

The Black Sea *Flexopecten* species-complex (Mollusca: Bivalvia: Pectinidae): Shell morphology and 16S rDNA variation

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Abstract

The taxonomic status and population variability of a Black Sea scallop (*Flexopecten*: Mollusca, Pectinidae) was evaluated in a DNA barcoding study using the 16S ribosomal DNA gene (=16S rDNA), in conjunction with seven morphological features of the shell. We conclude that the Black Sea form represents an eastern extension of the Mediterranean scallop *F. glaber* (Linnaeus, 1758). It is characterized by a significant lack of genetic variability when compared to the Mediterranean form, although it does contain new haplotypes not found in that species. There is also an increase in the mismatch in the distribution of nucleotides for the Black Sea form when compared with pooled samples of Mediterranean scallop. Plausible reasons for the observed phenomena are hypothesized from the standpoint of the epigenetic theory of evolution and the time since the scallop penetrated into the Black Sea. The lack of genetic variability is potentially due to founder effect and genetic drift.

Key words: adaptive compromise, Mediterranean Sea, morphological variation, ontogenetic channels, taxonomic diversity.

Introduction

Scallops of the Family Pectinidae are one of the most conspicuous groups of marine bivalves, and represent important components of commercial fisheries and aquaculture.

They are found across a wide range of habitats, and display a high level of morphological plasticity (Brand 2006). Mediterranean scallop (genus *Flexopecten*) in particular is a good representative of these traits in that it is widely employed as a commercial food product (Zenetos *et al.* 2005; Koutsoubas *et al.* 2007). The Atlantic group currently includes *F. felipponei* (Dall, 1922), *F. flexuosus* (Poli, 1795) (= *coarctatus*), *F. glaber* (Linnaeus, 1758) (= *ponticus*, = *proteus*), *F. hyalinus* (Poli, 1795). Only the last three species inhabit the Mediterranean basin (World Register of Marine Species 2019).

The resident scallop in the Black Sea is considered to be *F. glaber ponticus* (Bucquoy, Dautzenberg & Dollfus, 1889), a subspecies of the Mediterranean scallop *F. glaber* (Linnaeus, 1758) (Scarlatto &

Starobogatov 1972; World Register of Marine Species 2019). However, it is instead deemed by some researchers as a distinct species [*F. ponticus* (Bucquoy, Dautzenberg et Dolfus, 1889) (Milashevich 1916; Anistratenko et al. 2011)], based largely upon its endemism within the Black Sea (Revkov 2015).

However, geography can often be a confusing factor when employed as a mechanism to delineate species. For example, two closely related *Flexopecten* species, *F. glaber* (Atlantic Ocean) and *F. proteus* (Mediterranean basin) were found to represent but a single species (Imsiridou et al. 2012), based upon the analysis of several mtDNA genes (COI, 16S, 12S; Barucca et al. 2004; Saavedra & Pena 2006; Puslednik & Serb 2008; Pujolar et al. 2010; Imsiridou et al. 2012), and one nuclear gene (histone H3: Puslednik & Serb 2008; Pujolar et al. 2010). More recently, the results of a morphological study (Bondarev 2018) also identified the Black Sea form as a single species (*F. glaber*).

Species are recognized as the currency of biodiversity, and their identification is a necessary requirement for conservation and management (Dayrat 2005). Given this, our aim in this study is to clarify the taxonomy of scallop in the Black Sea, using as our descriptive tools both genetic and morphological analyses.

Materials and methods

Scallops were collected at a depth of 12-15 m from the mouth of Donuzlav Bay (Crimean peninsula, Black Sea), then relocated to growth cages at a mollusk cultivation farm (Sevastopol Bay: 44° 37' 13.3" N, 33° 30' 07.1" E). We collected 25 specimens of sexually mature mollusks older than 2 years, and subsequently employed 15 in our analyses.

Total DNA was isolated from adductor muscle using the innuPREP DNA Mini Kit (Analytik Jena, Germany). We amplified a 487 bp fragment of 16S rRNA using the following primers: direct 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and reverse 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi 1996). Ready-to-use lyophilized reaction mixtures (master mix) were employed in a 20 µL volume. The master mix contained all necessary components, including Taq DNA polymerase inhibited for the hot start, dNTP, and electrophoresis paint (Research-and-Production Company Genlab, Moscow).

PCR products were sequenced in the forward and reverse direction (*Evrogen Ru*, Moscow). Phylogenetic trees were derived using the Neighbor Joining (NJ) method (Saitou and Nei 1987) in MEGA 7.0 (Kumar et al. 2016), with subsequent bootstrap support (N=1000 replications). Genetic variability was calculated using DNASP 5.10 (Librado & Rozas 2009).

For comparative purposes, we downloaded the following 16S haplotypes from GenBank (NCBI, <http://www.ncbi.nlm.nih.gov/>): *F. glaber* (GU320280, HM627016, JQ611443, HQ197862), *F. proteus* (HM627045, HM627046, HM627048, HM627051, GU320283, GU320287). We also downloaded the following outgroups: *Chlamis glabra* (AJ243574), *F. flexuosus* (JQ611442), *Mirapekten moluccensis* (KP300558) and *Aequipecten opercularis* (AM494408) (see Table 1).

To identify individual growth ontogenies, we selected 25 individuals of the same age with a shell height 28–32 mm for morphological analysis using seven variables: Length (SL) of the scallops (maximum distance along the anteroposterior axis), height (H), width (W), length from the dorsal side to the basal groove (h), blade length (lz1), blade length from the notch to the opposite edge (lz2) and blade width (lh). The external characteristics were then derived as fractions of shell height (H). Morphological analyses were derived using Statistica 6.0 (TIBCO Software, Palo Alto CA). We employed a principal component analysis (PCA), with eigenvalues calculated from the covariance matrix. The contours of the scatter plot of individuals versus principal components represent ontogenetic trajectories (Mina et al. 1996; Mina 2001).

Results

Black Sea haplotypes derived in this study were found to cluster at 100% bootstrap probability with *F. glaber* and *F. proteus*, as well as *F. glaber*, *F. flexuosus* and *Chlamis glabra* (the latter three taxa separated at a very reduced bootstrap value; Fig. 1). They were completely identical in the nucleotide sequence along the entire length of this fragment and had a high degree of similarity with other haplotypes of *F. glaber*, as well as with *Chlamis glabra* and *F. flexuosus*.

Table 1. GenBank samples (number, location, reference) employed in this study.

GenBank Accession No	Collection localities	References
MH428573	Black Sea, Donuzlav Bay	This Study
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GU320280	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
HM627016	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
JQ611443	Rovinj, Croatia	Malkowsky Y. & Klussmann-Kolb A., 2012
HQ197862	Adriatic Sea	Pujolar J.M. et al., 2010
HM627045	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
HM627046	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
HM627048	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
HM627051	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
GU320283	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
GU320287	Aegean Sea, Thessaloniki	Chrysaeydi et al., 2010
JQ611442	Alcocebre, Spain	Malkowsky Y. & Klussmann-Kolb A., 2012
AJ243574	Mediterranean Sea	Canapa A. et al., 2000
KP300558	Philippines: Panglao Island, Bingag Spain: Malaga	Sherratt E. et al., 2016
AM494408		Arias A. et al., 2011

However, three separate Black Sea individuals represent new haplotypes [NCBI MH428573 (N=1) and MH428574 (N=2)], and cluster at a bootstrap value of 99% as an independent clade in the phylogenetic tree (Fig. 1). These three individuals are clearly distinct from *F. glaber*, *F. proteus*, *F. flexuosus*, and *Chlamis glabra*.

Black Sea specimens are separated from *F. glaber* at a *p*-distance = 0.1%, and from *F. proteus* at 0.08% (Table 2). In addition, *F. flexuosus* is geographically more remote from the Black Sea samples, yet is unusually close at a *p*-distance = 0.3%. Our results are consistent with results of previous studies (Pujolar *et al.* 2010; Imsiridou *et al.* 2012) that concluded *F. glaber* and *F. proteus* represent but a single species in the Mediterranean Sea.

When comparing scallops from the Mediterranean and, Black seas, we find the latter to display a significant reduction in genetic diversity (Table 3). It is likely that the decrease in haplotypic and nucleotide diversity, as well as a significant decrease in the number of polymorphic sites and mutations, is due to the assortative mating and selection of specimens for growth in the collector cages, similar to that found in the Pacific oyster (*Crassostrea gigas*), also cultivated from the Black Sea (Slynko *et al.* 2018). Moreover, the latter is most likely the primary source, particularly given that scallop is a synchronous hermaphrodite capable of self-fertilization (Jarne & Auld 2006; Pirkova & Ladygina 2017).

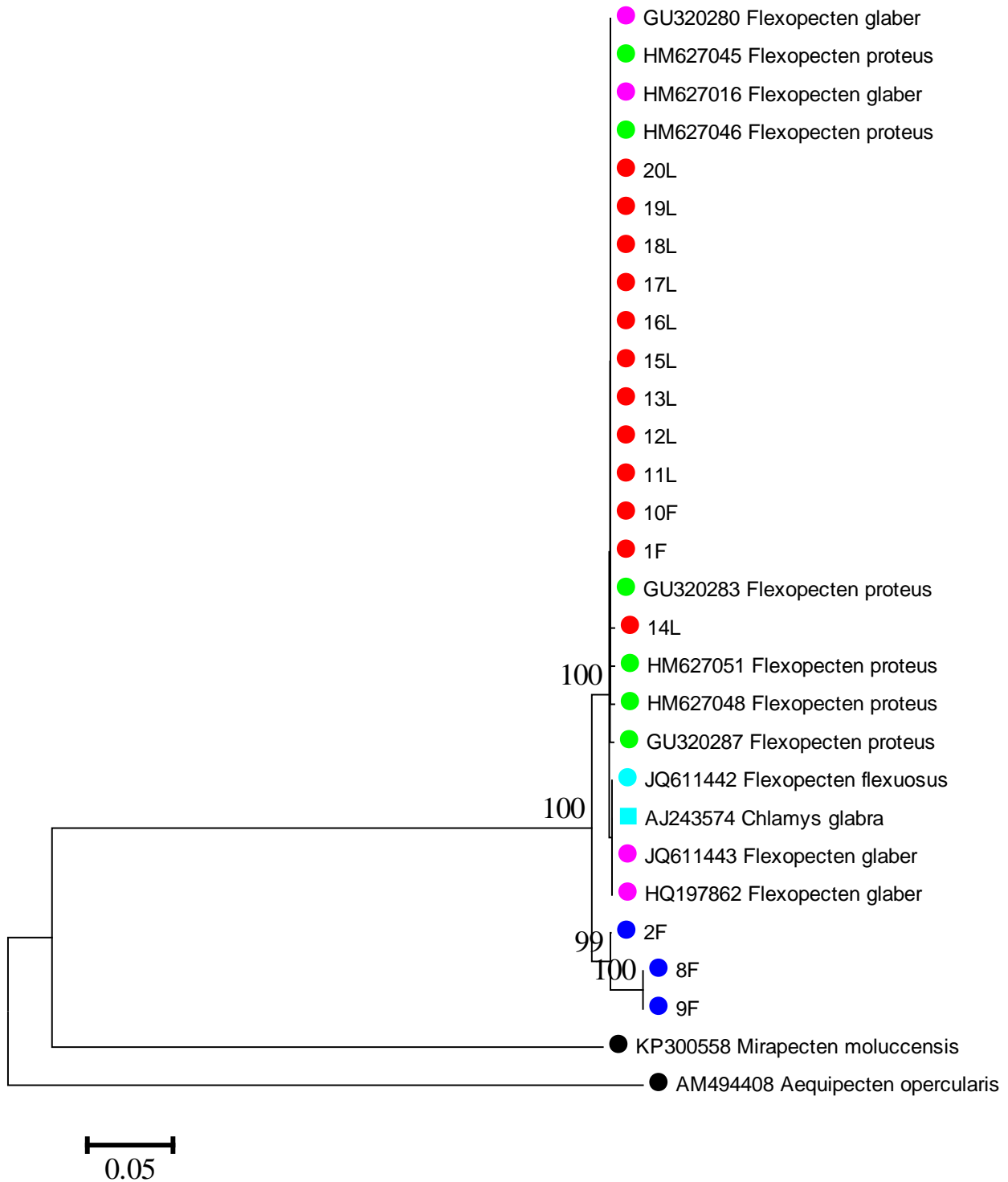


Figure 1. A Neighbor Joining (NJ) tree depicting phylogenetic relationships among *Flexopecten* haplotypes (GenBank sequences depicted as purple, green, and light blue circles) and Black Sea samples (red and dark blue circles, this study) derived from the 16S ribosomal DNA gene. Outgroups are depicted as light blue square and black circles. Values at branch nodes indicate bootstrap support. The unit of branch-length measurement = 0.05 nucleotides.

When analyzing the characteristics of the frequency distribution in pairwise comparisons of nucleotide variants of the 16S gene, we found observed and expected distributions were strongly congruent for Mediterranean *F. glaber*, with but two peaks of excess (Fig. 2B), whereas three equilibrium violations occurred for Black Sea *F. glaber* (Fig. 2A). Scallop in the Black Sea is a relatively recent migrant, following

the merger of the Mediterranean and the Black seas circa eight kya (thousands of years ago) (Zaitsev & Ozturk 2001; Bondarev 2018). An indirect confirmation of this is the relatively high level of morphological variability in Black Sea *Flexopecten*, similar to that found within the entire *Flexopecten* clade (Pujolar *et al.* 2010; Bondarev 2018).

Table 2. Matrix of p -distances derived among *Flexopecten* scallop from the Black Sea, Mediterranean Sea, other species of *Flexopecten*, and outgroups (where p -distance = the proportion (p) of nucleotide sites at which two sequences being compared are different. It is derived as number of nucleotide differences divided by total number of nucleotides compared; values presented as %).

Species	<i>Flexopecten</i> (Black Sea)	<i>F. glaber</i>	<i>F.</i> <i>proteus</i>	<i>F.</i> <i>flexuosus</i>	<i>Mirapecten</i> <i>moluccensis</i>	<i>Aequipecten</i> <i>opercularis</i>
<i>Flexopecten</i> (Black Sea)	–					
<i>F. glaber</i>	0.1	–				
<i>F. proteus</i>	0.08	0.07	–			
<i>F. flexuosus</i>	0.3	0.02	0.2	–		
<i>Mirapecten</i> <i>moluccensis</i>	64	65	65	65	–	
<i>Aequipecten</i> <i>opercularis</i>	72	72	72	72	72	–

Table 3. Genetic variability of the 16S ribosomal DNA gene derived in this study for *Flexopecten* scallop from Black Sea and Mediterranean seas. N = number of specimens, S = number of polymorphic sites, m = total number of mutations, h = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, K = intragroup nucleotide differentiation. *Flexopecten* from the Mediterranean Sea includes all of the *Flexopecten* and *Chlamys* individuals presented in Materials and Methods.

Species group	n	S	m	h	Hd	π	K
<i>Flexopecten</i> from the Black Sea	15	18	18	4	0.467	0.012	5.2
<i>Flexopecten</i> from the Mediterranean Sea	14	294	296	7	0.824	0.186	77.9

To confirm this point of view, we analyze the existence of ontogenetic trajectories for morphological data of mollusk shells. When eigenvalues of the first principal component are plotted against shell height, two developmental paths were depicted (Fig. 3). Eight individuals (4, 6, 7, 13, 18, 23-25; Fig. 2A lower) represent smaller individuals, whereas 10 (1-3, 10, 12, 14, 16, 17, 19, 22; Fig. 2A upper) represent larger individuals. An additional seven (i.e., 5, 8, 9, 11, 15, 20, 21; Fig. 2A midline) cannot be reliably assigned to either group.

Discussion

Our results demonstrate that *Flexopecten* from the Black Sea is identical with *F. glaber* from the Mediterranean Sea, which now seemingly includes species previously considered distinct. These are: *F. glaber*, *F. proteus*, and *Chlamis glabra* (Pujolar *et al.* 2010). It also follows from our results that *F. flexuosus* should also be included within this single taxonomic group, at least according to data derived from the 16S rDNA gene.

The reliability of this gene in establishing taxonomic relationships within scallops has been previously demonstrated (Imsiridou *et al.* 2012). In our study, several features that pertain to Black Sea

scallop are revealed: Those collected in Donuzlav Bay and cultivated on the marine farm show a significant decrease in molecular variability when compared with the pooled sample of Mediterranean scallops. These values are: a two-fold decrease in haplotype diversity, 15-fold in nucleotide diversity, 15-16-fold in number of polymorphic sites and mutations, and four-fold for intragroup nucleotide differentiation. Second, new haplotypes are present in the Black Sea samples. Third, there are violations of the nucleotide distribution at equilibrium.

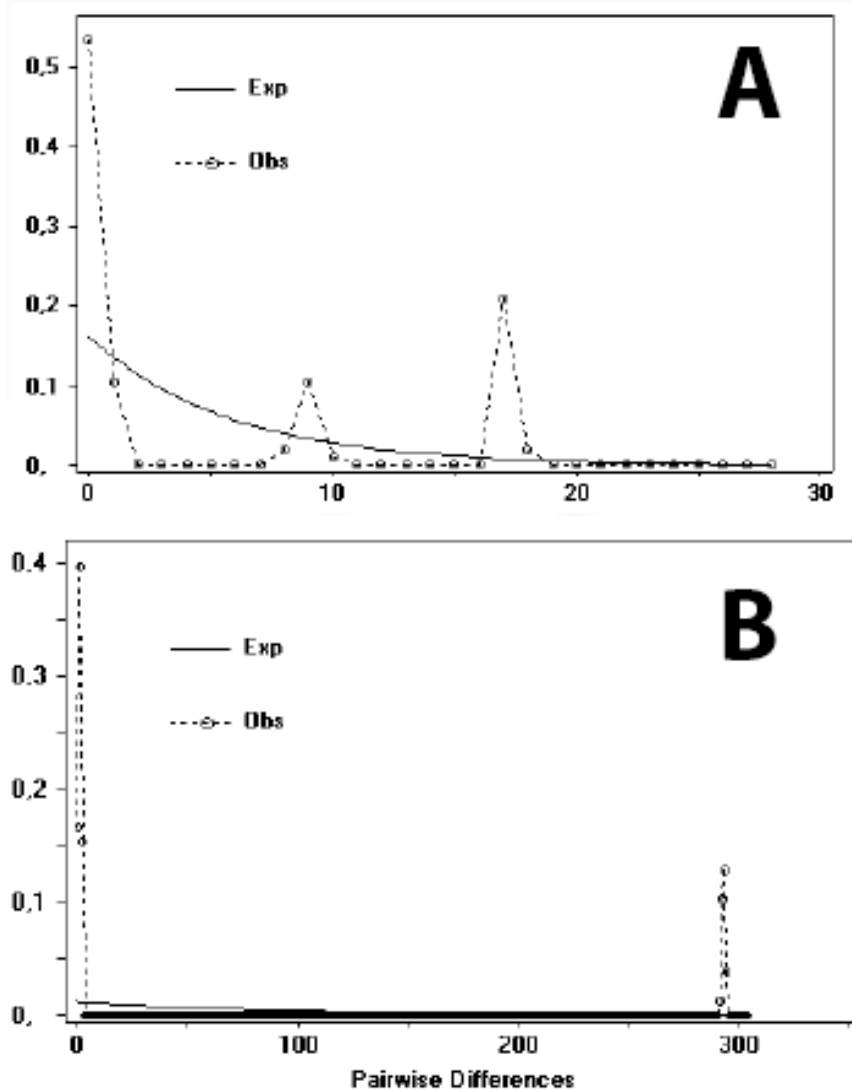


Figure 2. Frequency of occurrence depicted for 16S ribosomal DNA variants among individuals of *Flexopecten glaber*. A: Black Sea population, B: Mediterranean population.

On the one hand, our data demonstrate the potential for inbreeding depression and / or the founder effect in the Black Sea sample, despite the fact that the samples were randomly taken from the natural population. We observe a similar pattern when analyzing genetic variation in *Rapana venosa*, a recent invader in the Black Sea (Slynko et al., 2020). The relatively recent emergence of scallops in the Black Sea and its subsequent adaptation to a completely different series of hydrological and hydrochemical conditions inherent in the Black Sea should also be taken into account (Bondarev, 2018). Indirect support for the latter may be found in our ontogenetic data. As a rule, similar effects (i.e., endogenous developmental aberrations) often occur when environmental conditions are disturbed (Rasnitsyn 2006). The fact that this process has occurred during the contemporary period is confirmed by the presence of specimens not differentiated in any of the development channels, and reflects the concept of adaptive compromise (Mayr 1974; Schwartz 1980). The identification of new haplotypes, and the mismatch in the distribution of nucleotides, also support this hypothesis.

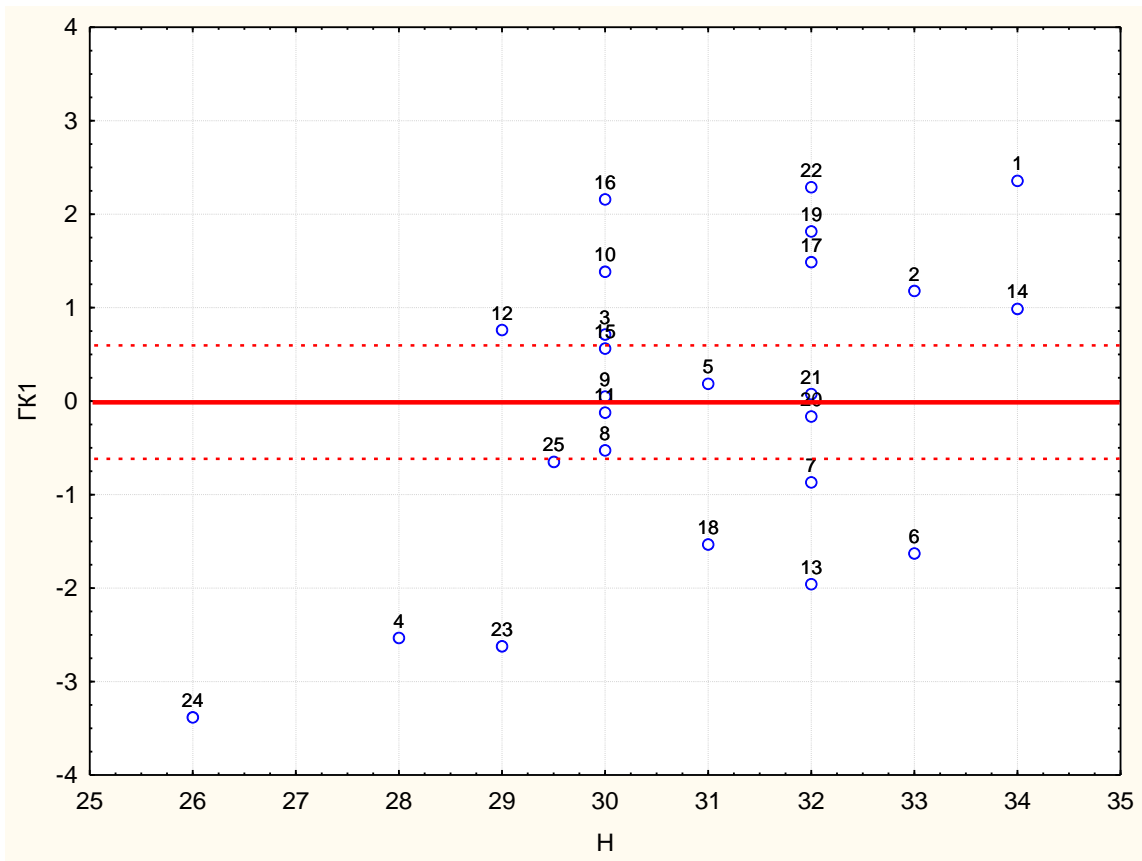


Figure 3. Plot of shell length (H: in mm) versus Index of Morphological Plasticity (ГK1).

Thus, the Mediterranean *Flexopecten glaber* has relatively recently settled in the Black Sea, and has yet to differ taxonomically from ancestral stock. However, in the specific conditions of the Black Sea, it is now characterized by a well-defined process of genetic adaptation (i.e., the appearance of new haplotypes) as well as morphological accommodations (i.e., the formation of different ontogenetic channels).

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