the Mediterranean Sea, the North Sea, and the Baltic Sea (Russell and Petersen 1973; Freire, 2010), the Caspian Sea, the Sea of Azov (Brock, 1979) and there is some evidence that this species entered also into the Red Sea (Brock 1987).This species is particularly abundant in the Gulf of Gabes, the north coast of Sfax in Tunisia (Derbali et al., 2008; 2009) and several European coastal lagoons such as the ones in the south and south-east of Britain (McArthur, 1998), Portugal (Machado and Costa 1994), Greece (Gontikaki, 2003) and Italy (Ivell, 1979; Trotta and Cordisco, 1998), (fig.2). *C. glaucum* inhabits soft-sand or sand-muddy bottoms (Sara, 2007; Derbali et al., 2009). It is a proper euryhaline and eurythermic species therefore even extreme temperature and salinities do not form a barrier against its dispersal. *C. glaucum* is tolerant to salinities ranging from 5 PSU to 84 PSU (Boyden and Russel, 1972; Labourg and Lasserre 1980) and temperatures from 0°C to over 45°C (Russell and Petersen 1973; Wilson and Elkaim, 1997). Despite its eurytopy *C. glaucum* populations can be found only in specific coastal areas, mainly lagoons, estuaries, bays and lakes. It inhabits sandy sediments not over the 30-35 m isobaths (Krzyminska, 1993). Although Russell (1972) supposed that the major limiting factor for the cockle was the intolerance to air exposure provoked by tides *C. glaucum* can be easily found in tidal flats. It is more reasonable that sediment texture is more important than air exposure for this species, as it is never found in areas with "loose" sediment structure i.e. well-sorted sand which results from currents and wave exposure (Brock, 1979).

Fig.2 European distribution of *Cerastoderma glaucum* (Poiret, 1789)

3. Cerastoderma glaucum and Cerastoderma edule – differences

The lagoon cockle (*Cerastoderma glaucum*) has a very close relative which is the species known as the common cockle (*Cerastoderma edule*). These two species have been described along the time as being varieties of the same specie namely *C. edule* (Rygg, 1970).

From the Ponto-Caspian region, and the Black Sea, there are six varietes of *C. edule* described (*C. edule major*, *C. edule lamarcki*, *C. edule batesoni*, *C. edule maeotica*, *C. edule nuciformis*, *C. edule quadrata*) (Grossu, 1962). Out of these, *batesoni*, *lamarcki* and *quadrata* are considered to be varieties of *C. glaucum* like *maeotica*, *nuciformis* and *major* that would also belong to the same species, *C. glaucum*, by looking at drawings and the hard substrate they were collected from (Rygg, 1970).

C glaucum and *C. edule* show different ecology (Hummel *et al.* 1994; Machado and Costa 1994). However, in some areas due to peculiar environmental conditions, sympatric populations can be found like in Portugal (Machado and Costa 1994), Finland (Rygg 1970), Norway, Sweden, Denmark, Netherlands, England and France (Brock 1987).

Machado and Costa (1994) identified a set morphological characters in which make the two species distinguishable (Tab 1; Fig. 3). They used both morphological and enzymatic criteria (i.e. the hepatopancreatic malate dehydrogenase). A new morphological trait to be considered is the ventral valve junction (crenulate in *C. edule* and typically straight in *C. glaucum*). The prominence of the ligament cannot be used for distinguishing specimens from the Portuguese coast, as it appeared not valid for more than 50% of individuals in some samples.

Table 1. Morphological differences between the two *Cerastoderma* species identified by Machado and Costa (1994).

Character	*Cerastoderma edule*	*Cerastoderma glaucum*
Valve profile	smooth	Sharp
Ventral valve junction	crenulate	typically straight
Type of calcareous scales	laminar type, perpendicularly inserted in valves	typically non existent or thick bent to umbonal region
Shell colour	variable, different from colours typical of *C. glaucum*	typically glaucum yellow or salt-pit green
Posterior valve junction	typically crenulate	typically straight or slightly crenulate
Visibility of ligament	generally visible or at the limit of visibility	typically not visible
Amount of periostracum	variable	typically large amount
Shell shape	variable	Variable
Internal brown spot	typically present	typically present

a.
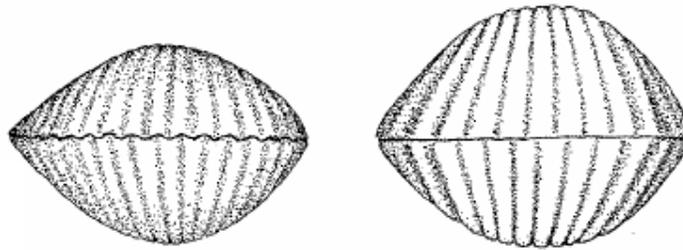
b.
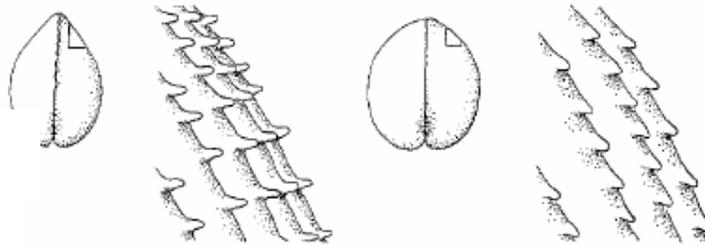
Fig.3 Differences between *C. edule* (left) and *C. glaucum* (right) (from Machado and Costa, 1994) a. Ventral views showing characteristic valve profile and ventral valve junction in both species; b. schematic representation of characteristic shape and type of valve insertion of calcareous scales in both species (front view). Picton, B.E. & Morrow, C.C., 2010. *[In] Encyclopedia of Marine Life of Britain and Ireland*

II. MATERIAL AND METHODS

4. Sample collection

The bivalve mollusks samples were manually collected in depths of 1-10 m by scuba diving at seven sites along the southern Romanian Black Sea sector during 2009 (Fig. 4).
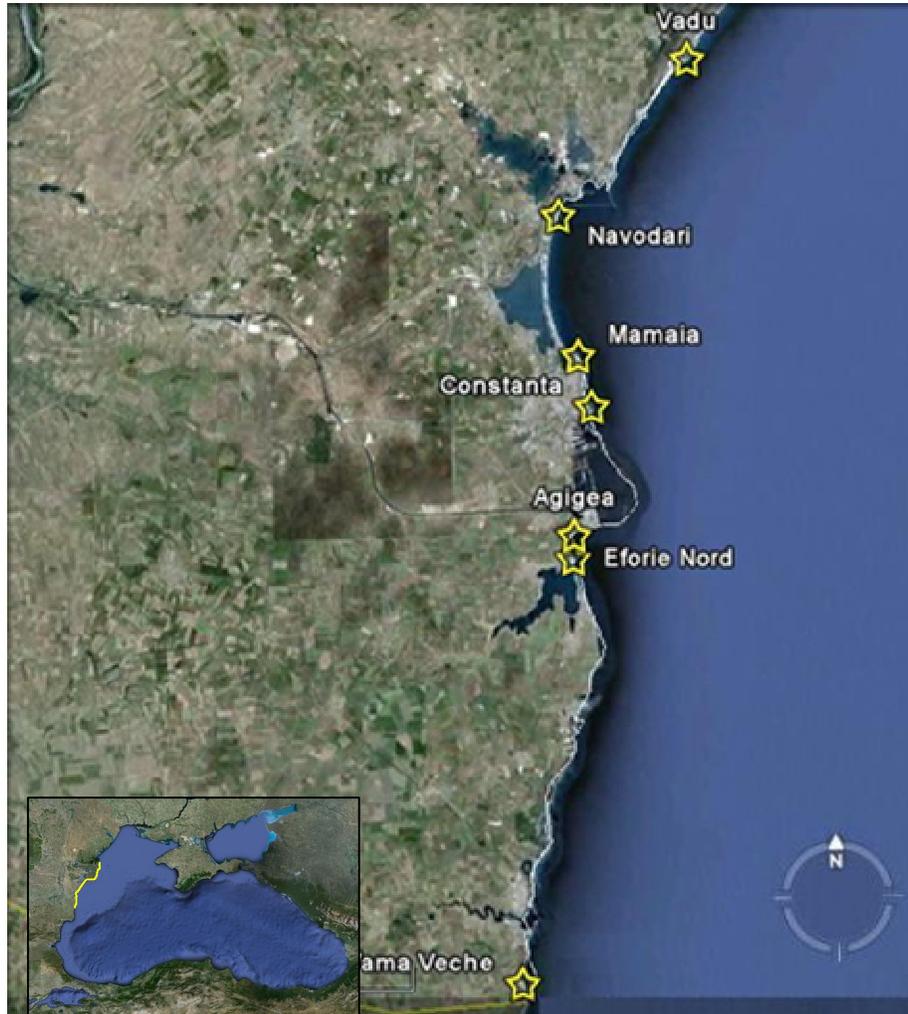
Fig. 4. Location of sampling sites for *C. glaucum* in the Romanian Black Sea sector (2011 Google – Imagery 2011 TerraMetrics, Map Data 2011 Basarsoft, Geocentre Consulting, Tele Atlas, Transnavicom, Google)

5. Sample preparation and analysis

Molecular analyses of individual bivalve samples were performed according to the following procedures (Fig.5):
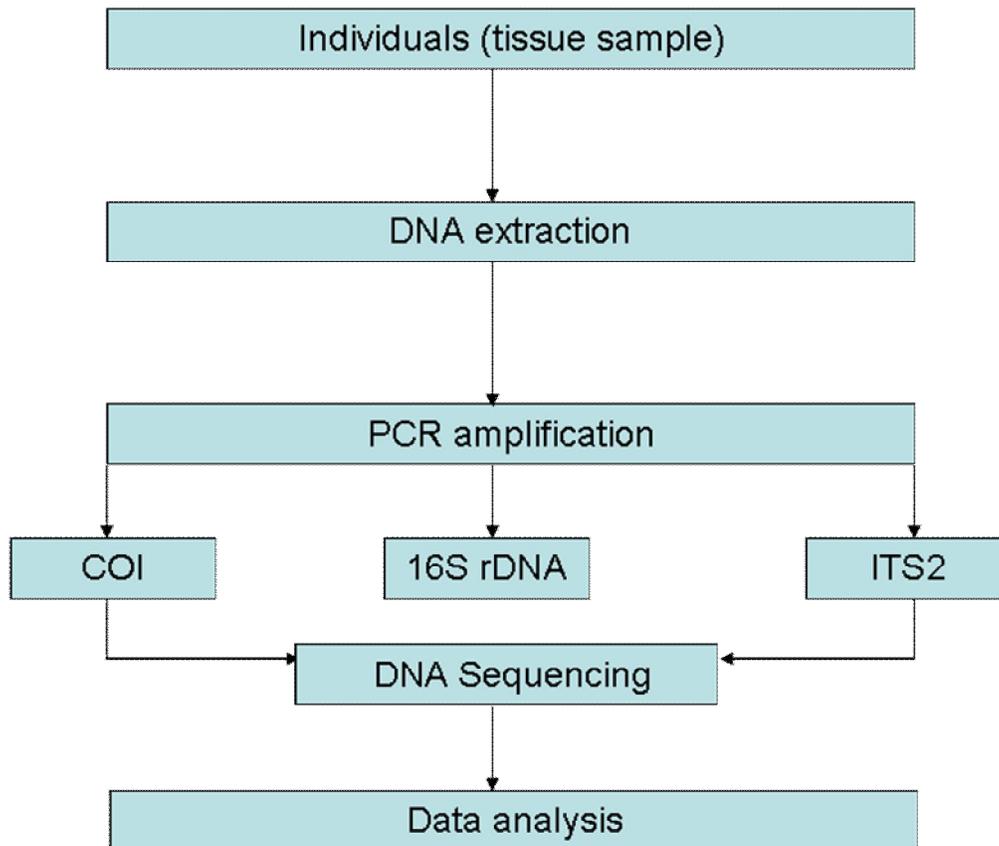

Fig. 5 The main steps involved in sample analysis of the *Cerastoderma glaucum* individuals

III. RESULTS

6. Cytochrome oxidase subunit I (COI) data analysis

Founded haplotypes

A number of 8 haplotypes have been identified from the Romanian littoral of the Black Sea (Tab.2). The most common haplotype, represented in all samples across the study sites is number 4, followed by 1 and 2. Two individuals, one from Năvodari, the second from Vama Veche showed peculiar haplotypes that are not present in the rest of the stations n7 and n8, respectively.

Variation amongst individuals was observed in loci: 139 (C→A), 190 (A→G), 241 (C→T), 244 (G→A), 271 (G→A), 256 (T→G), 364 (T→C), 404 (C→T) and 493 (A→C). In loci139 and 493 a transversion from C to A and A to C occurs. Other mutations appear to be "transitions" a more common condition than transversions.

Table 2 Cytochrome oxidase subunit I haplotypes of Romanian *C. glaucum* populations

		Stations (number of individuals)				
		Navodari (6)	Mamaia (13)	Agigea (7)	Eforie Nord (2)	Vama Veche (4)
Haplotypes	1	2	4	2	1	-
	2	1	1	1	-	2
	3	1	-	1	-	-
	4	1	8	1	1	1
	5	-	-	1	-	-
	6	-	-	1	-	-
	7	1	-	-	-	-
	8	-	-	-	-	1

Molecular basic indices

The table 4 shows the molecular basic index. The highest number of haplotypes (6) per sampling site was obtained from Agigea (7 individuals), while the lowest number (2) from Mamaia (13 individuals). Haplotype diversity (H_d) was higher in Eforie Nord individuals, even if this datum can be biased by the very low number of individuals (2) analysed from this locality. In the case of Agigea (0.952) and Vama Veche (0.833) diversity is rather high in comparison to Mamaia (0.564).

In Vama Veche 3 haplotypes out of four can be found. This sampling site is very different from the others mainly in relation to the mean number of pairwise differences (2.333) therefore there are significant nucleotide differences between pairs sampled. This is also confirmed by the nucleotide diversity which is high in Vama Veche (0.004).

Table 3 Molecular basic indices of cytochrome oxidase subunit I

	Location	N	N_H	S	H_d	K	π
Navodari	44°19'42.45"N 28°40'14.87"E	6	5	5	0.933	1.870	0.004
Mamaia	44°12'40.29"N 28°39'11.15E	13	3	3	0.564	0.769	0.001
Agigea	44°4'57.91"N 28°38'30.47"E	7	6	6	0.952	1.905	0.004
Eforie Nord	44°3'14.96"N 28°38'37.44"E	2	2	1	1.000	1.000	0.001
Vama Veche	43°46'9.30"N 28°35'5.40"E	4	3	4	0.833	2.333	0.004

N: number of individuals; N_H: number of haplotypes; S: number of polymorphic sites; H_d: haplotype diversity; K: mean number of pairwise differences; π: nucleotide diversity

Haplotype Network

The haplotypes distribution suggests a star-like structure, typical of populations in expansion (Fig. 6). This type of network can be interpreted as an expression of a founder effect. The majority of haplotypes differ by only 1 mutation from the central haplotype (75), while only few haplotypes differ by 2 mutations. Haplotype 88 is shared by samples coming from all the other sites except Vama Veche and is ranking as second of importance concerning the number of individuals per station.
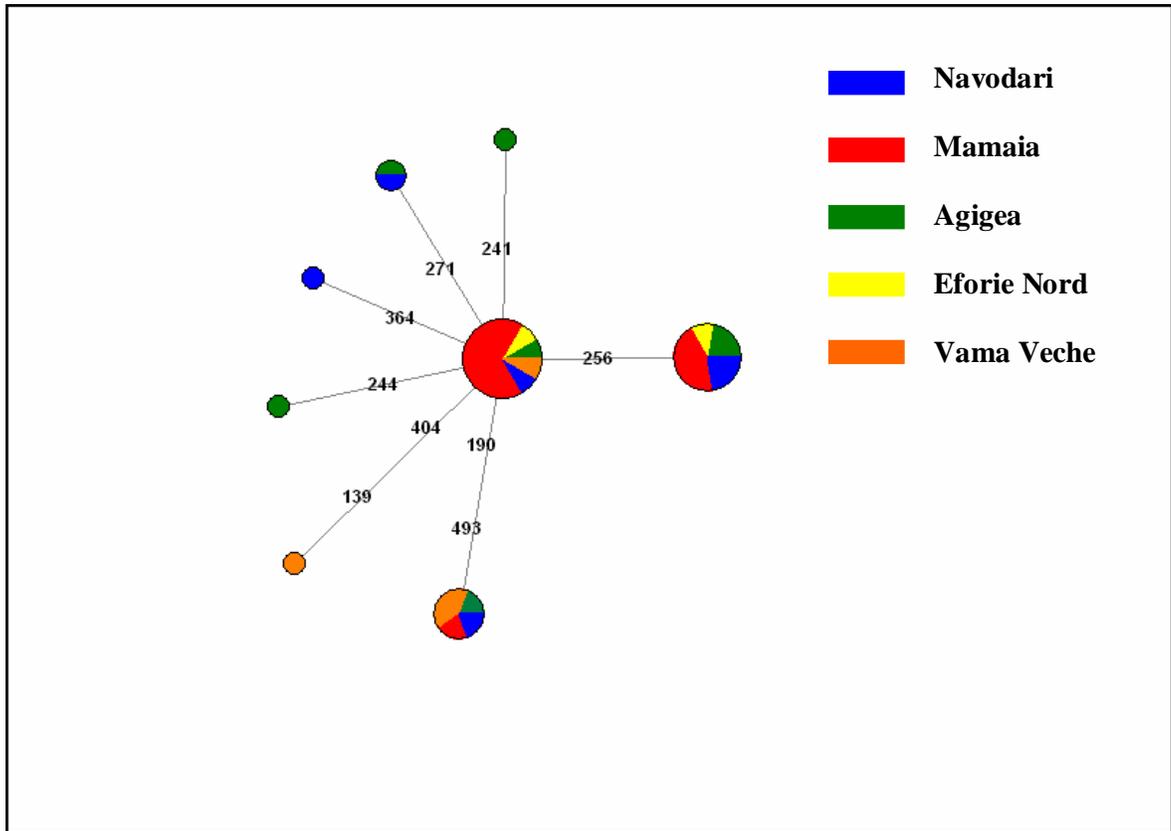
Fig. 6 Haplotype network of *COI*. Circle sizes are proportional to frequencies of each haplotype. Numbers on branches represent mutation steps.

Neutrality tests

The application of Tajima D and F of Fu statistic of neutrality was not applicable to Eforie Nord samples due to the small sample size (2 individuals). In the case of Navodari and Agigea the test is significant indicating population expansion (p value < 0.05) contrarily toVama Veche. This result can be related to the fact that the population from Vama Veche is the older one bearing 2 mutations, possibly the founder population.

Table 4 Tajima D and Fu's F neutrality tests based on *COI*

	Location	Tajima D	Fu's F
Navodari	44°19'N 28°36'E	- 0.826 (p: 0.258)	- 2.263 (p: 0.023)
Mamaia	44°14'N 28°38'E	- 0.646 (p: 0.285)	0.250 (p: 0.491)
Agigea	44°5'N 28°36'E	-1.129 (p: 0.153)	- 3.408 (p: 0.005)
Eforie Nord	44°2'N 28°39'E	-	-
Vama Veche	43°45'N 28°36'E	0.650 (p: 0.253)	0.360 (p: 0.471)

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Genetic distance analysis

We perform pairwise Fst for testing the differentiation between populations. Significance of pairwise differentiation was tested using 5040 permutations. The population pairwise F_{ST} for *COI* sequences showed that the only significant difference is between populations from Mamaia and Vama Veche (p<0.05).

The NJ dendrogram based on the molecular distance matrix of *COI* sequences (Fig 7), clustered the populations from stations according to similarities and geographical distribution. The northern populations (AG, M, N) form a separate cluster from the southern ones (E, VV). The southernmost population from Vama Veche is also distant from the one in Eforie Nord.

Table 5 F_{ST} values between populations. In red values with statistical significance p<0.05, in blue the p-values

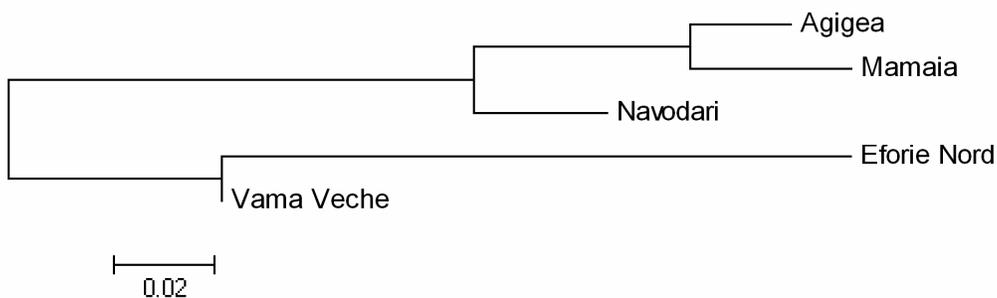| Populations	Navodari	Mamaia	Agigea	Eforie Nord	Vama Veche
Navodari		0.757	0.983	0.999	0.279
Mamaia	0.059		0.746	0.999	**0.018**
Agigea	0.132	0.052		0.999	0.274
Eforie Nord	0.339	0.293	0.313		0.393
Vama Veche	0.042	**0.27**	0.056	0.058	

Fig. 7 NJ dendrogram based on the molecular distance matrix of *COI* sequences (Kimura 2-P)

Relation between genetic and geographic distance

We perform the mantel test with the objective of examining microevolutionary processes such as isolation-by-distance (IBD).

Table 6 F_{ST} based genetic distances (Fst/ (1-F_{ST}), blue) and geographic distances (km, bold) among the five of the studied populations

Populations	Agigea	Eforie Nord	Mamaia	Navodari	Vama Veche
Agigea		0.4556	0.0537	0.1521	0.0593
Eforie Nord	4.7		0.4144	0.5129	0.0616
Mamaia	17.5	22.2		0.0627	0.3699
Navodari	30.1	34.8	12.6		0.0438
Vama Veche	44.3	39.6	61.8	74.7	

$$y = -0.0019x + 0.2845$$
$$R^2 = 0.0467$$
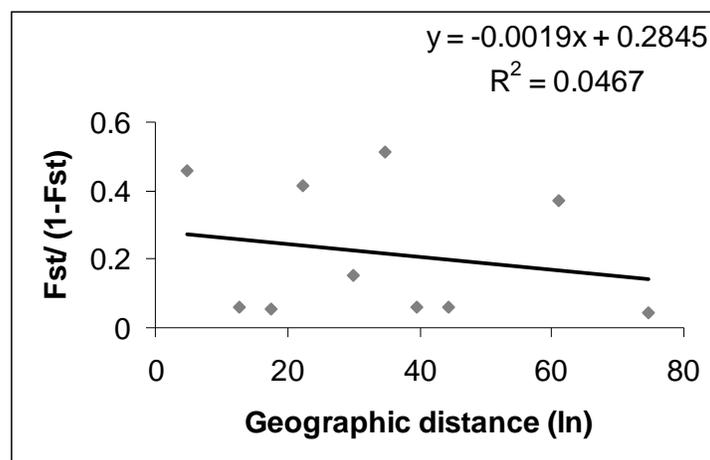
Fig. 8 Results of Mantel test between geographic distance of sampling sites (km) and genetic distances based on pairwise F_{ST} *COI* haplotypes

Analysis of Molecular Variance (AMOVA)

In order to understand the partition of genetic variation between the *C. glaucum* populations there were three hypotheses formulated under AMOVA (Tab.7). In the first hypotheses, where Vama Veche population is tested against populations of the other sites the highest proportion of molecular variation (26.07) is significantly explained (p <0.001). The other hypotheses (Mamaia Vs others) and (Vama Veche, Eforie, Agigea Vs Mamaia, Navodari, Vadu) are not significant.

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Table 7 AMOVA analysis based on cytochrome oxidase subunit I sequences

	Hypothesis 1	Hypothesis 2	Hypothesis 3
Percentage of variance	Group1: Vama Veche Group2: Agigea, Eforie Nord, Mamaia, Navodari, Vadu	Group1: Mamaia Group2: Agigea, Eforie Nord, Vama Veche, Navodari, Vadu	Group1: Vama Veche, Eforie Nord, Agigea Group2: Mamaia, Navodari, Vadu
Among Groups	**26.07** (p=0.000)	**-2.35** (p=0.201)	**3.84** (p=0.094)
Among populations within groups	**-8.73** (p=0.413)	**0.38** (p=0.256)	**-2.73** (p=0.760)
Within populations	**82.66** (p=0.506)	**101.97** (p=0.502)	**98.89** (p=0.521)

Phylogenetic Analysis

Bayesian method

The phylogenetic tree obtained with the aid of Mr. Bayes software was just partially resolved due to the close similarities of the sequences.

The only significant differences between the sequences are visible in the haplotypes 78 – AG4, M1, N12, VV7, VV10; 79 – AG2, N13; 88 – AG1, AG8, E3, M12, M14, M21, M2, N5, N11. It is easily observable that by choosing *C. edule* as outgroup, this species is very distant from the rest of *C. glaucum* individuals.

Fig. 9 Bayesian inference of *Cerastoderma glaucum COI* sequences (phylogenetic tree) with *C. edule* as an outgroup (specified model: GTR; number of generations: 50 000 000)
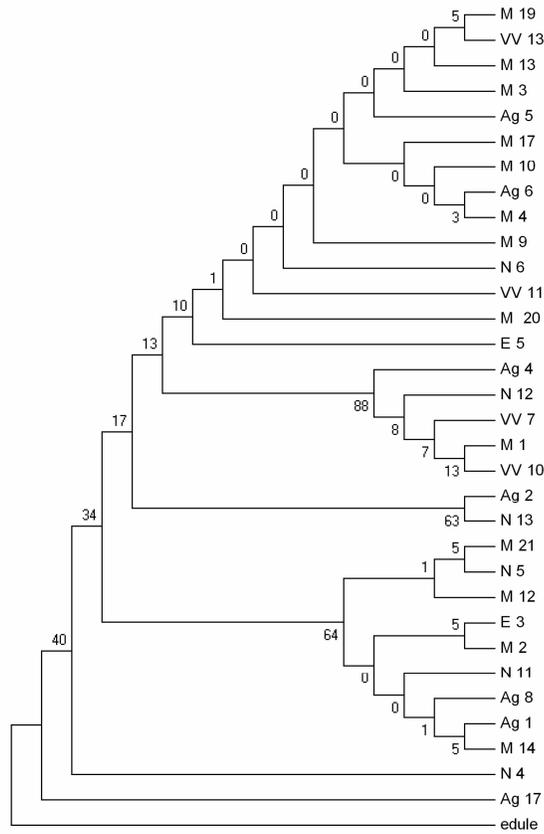
Neighbor Joining Method

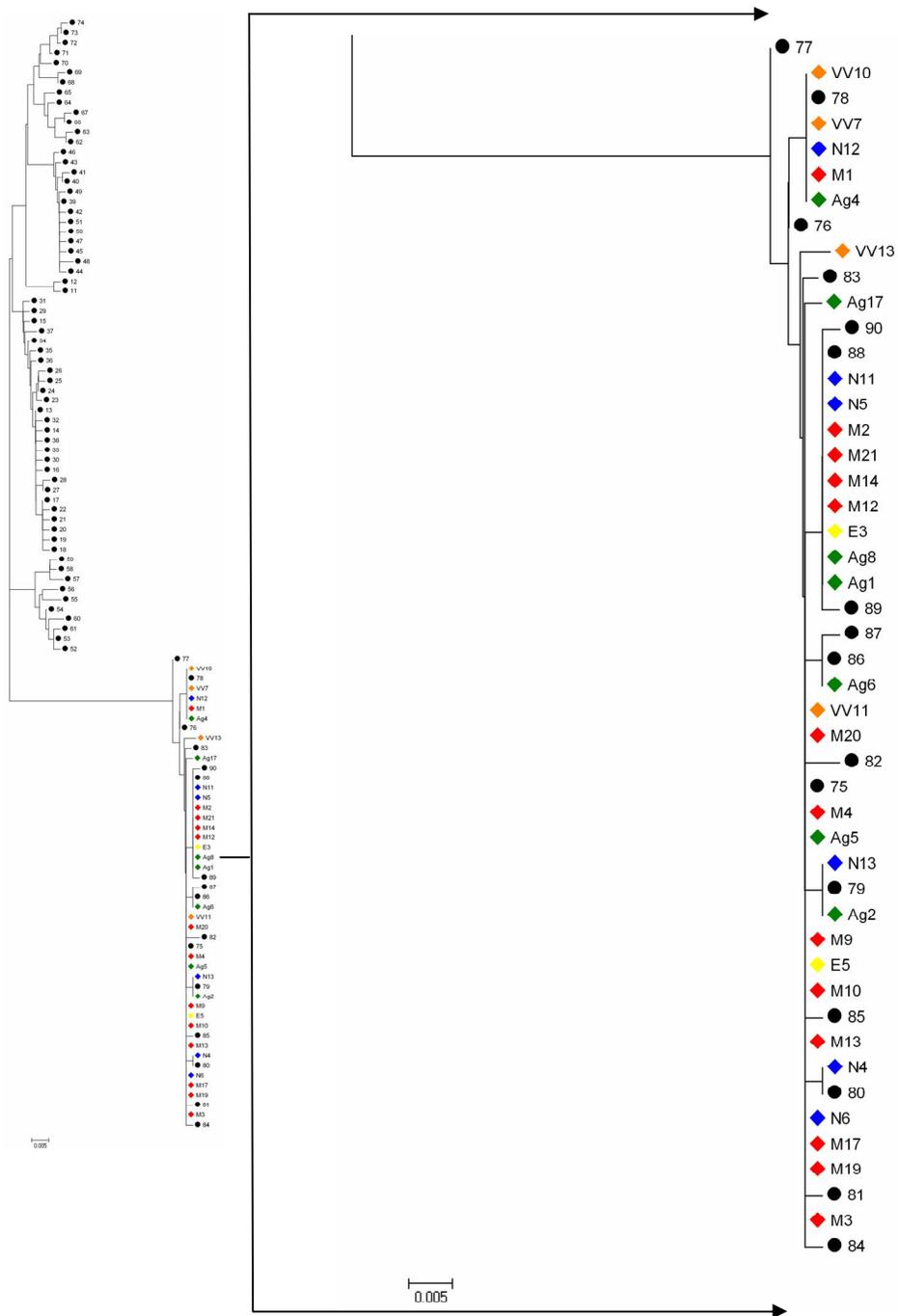Fig. 10 NJ dendrogram based on *COI* sequences rooted with *C. edule*

Fig. 11 NJ dendrogram based on *COI* seq: original data (in color) combined with data from the literature (black circle: Tarnowska et al. 2010).

7. 16S rDNA data analysis

Founded haplotypes

A number of 5 haplotypes based on *16S rDNA* mitochondrial marker have been identified from the Romanian littoral of the Black Sea. The most common haplotypes were 1 and 2.Variations between individuals have been observed at sites: 111 (G→A), 191 (C→T), 201 (T→C), 203 (T→C).

All these mutations represent transitions and they are responsible for the haplotypes found along the Romanian littoral.

Table 8 *16S rDNA* haplotypes of Romanian *C. glaucum* populations

		Stations (number of individuals)					
		Vadu (2)	Navodari (5)	Mamaia (12)	Agigea (8)	Eforie Nord (2)	Vama Veche (8)
Haplotypes	1	2	1	2	2	1	-
	2	-	4	8	5	1	5
	3	-	-	1	1	-	2
	4	-	-	1	-	-	-
	5	-	-	-	-	-	1

Molecular basic indices

The highest number of haplotypes revealed by the analysis of a 404 bp sequence of the *16S rDNA* compared to the number of individuals was obtained from Agigea and Vama Veche (3 haplotypes from 8 individuals). In contrast, the lowest number of haplotypes is found in Mamaia (4 haplotypes from 12 individuals).

As it is observed in the cases of Vadu and Eforie Nord, there are only two individuals/station to be investigated and this fact proves to be not appropriate to obtain the genetic parameters. The values of these two stations are not enough to draw some conclusions about these populations.

The number of polymorphic sites and also the mean number of pairwise differences support the more diverse population from Vama Veche.

Table 9 Molecular basic indices of *16S rDNA*

Station	Location	N	N$_H$	S	H$_d$	K	π
Vadu	44°25'42.63"N 28°46'2.97"E	2	1	0	0.000	0.000	0.0000
Navodari	44°19'42.45"N 28°40'14.87"E	5	2	1	0.400	0.400	0.0009
Mamaia	44°12'40.29"N 28°39'11.15E	12	4	3	0.561	0.742	0.0018
Agigea	44°4'57.91"N 28°38'30.47"E	8	3	2	0.607	0.786	0.0019
Eforie Nord	44°3'14.96"N 28°38'37.44"E	2	2	1	1.000	1.000	0.0024
Vama Veche	43°46'9.30"N 28°35'5.40"E	8	3	3	0.607	1.107	0.0027

N: number of individuals; N$_H$: number of haplotypes; S: number of polymorphic sites; H$_d$: haplotype diversity; K: mean number of pairwise differences; π: nucleotide diversity
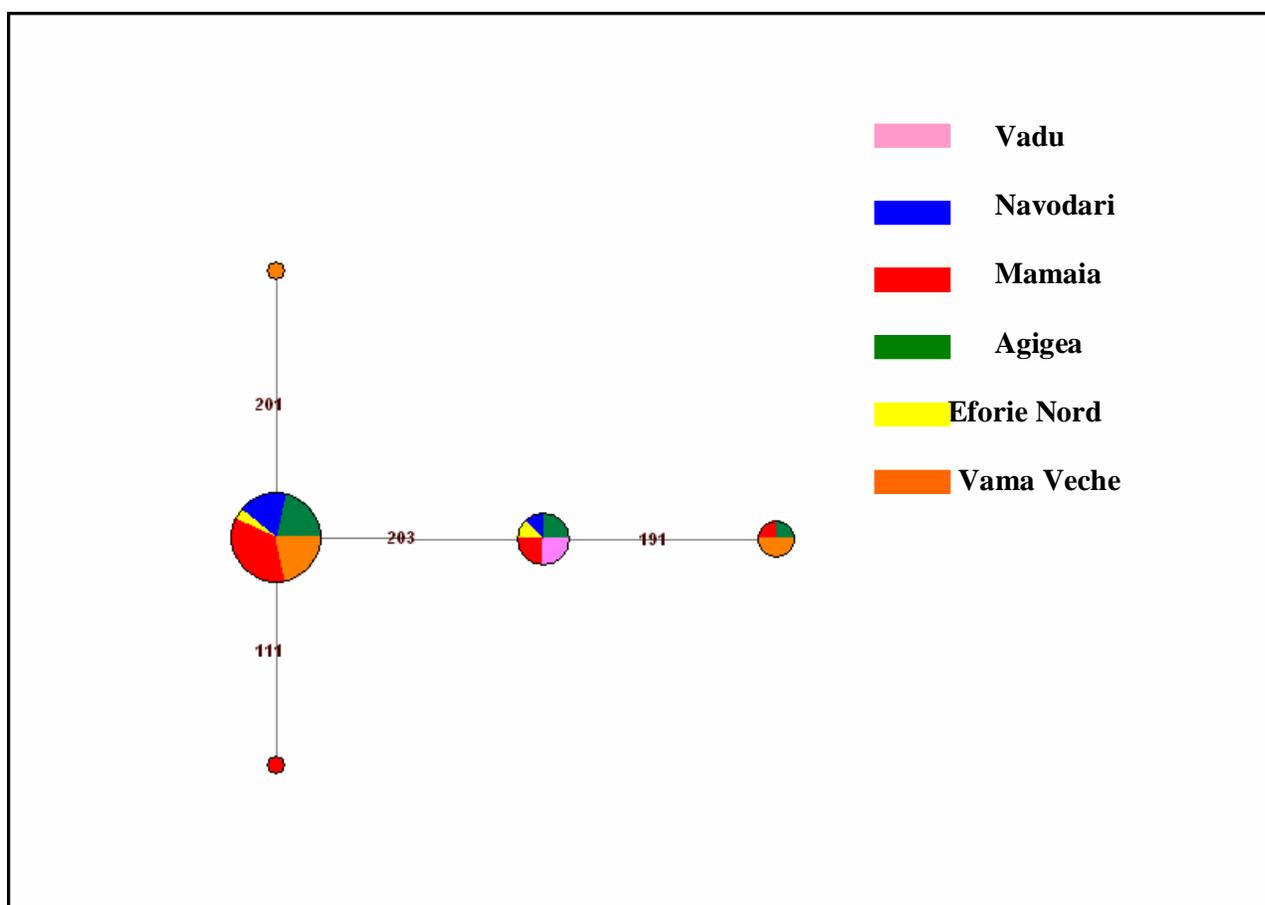
Haplotype network

Fig. 12 Haplotype network of *16S rDNA* haplotypes. Circle sizes are proportional to frequencies of each haplotype.

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Neutrality tests

The neutrality test in the case of the *16S rDNA* gene proved to be statistically insignificant. As shown previously, the populations from Vadu and Eforie Nord are lacking the necessary data, so they are not valid in this case.

Table 10 Tajima D and Fu's F neutrality tests based on *16S rDNA*

	Location	Tajima D	Fu's F
Vadu	44°25'42.63"N 28°46'2.97"E	-	-
Navodari	44°19'42.45"N 28°40'14.87"E	- 0.817 (p:0.297)	0.090 (p: 0.302)
Mamaia	44°12'40.29"N 28°39'11.15E	- 0.829 (p: 0.228)	-1.256 (p: 0.067)
Agigea	44°4'57.91"N 28°38'30.47"E	0.069 (p: 0.455)	- 0.223 (p: 0.193)
Eforie Nord	44°3'14.96"N 28°38'37.44"E	-	-
Vama Veche	43°46'9.30"N 28°35'5.40"E	0.178 (p: 0.460)	0.390 (p: 0.514)

Genetic distance analysis

The population pairwise F_{ST} for *16S rDNA* sequences showed no significant difference between populations from the studied area (F_{ST} p<0.05)

Table 11 F_{ST} values between populations based on *16S rDNA*. In blue the p-values

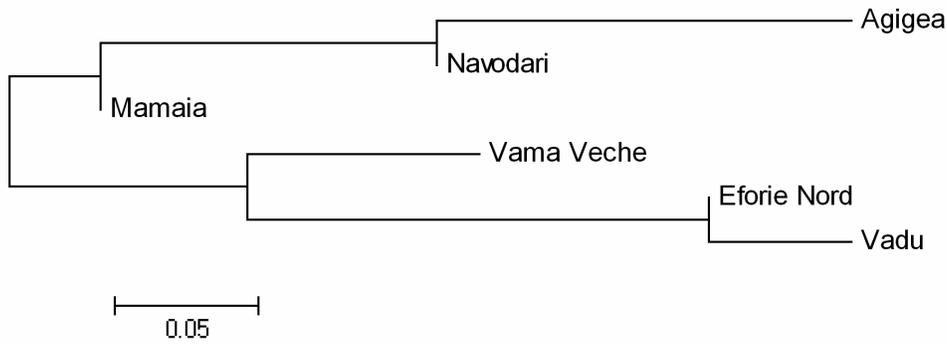| Populations	Vadu	Navodari	Mamaia	Agigea	Eforie Nord	Vama Veche
Vadu		0.149	0.104	0.437	0.999	0.138
Navodari	0.149		0.999	0.807	0.999	0.674
Mamaia	0.104	0.999		0.808	0.999	0.709
Agigea	0.437	0.099	0.078		0.999	0.999
Eforie Nord	0.999	0.215	0.237	0.378		0.999
Vama Veche	0.257	0.073	0.050	0.081	0.226	

Fig. 13 NJ dendrogram based on the molecular distance matrix of *16S rDNA* sequences

Relation between genetic and geographic distance

Table 12 F_{ST} based genetic distances (Fst/ (1-F_{ST}), blue) and geographic distances km, bold) among the six of the studied populations based on *16S rDNA*

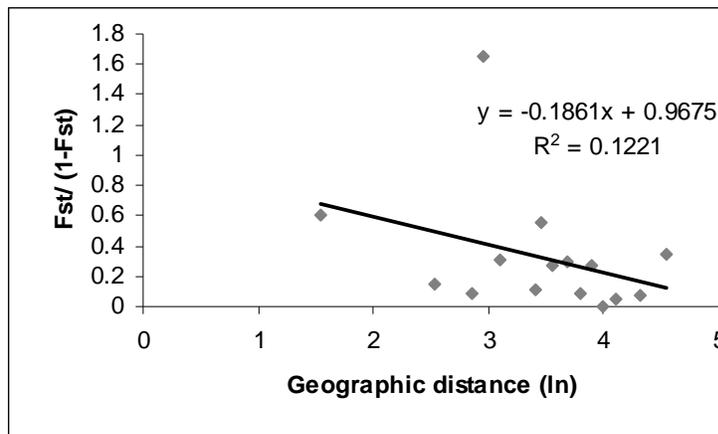| Populations	Vadu	Navodari	Mamaia	Agigea	Eforie Nord	Vama Veche
Vadu		1.6525	0.5552	0.2771	0.0000	0.3459
Navodari	19.32		0.1521	0.1099	0.2739	0.0787
Mamaia	31.92	12.6		0.0846	0.3106	0.0526
Agigea	49.4	30.1	17.5		0.6077	0.0881
Eforie Nord	54.12	34.8	22.2	4.7		0.2920
Vama Veche	94.02	74.7	61.08	44.3	39.6	

Fig. 14 Results of Mantel test between geographic distance of sampling sites (km) and genetic distances based on pairwise F_{ST} *16S rDNA* haplotypes

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The Mantel test seems not to be statistically significant regarding the *16S rDNA* gene (p<0.05, r = 0.1221).

Analysis of Molecular Variance (AMOVA)

The AMOVA hypothesis also supports the affirmation that the gene for *16S rDNA* is not so good in discriminating between populations of the same species. Despite this fact, a slight differentiation can be observed from the first hypothesis when the population of Vama Veche is grouped apart from the others (6.61).

Table 13 AMOVA analysis based on *16S rDNA* sequences

Percentage of variance	Hypothesis 1	Hypothesis 2	Hypothesis 3
	Group1: Vama Veche Group2: Agigea, Eforie Nord, Mamaia, Navodari, Vadu	Group1: Mamaia Group2: Agigea, Eforie Nord, Vama Veche, Navodari, Vadu	Group1: Vama Veche, Eforie Nord, Agigea Group2: Mamaia, Navodari, Vadu
Among Groups	**6.61** (p=0.000)	**2.56** (p=0.194)	**1.81** (p=0.300)
Among populations within groups	**-12.98** (p=0.972)	**-11.67** (p=0.909)	**-11.14** (p=0.990)
Within populations	**106.36** (p=0.957)	**109.11** (p=0.961)	**109.33** (p=0.960)

Phylogenetic Analysis

Bayesian method

Fig. 15 Bayesian inference of *Cerastoderma glaucum 16S rDNA* sequences (phylogenetic tree) with *C. edule* as an outgroup (specified model: GTR; number of generations: 50 000 000)

By running a Bayesian inference of a phylogenetic tree, only two clustering could be observed. The individuals that could not be resolved are in the same line, and the others are represented separately.

Neighbor Joining Method

Fig. 16 NJ dendrogram (Kimura 2P - parameter) of *16S rDNA* sequences rooted with *C. edule*.

8. Cytochrome oxidase subunit I (COI) and 16S rDNA combined data analysis

Founded haplotypes

The combined data set contained 918 characters, among which a number of 10 haplotypes have been identified. The common haplotype shared by all stations was 2 (N, M, AG, VV), followed by haplotype 1 (N, M, AG). Haplotypes 3 (N, AG) and 4 (M, AG) were present in two populations each. The rest of the haplotypes from 5 – 10 were present in only one population.each.

Variations between individuals have been observed at following sites: 111 (G→A), 191 (C→T), 201 (T→C), 203 (T→C), 543 (C→A), 594 (A→G), 645 (C→T), 660 (T→G), 675 (G→A), 808 (C→T) and 897 (A→G). In the positions 543 and 660 there is a transversion from C to A and respectively T to G. The rest of the mutations show transitions which are more common than transversions. These mutations are responsible for the haplotypes found along the Romanian littoral.

Table 14 Cytochrome oxidase subunit I (*COI*) and *16S rDNA* combined data analysis haplotypes

		Stations (number of individuals)			
		Navodari (3)	Mamaia (8)	Agigea (6)	Vama Veche (4)
Haplotypes	1	1	2	1	-
	2	1	1	1	2
	3	1	-	1	-
	4	-	1	1	-
	5	-	-	1	-
	6	-	-	1	-
	7	-	1	-	-
	8	-	3	-	-
	9	-	-	-	1
	10	-	-	-	1

Molecular basic indices

The combined results of the two mitochondrial markers show a low haplotype number in Mamaia population compared to the populations from other stations. The population from Vama Veche although has only 3 haplotypes compared to the number of individuals, present a high mean number of pairwise differences (3.853).

Table 15 Molecular basic indices of combined *COI* and *16S rDNA*

	Location	N	N_H	S	H_d	K	π
Navodari	44°19'42.45"N 28°40'14.87"E	3	3	5	1.000	3.344	0.004
Mamaia	44°12'40.29"N 28°39'11.15E	8	5	6	0.858	1.967	0.002
Agigea	44°4'57.91"N 28°38'30.47"E	6	6	7	1.000	2.808	0.003
Vama Veche	43°46'9.30"N 28°35'5.40"E	4	3	7	0.833	3.852	0.004

N: number of individuals; N_H: number of haplotypes; S: number of polymorphic sites; H_d: haplotype diversity; K: mean number of pairwise differences; π: nucleotide diversity

Haplotype network

The haplotype network of the combined mitochondrial markers has a median vector (mv1) that represent a hypothesized (often ancestral) sequence which is required to connect the existing sequences within the network. At some point, the characters 203 and 660 occur twice, forming a cycle (box-like) that is called "a cube". In this case we are dealing with parallel mutations or homoplasy.
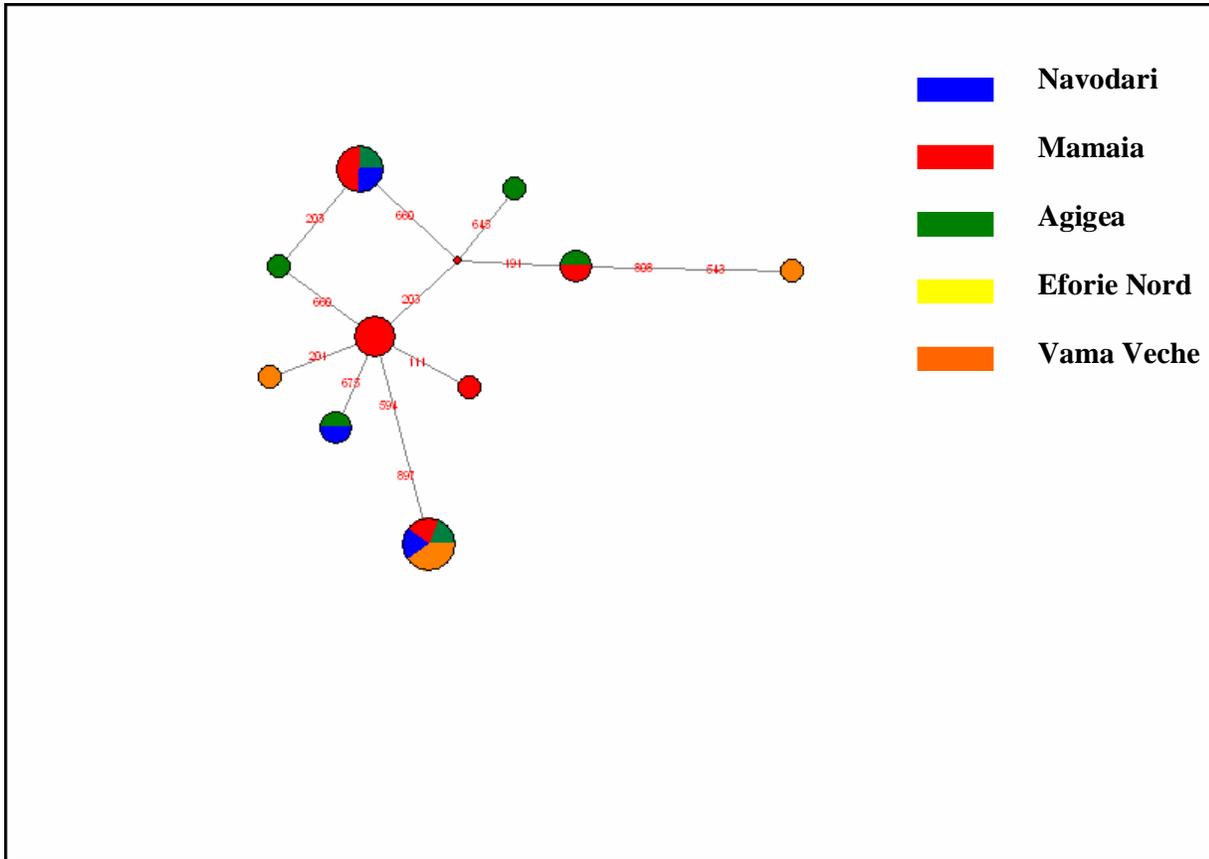

Fig. 17 Haplotype network of *COI* and *16S rDNA* combined. Circle sizes are proportional to frequencies of each haplotype.

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Neutrality tests

The neutrality tests were not significant even in a combined analysis of the two markers.

Table 16 Tajima D and Fu's F neutrality tests based on the combined analysis of *COI* and *16S rDNA*

	Location	Tajima D	Fu's F
Navodari	44°19'42.45"N 28°40'14.87"E	0.000 (p: 0.000)	- 0.077 (p: 0.231)
Mamaia	44°12'40.29"N 28°39'11.15E	- 0.704 (p: 0.280)	- 1.191 (p: 0.136)
Agigea	44°4'57.91"N 28°38'30.47"E	- 0.504 (p: 0.371)	- 3.453 (p: 0.005)
Vama Veche	43°46'9.30"N 28°35'5.40"E	0.039 (p: - 0.363)	1.099 (p: 0.624)

Genetic distance analysis

Table 17 F_{ST} values between populations based on combined *COI* and *16S rDNA*. In blue the p-values

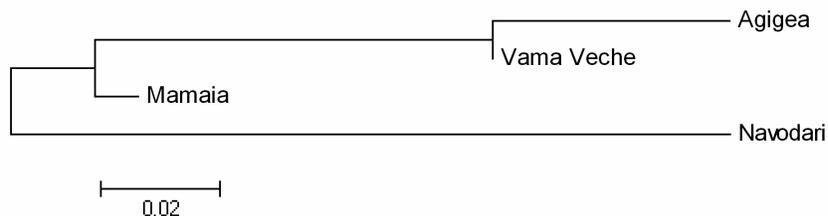| Populations	Navodari	Mamaia	Agigea	Vama Veche
Navodari		0.846	0.999	0.999
Mamaia	0.142		0.934	0.294
Agigea	0.24	0.115		0.398
Vama Veche	0.169	0.04	0.06	

Fig 18 Neighbor-Joining dendrogram based on the molecular matrix distances of *COI* and *16S rDNA* combined sequences

Analysis of Molecular Variance (AMOVA)

AMOVA analysis discriminates between Vama Veche and the other sites (Hypothesis 1), explaining 13.92 percentage of variation. In the other cases, when Mamaia is grouped alone and when southern populations (M, VV) are compared to northern ones (N, Ag) the percentage of variation explained is low and not significant (p > 0.05) and 0.54 respectively 1.20.

Table 18 Combined *COI* and *16S rDNA* AMOVA

|	Hypothesis 1	Hypothesis 2	Hypothesis 3
Percentage of variance	Group1: Vama Veche Group2: Agigea, Mamaia, Navodari	Group1: Mamaia Group2: Agigea, Vama Veche, Navodari	Group1: Navodari, Agigea Group2: Mamaia Vama Veche
Among Groups	**13.92** (p=0.000)	**0.54** (p=0.243)	**1.20** (p=0.331)
Among populations within groups	**13.28** (p=0.744)	**7.57** (p=0.751)	**7.10** (p=0.881)
Within populations	**99.36** (p=0.857)	**108.11** (p=0.860)	**108.32** (p=0.856)

Neighbour Joining Method
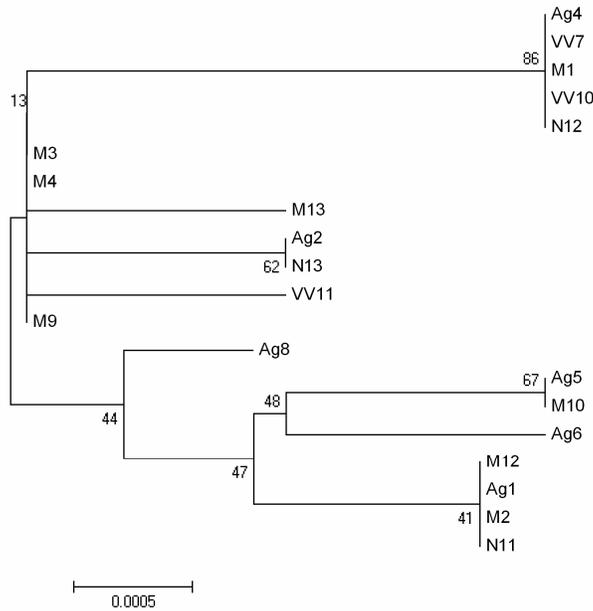
Fig. 19 NJ dendrogram (Kimura 2P - parameter) of combined *16S rDNA* and *COI* sequences

IV. Discussion

9. Discussion on mitochondrial markers

The 8 haplotypes detected at the Romanian littoral of the Black Sea analyzed with the aid of *COI* are belonging to the Ponto-Caspian phylogroup as shown in previous studies upon populations of *Cerastoderma glaucum* (Nikula and Väinölä, 2003; Tarnowska et al., 2010). Nikula and Väinölä, 2003 investigating populations of this specie by employing mitochondrial markers (*COI*) and allozymes, discovered two distinct phylogroups, respectively Mediterranean-Atlantic and Ponto-Caspian.

This view was shared also by Tarnowska et al., 2010 who studied populations from a wider range of the same specie by means of cytochrome oxidase subunit I (*COI*) and microsatellites. In both studies, the more diverse phylogroup regarding the nucleotide diversity, number of haplotypes, was the Mediterranean-Atlantic one. The Ponto-Caspian phylogroup instead registered low diversity of molecular indices.

Our study tends to complete these previous ones by adding new information regarding the genetic structure and distribution of *Cerastoderma glaucum* in the Black Sea. The only information available from Black Sea region regarding this specie was done by samplings in Varna (Bulgaria) (Nikula and Väinölä, 2003), Constanta (Romania) and (Seihierovka) Ukraine (Tarnowska et al., 2010). From Varna, a number of 4 haplotypes (75, 78, 87, 88), Constanta 5 haplotypes (75, 78, 79, 88, 89) and Seihierovka 7 haplotypes (75, 80, 81, 86, 87, 88, 90) have been identified. By adding populations from Romanian littoral, the following haplotypes are reported: 75, 78, 79, 80, 86, 88 and two new unknown haplotypes (Hap Ag and Hap VV).

The Authors used different nomenclature for the identified haplotypes in the Ponto-Caspian region. In consequence, the same haplotype had in some cases two nominations, as follows: 24 (= 75); 28 (=87); 30 (=78); 32 (88). These haplotypes were common in both studies. In order to have the same nomenclature for the haplotypes we adopted the nomenclature from Tarnowska et al., 2010, due to the more recent and newly reported haplotypes.

The overall distribution of genetic variability in Romanian *C. glaucum* populations is low, which is in agreement with the previous studies on this specie in the Ponto-Caspian region. The relations between haplotypes suggest a founder effect like otherwise every result of the *COI* analysis. There is a central haplotype, namely 75 present in every station. The 2 mutations suffered by the Vama Veche population and only one present in the rest, makes it the possible candidate population of the founder effect and thus the source for other populations present at the Romanian littoral.

This view could be explained by both linking the presence of the specie to the formation of the Black Sea and by understanding the pattern of the Black Sea currents. According to Ryan et al., 1997, a massive flood from Mediterranean occurred about 7500-8000 years ago, transforming the former freshwater environment of the Black Sea into a salty one. If the flood hypothesis is correct than the low genetic diversity of *C. glaucum* populations may be explained in terms of short period of time that passed since the specie entered the Black Sea and did not had the necessary time to become more diverse.

Regarding the *16S rDNA* gene, this could not be a good marker that can discriminate between populations of the same species. This is observable from genetic distance analysis of pairwise differences (FST) and the analysis of molecular variance (AMOVA).

10. How can upwelling and downwelling phenomenon in the Black Sea influence the distribution of the specie?

The distribution of the specie at the Romanian littoral seems to agree to the specific spectrum of reproductive strategies that are possible to act as forces influencing the gene flow and the genetic differentiation among populations (Palumbi, 1994). Species with planktotrophic larvae, which have high dispersal capacity and have a longer life, are capable of connecting the adult populations across spatial scales (Palumbi, 2003). The lecitotrophic larvae that are short-living are likely to show less gene flow between populations (Hoare et al., 1999), and in contrast, the species that lack a larval stage, are possible to have the lowest levels of genetic diversity.

The last two categories of larvae are more plausible to follow the pattern of *C. glaucum* due to the fact that the larval stage in this specie is not very well known, with some authors stating that the stage is very short about 1 week (Boyden and Russell, 1972) or others that is absent.

According to the cytochrome oxidase subunit I (*COI*) all the populations from the Romanian littoral act as only one big population without any genetic pattern.
It is known that the winds from the Black Sea region have an influence upon surface salinity, the way nutrients are distributed and the level of chlorophyll from the water mass (Staneva et al., 2010). The downwelling phenomenon is favored by the winds from north and east that push the water surface rich in nutrients and with a low salinity toward the coast. In opposition, the winds from south and west influence the upwelling phenomenon that brings offshore the riverine waters (Staneva et al., 2010).

Shanks and Brink, 2005 tested the hypothesis according to which the bivalve larvae that swim slow are brought into great distances from the shore during upwelling and brought back to the shore during downwelling. They showed that when the larvae are situated below the

thermocline, the hypothesis was not confirmed, the larvae being swept onshore during upwelling and into the open sea in downwelling. The hypothesis was confirmed when the larvae were above the thermocline, leading to the conclusion that the influence of upwelling and downwelling on bivalve larvae varies depending on the behaviour and the vertical distribution. (Shanks and Brink, 2005).

Wave action can have a limiting effect upon the larvae of C. glaucum during their settlement. The hypothesis states that the specie is limited by areas where the influences of the waves are not possible and also by low tidal level when air exposure is infrequent (Boyden and Russell, 1972).

11. The presence of the Constanta harbor does not seem to influence the populations of C. glaucum.

The Romanian littoral of the Black Sea suffered different changes due to the human activities among which harbour construction play a major role (Ungureanu and Stanica, 2000). The Constanta harbour is the biggest harbour from the Romanian Black Sea and also the major gateway for alien species (Anastasiu et al., 2009). Despite the impact of the harbour activites upon the Romanian littoral, this seems not to influence the distribution of *Cerastoderma glaucum* as observed from genetic structure. In fact the results may indicate that no genetic pattern can be found in the populations of the bivalve along the littoral. If this is true than we must look at the way the harbour could induce indirectly this scenario.

Beside the Constanta harbour, there are also the small Midia and Mangalia harbours, all having protection jetties. It is known that such structures like jetties could modify the distribution of the marine currents and thus the transportation of the sediments (Ungureanu and Stanica, 2000). For example the most important jetty is the one from Midia which does not allow the transportation of the sediments from the Danube towards south, leading to sediment deprivation of the southern part of the Romanian littoral. This is reflected also by the rocky bottom present in this part. These kinds of constructions are responsible for the distribution of sediments and it is possible they also indirectly influence the *Cerastoderma* populations from the littoral by the type of sediments the specie lives on.

12. Aspects about the morphology of the shell and the haplotypes

The study of C. glaucum shell morphology lead Mariani et al., 2002 to the conclusion that allometric differences exist between the shells of the two *Cerastoderma* species. While C. glaucum has a positive allometry meaning that if the animal grows, the breadth, weight and height increase faster than length leading to a round shape, in *C. edule* there is a negative

allometric growth resulating in a more elongated shape. In the case of the Romanian populations of *C. glaucum* , since no correlation can be made between mitochondrial DNA and shell shape, it is possible that the only factors shaping the morphology of the shell are the environmental factors such as salinity, temperature and substrate.

13. The conection between C. glaucum and Limnocardiidae family – Razelm–Sinoe complex (when it became closed, how the shells evolved there)

An interesting case is the relation between *Cerastoderma glaucum* and the Limnocardiidae family with its representatives living in lakes near the Romanian Black Sea. Altough in the past, this relict family Limnocardiidae, had a very wide distribution encompassing the whole Ponto-Caspian region, in the present their distribution is limited to the Caspian Sea and a few lagoons of the Black Sea (Popa et al., 2009). There is a close similarity between the *Cerastoderma glaucum* and members of the Limnocardiidae which is maybe suggesting the close origin of the families. In fact Croghan, 1983, speaking about the evolution of brackish and freshwater fauna, gives an example of a very particular evolution in this Ponto-Caspian region. He is mentioning that family Limnocardiidae evolved from a marine ancestor, namely *Cerastoderma* during the Pliocene period thus leading to a diverse radiation of this family comprising about 30 endemic genera. In Romania, the distribution of the extant Limnocardiidae is limited to the Razelm Complex (Popa et al., 2010) with only two extant genera still inhabiting these lakes.

The Razelm complex is a lagoon system that has been shaped to its present day structure by the influence of Danube Delta and the Black Sea. The complex lies at the interface of both water masses being separated by a wetland from the Delta and by sand bars from the Black Sea (Vadineanu et al., 1997) and it has appeared in a former marine gulf (Bretcan et al., 2009). It is highly possible that *Cerastoderma glaucum* shared the same habitat with these Limnocardiidae, given the fact that it was observed in this lake under the name of *Cerastoderma edule var. nuciformis* (Grossu, 1962). This possible coexistence of *Cerastoderma* and the Limnocardiidae, could be explained by the existence of a communication of the Razelm complex with the Black Sea.

The normal drainage was from the lakes towards the Black Sea, but in some accidental cases due to storms a high percentage of marine water was brought to the lake. It is known that before some hydrotechnical works the salinity of the lake was almost close to the salinity of the Black Sea (Bretcan et al., 2009), meaning that over 70% of the zoobenthos was made up of Ponto-Caspian relicts and only 30% were fresh and brackish water species (Dimitriu et al., 2008), this being a good reason for the presence of *Cerastoderma* in the lake. After the year

1970, the connection with the sea was interrupted and the freshwater from Danube modified the salinity to a very low level of about 0.4-0.6 g/l (Bretcan et al., 2009) which favored the disappearance of the benthic mollusk among which those from the family Cardiidae that were euryhaline specie. This change in the salinity of water brought an almost total replace of the former fauna, with 90% freshwater species, only 7% Ponto-Caspian relicts and 3% brackish and marine species (Dimitriu et al., 2008). This very low salinity made the water from the lake to be maybe not proper for *Cerastoderma* to survive in, but good enough for the members of the Limnocardiidae which are tolerant to freshwater.

14. The influence of Vama Veche reservation upon C. glaucum

According to the molecular basic indices, the individuals from Vama Veche are the most diverse in respect to the other stations. This may be found in the nucleotide diversity (0.004) and the number of pairwise differences (2.333). Also the individuals from Vama Veche have two mutations in the following positions (139-404; 190-493) while the other individuals have only one mutation each. By looking at the way the mutations evolve, it is highly possible that the population/individuals from Vama Veche are older, which may lead them to the status of founder population. The population/individuals having only one mutation are more new on an evolutionary scale. The closest station that share almost the same diversity is Agigea, with nucleotide diversity equal to that of Vama Veche (0.004), but the mean number of pairwise differences is lower (1.905).

Vama Veche is a little more genetically diverse than the other stations, regarding the individuals sampled, and the diversity could be linked to the status of the "Littoral Marine Aquatory 2 Mai-Vama Veche" marine reserve. There were numerous measures and actions for protecting biodiversity in this reservation since its designation by Law No. 5/2000 as a marine protected area (Zaharia et al., 2008) all these contributing to the limitation of the anthropic impact. It has been stated that this reservation is a combination of different habitats, serving as a refugee and reproduction zone for marine species (Zaharia et al., 2008) which could possibly be also the case for the *Cerastoderma glaucum* population living in the perimeter of the reserve.

V. Conclusions

- The sequences obtained from *Cerastoderma glaucum* individuals supports the presence of only this species at the Romanian littoral and not the presence of *C. edule* Linnaeus, 1758 described from the speciality literature from this area.

- The genetic structure of the Romanian populations of *Cerastoderma glaucum* seems to have no patterns across its distribution range

- The Constanta harbour is likely not to influence the genetic structure of the populations

- The haplotypes encountered in our study demonstrates that the Romanian population is a part of the Ponto-Caspian phylogroup

- The results indicates low genetic variability of this specie at the Romanian littoral, a view shared also by other species from the Black Sea

- Amongst the three genetic markers used in investigating the genetic structure of populations, cytochrome oxydase subunit I (*COI*) seems to be the most proper one.

- *16S rDNA* could not be a proper markers to discriminate between populations of the same specie

- The combined analysis of *COI* and *16S rDNA* showed 10 haplotypes for all the investigated stations which is more than each marker investigated separately, and maybe for the future studies they could be employed on a bigger number of samples

- The main haplotype 75 was present in all populations of Romanian *C. glaucum*

- Vama Veche showed the greatest haplotype diversity off all stations as well as the nucleotide diversity and mean number of pairwise differences

- The haplotypes from Vama Veche have two mutations, while the other haplotypes have only one mutation

- We suppose that that by having two mutations, the population from Vama Veche is the most older one and could be taken into consideration as playing a role as the founder effect.

- The greatest diversity of Vama Veche could be linked to it status as a marine reserve, thus the anthropic influences are very low

- Mamaia was the least diverse station regarding the basic molecular indices, despite the great number of individuals examined fom the station

- The morphology of the specie does not appear to have any connection with the mitochondrial DNA, thus the only factors that are most likely affecting it are temperature, salinity and the substrate.

- There could be a link between the marine *Cerastoderma glaucum* and the freshwater relict family Limnocardiidae, as they might evolve together in the same environment

- The present study tends to complete the knowledges about the distribution and genetic structure of *Cerastoderma glaucum* from the Black Sea

- The data of our research open a way for future studies in a more complex investigation of this species from the Black Sea, the reconstitution of the whole natural history of the species and its possible impact upon human society by using it as an alternative source food.

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