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RESEARCH ARTICLE

Population Dynamics of the Demosponge *Aplysina Aerophoba* (Nardo 1833) in the Mediterranean Sea Using MtDNA COI Gene

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Abstract

Aplysina Aerophoba is one of the most representative sponge species of the Mediterranean, while it is considered an indigenous species of the basin. Its importance lies not only in the fact that it is a main species of the benthic fauna, but many biomolecules with potential medicinal effects are extracted from it, which monopolized the interest of researchers. In the present research work, *Aplysina Aerophoba* population from the Aegean Sea was examined and compared with other Mediterranean populations, using the cytochrome oxidase (COI) subunit I gene, the use of which has a multitude of advantages. Regarding the population of the sponge from the Aegean Sea, it presented five different haplotypes, while a high diversity is observed based on the fixation index and molecular variance analysis. Among Mediterranean populations, there appears to be genetic differentiation between populations, while those from Italy and Spain showed a common haplotype. This result agrees with several works, which support the differentiation of populations of a species between the eastern and western Mediterranean Sea. This fact may be due to the seasonally changing climatic and geomorphological conditions of the basin. Moreover, by calculating the Tajima's D index, the neutrality theory is confirmed, as the mutations do not change the allelic frequencies of the *Aplysina Aerophoba* populations under consideration. Finally, from the demographic study of the Aegean population, it emerged that it did not undergo any strong change in evolutionary time.

Key words: *Aplysina Aerophoba*, Mediterranean Sea, Populations genetics, COI, Demographics.

1. Introduction

Sponges are the first and simplest phylum of metazoans (Bell et al., 2015) and consist of one of the most diverse phyla of animals, with a global estimation of approximately 5,000 species. This extremely high abundance and distribution classify them as one of the most important species with respect to the ecological impact on their site fidelity zones (Idan et al., 2017; Coll et al., 2010). Various marine organisms use sponges' communities for reproduction, food resources or occasionally shelter. Moreover, sponges have a great economic importance regarding chemical compounds that have beneficial effects via pharmaceutical business (Lee et al., 2001). Their wide distribution has led to the evolution of various adaptive traits, with the most profound being the efficient filtration of water to absorb the necessary nutrients. Taxonomically speaking, sponges are classified in four genera (Calcarea, Hexactinellida, Demospongiae and Homoscleromorpha – Cardenas et al., 2012; Wörheide et al., 2012), with almost the 85% of the global and Mediterranean records being demosponges (Systema Porifera, Hooper & van Soest, 2002). Across the globe, sponges can appear widely distributed (van Soest et al.,2012),

nevertheless presenting the common animal pattern of distribution, in which a decrease in sponge fauna is observed from the Equator towards the poles. The temperate Mediterranean Sea is both a centre of abundance and biodiversity (Xavier et al., 2012), while the Mediterranean sponge fauna represents 11% of global biodiversity (Pansini et al., 2003). In the Mediterranean basin, sponges are distributed from the northwest to the southeast (Pansini et al., 2003; Voultsiadou, 2009), due to the prevailing physicochemical characteristics (Idan et al., 2017) but also of the non-viability of the larvae for a long time. An exception is cleaning sponges, which show an opposite pattern of abundance (Voultsiadou et al., 2009).

The high abundance of species, combined with the rapid geological changes it has undergone, turn the Mediterranean into a field of study of the population genetics of these key organisms. These environmental changes probably led to different evolutionary forces in the population dynamics of the species (Petit, 2003), and therefore speciation or even bottleneck effect phenomena may drove in species-specific population shrinks (Riesgo et al., 2016). The most important period was during the Pleistocene glaciations (Hewit et al., 2000), where barriers to gene flow and isolated refugia were created (Moura et al., 2014; Feidantsis et al., 2022; Konstantinidis et al., 2022). The later has been argued that, within the Mediterranean Sea various marine organisms show a differentiation pattern between the eastern and the western basin (Moura et al., 2013; Gkafas et al., 2017; Trajer et al., 2021).

Sponge populations show a fine population structure within the Mediterranean Sea. *Chondrosia reniformis* appeared to show differences in the COI gene between populations of eastern and western Tunisia (Mussa et al., 2022). *Ircinia fasciculata*, in addition, is a characteristic study species, as the potential barrier to gene flow seems to be in three places in the Mediterranean, namely in North Balearic Sea, Ligurian Sea and Alboran Sea (Riesgo et al., 2016). Additionally, it is argued that the populations of many sponges and cnidozoa show a similar pattern (Duran et al., 2004; Mokhtar-jamai et al., 2011), while in Alboran Sea it is clarified a barrier in gene flow between North-East Atlantic and Mediterranean Sea. On the other hand, there are sponges characterized by genetic panmixia such as *Crambe crambe* (Bell et al., 2015) and *Spongia officinalis* (Dalianis et al., 2011).

Aplysina Aerophoba is a sponge species with distribution throughout the Mediterranean basin (Coppari et al., 2016). Its preferred habitats are characterized by shallow water up to 40 meters and abundant sunshine (Idan et al., 2018). This is due to mesohyl hosts a variety of microorganisms including cyanobacteria, the metabolism of which produces several primary and secondary metabolites (Binnewerg et al., 2020), which led to the continuous study of the sponge's microbiome and symbiotic relationships. However, despite the wide research effort in this direction, the biology and evolution of the sponge has not been extensively studied.

A. Aerophoba, gained high attention due to taxonomically reasons, suggesting its closely relation to the species *Aplysina cavernicola* (Checaldonne et al., 2006) using morphological data. At the same time, the use of MtDNA for such studies was under consideration, since studies argue that the MtDNA genes are not properly conserved (Chen, 2012; Deagle et al., 2014). Nevertheless, the cytochrome oxidase (COI) subunit I gene is currently widely used for population genetics and phylogeny studies (Heim et al., 2017), as applied in the present work, and it has a multitude of advantages. In particular, in a cell there are many copies of this conserved gene, which do not carry internal non-transcribed regions (Avise et al., 1987). At the same time, it is much more studied compared to nuclear DNA genes (Hagelberg, 1994), while it has the ability to distinguish *A. Aerophoba* from *Aplysina cavernicola* (Costa et al., 2020). Finally, its use is also supported as it can distinguish differences within a population (Deagle et al., 2014), while it is considered suitable for metazoans (Andujar et al., 2018).

To the best of our knowledge population genetics of *A. Aerophoba* have not been studied throughout the Mediterranean, despite the high representativeness of the sponge in the area. Including the fact that many sponges show clear genetic differentiation and disruption of gene flow between their populations, as in *Chondrosia reniformis* (Mussa et al., 2022), *Ircinia fasciculata* (Riesgo et al., 2016) and *Spongia officinalis* (Dalianis et al., 2011), it is raised the question of whether panmixia or differentiation is observed in the populations of *A. Aerophoba* mainly in the East-West Mediterranean axis. This issue is further strengthened by the fact that the distribution pattern of many benthic species is the same as that of *A. Aerophoba* (Mokhtar-jamai et al., 2011). Finally,

can be estimated the effect of environmental crises on the populations o *A. Aerophoba*, the origins of which derives possibly from the Tethyan Sea (Schmitt et al., 2005). Additionally, the demography of the population from the Aegean Sea was examined in detail in order to ascertain the change of the effective population size on that side of the Mediterranean which is characterized not only by different climatic and physicochemical characteristics but also by a deeper water column (Donavaro et al., 1999).

The aim of the study is to examine the population dynamics of the species in question and test the hypothesis that *A. Aerophoba* follow the fine population pattern and constitute a single cluster in the Mediterranean basin, so the populations are genetically connected, as it is demonstrated on the level of *Aplysinidae* (Schmitt et al., 2005). This is tested with the comparison of sponge populations from different regions of the Mediterranean, so as to establish the uniformity of the sequences of the conserved COI gene and draw conclusions about their evolution.

2. Materials and Methods

2.1 Sampling, DNA Extraction and PCR Amplification

Initially, six specimens of *A. Aerophoba* were collected from Aegean Sea by scuba diving, which were preserved at -20 °C, without any further processing. Pinacodermis and mesohyl tissues were used for the DNA extraction using CTAB protocol by Doyle and Doyle (1987). DNA fragments was verified by 0.8% agarose gel electrophoresis.

Polymerase chain reaction (PCR) was applied to amplify the COI gene from each sample. Species-specific primers were designed to amplify the COI gene of *A. Aerophoba*, with the following sequences: forward 5´- AATTAGACTGGAGTTATCTGC - 3´ and reverse 3´- GCATAGTAATTGCTCCAGC - 5´.

Amplification reaction mixtures consisted of ~10 ng DNA template, 0.2 µM of each primer, 6 µl of 10× reaction buffer and 1 unit of polymerse taq using the GoTaq Green Master Mix (Wisconsin, USA) in a final volume of 20 µl. PCR amplifications conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 thermal cycles of denaturation at 94 °C for 45 s, annealing at 50 for 1 min, and extension at 72 °C for 1 min 30 s, with a final extension at 72 °C for 10 min. All PCR products were separated by electrophoresis on 1.2% agarose gels. Sanger sequencing was applied on an ABI PRISM ® 3700 DNA Analyzer (Applied Biosystems, Waltham, MA, USA).

2.2 Data Analysis

Sequences were tested through BLAST search in GenBank NCBI (GenBank®—www.ncbi.nlm.nih.gov/) in order to verify sequence orthology. Additional sequences were used (accessed from GenBank on 30 May 2023) from Spain (Gulf of Rosses) (Steinert et al., 2017) and Italy (Costa et al., 2020). All datasets were aligned and trimmed using the AliView software (Larsson, 2014).

FABOX v.1.61 (Villesen, 2007) was used to extract the haplotypes per population. Arlequin v.3.5
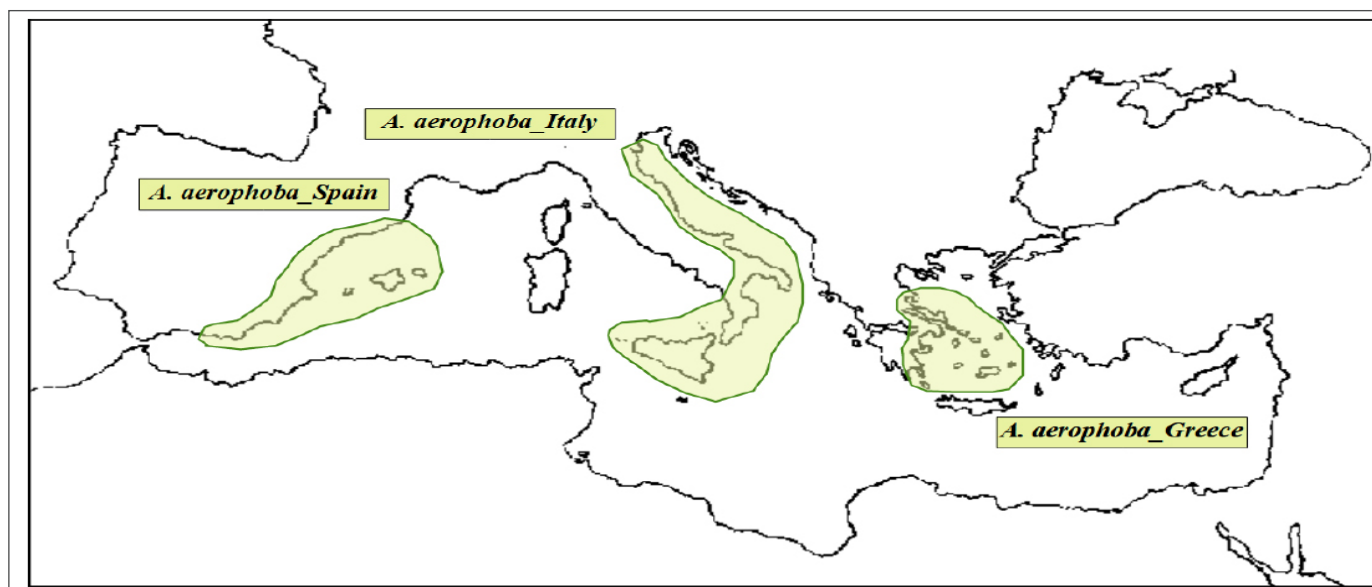

Figure 1. *Visualization of the three Mediterranean regions from which the COI gene sequences were obtained from the Aplysina Aerophoba sponges.*

software (Excoffier & Lischer, 2010) was used to calculate linkage disequilibrium. AMOVA (analysis of molecular variance), neutrality test (Tajima's test) and F_{ST} pairwise index was also calculated.

Finally, DIY-ABC v.2.1.0 software was used, to infer demographic history based on the Approximate Bayesian Computation (ABC) analysis. We tested four scenarios as follows: (1.) Scenario 1: Constant population size model; (2.) Scenario 2: Bottleneck with size recovery model, where ancestral population experienced a bottleneck followed by population size recovery; (3.) Scenario 3: Population expansion model of the ancestral population; (4.) Scenario 4: Bottleneck and expansion model, where the ancestral population favored an expansion, followed by a bottleneck, and finally current population experiences a size recovery; (5.) Scenario 5: Bottleneck without size recovery model, where the ancestral population after bottleneck did not recover in current times.

Table 1. *Summary report of the demographic scenarios used in the diy-abc v.2.1.0 application with approximate bayesian computation, to verify the applicable demographic model for Aplysina Aerophoba sponges in the Greek samples, in which the thickness of each section is proportional to the size of the population there. The following are clarified:*
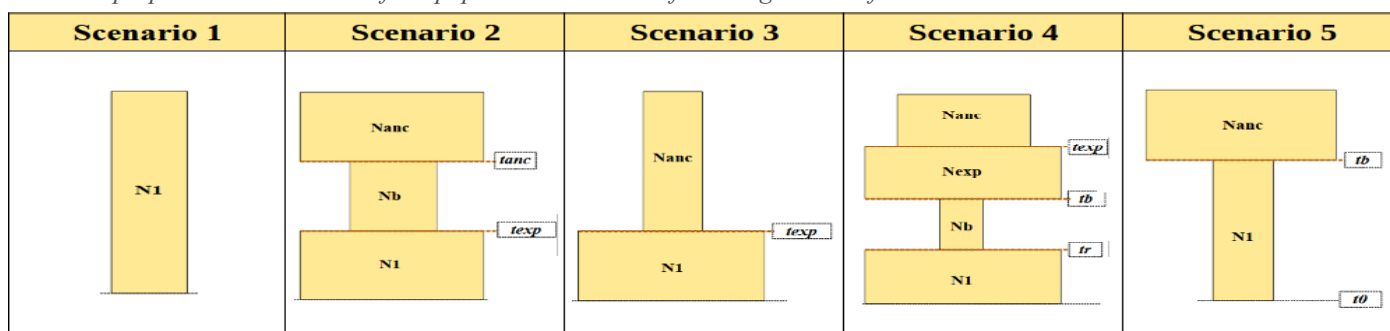
N1→existing population, t0→time at t=0, tb→bottleneck effect time, tr→population recovery time, Nanc→ancestral population, Nb→population that has undergone bottleneck effect, texp→time when population growth was observed, tanc→time at which the ancestral population first suffered for the first time, VarNe→change in population size.

Table 2. *Defined priors in the historical mode of diy-abc v.2.1.0 and the rations between parameters.*

Priors	N1	t0	texp	Nb	tanc	Nanc	Nexp	tr	tb
Minimum	10	10	10	10	10	10	10	10	10
Maximum	10^6	10^4	10^8	10^3	10^8	10^5	10^4	10^5	10^6
Rations	tanc > texp > tb > tr > t0, Nexp > Nanc > N1 > Nb								

3. Results

Analysis, demonstrated the existence of six haplotypes, where the MED-1 haplotype was common among the sequences from Spain and Italy, while in Greece the remaining five haplotypes (MED-2, 3, 4, 5, 6). The sequences of each specimens of Aegean Sea were deposited in NCBI with accession numbers SUB13503082OR100697, SUB13503082OR100698, SUB13503082OR100699, SUB13503082OR100700, SUB13503082 OR100701 and SUB13503082 OR100702.

AMOVA (Table 3) show no genetic differentiation within a population or among the examined populations (p-value > 0.05), while a statistically significant genetic differentiation was calculated between the EAST and WEST populations (p-value = 0.024). Arlequin v.3.5 statistical processing showed that there is genetic differentiation between the EAST and WEST populations (F_{ST} = 0.49, p-value = 0.03 – Table 4), which was also confirmed from analysis of variance.

Table 3: *Amplification of hierarchical analysis of molecular variance (AMOVA) for EAST and WEST populations for A. Aerophoba. The WEST population is represented by the Spanish and Italian haplotypes as extracted from NCBI, while the EAST population is represented by the Aegean Sea haplotypes.*

Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation index
Among populations	1	7.200	1.24Va	49.79	
Within populations	8	10.000	1.25Vb	50.21	F_{ST} = 0.49
Total	9	17.000	248.96		

Table 4. *FST and p-value among EAST and WEST populations of A. Aerophoba. Significance rate is equal to 0.05.*

Populations	EAST	WEST
EAST	0	
WEST	F_{ST} = 0.49 (49%), p = 0.03 ± 0.01	0

For the Tajima's D index, the null hypothesis (H0) means that evolution acted in the population according to the Kimoora's neutral theory, where polymorphisms are silent and do not affect the allelic frequencies of individuals (p-value > 0.05), while according to the alternative hypothesis H1 polymorphisms lead to a change in allelic frequencies of the population and thus natural selection acts (p-value < 0.05). In this case, it seems that for the two populations of A. Aerophoba, EAST and WEST, hypothesis H1 is rejected, which means that random mutations do not lead to a change in the allelic frequencies of the populations. Regarding the WEST population, where Tajima's D is equal to zero, the observed genetic differentiation is the same as expected (Table 5).

Table 5. *Tajima's D index and its p-value for the two populations of Aplysina Aerophoba under study, where in the WEST population there are haplotypes from Spain and Italy and in the EAST population haplotypes from Greece. It is defined that the confidence level of the statistical results is defined as 95%, p=0,05.*

Statistics	Population EAST	Population WEST	Mean	Standard deviation
Sample size	6	4	5	1.41
S	9	0	4.5	6.36
Pi	4	0	2	2.83
Tajima's D	0.09	0	0.04	0.06
p-value	0.52	1	0.76	0.34

In the last stage, by application of demographic analysis, scenario 1 was confirmed for the population of the particular sponge in the central Aegean Sea. The numerical features, that were used for defining the priors in the historical mode of the application are demonstrated on the table 2, where the relationship between the parameters are also shown in table 1. Figure 3 shows that the scenario 1 applies with a probability of 60%. Then, a posterior distribution of parameters was estimated for this scenario, where for that the parameters were t0 and N1. The results of this analysis is shown in figure 4. Finally, the statistical parameters median and mode for both t0 and N1 are demonstrated on the table 6, where the mode is the average of the values and the median informs about how many individuals there are on average in the generation (for N1) and how long is the duration of the generation of A. Aerophoba (for t0).
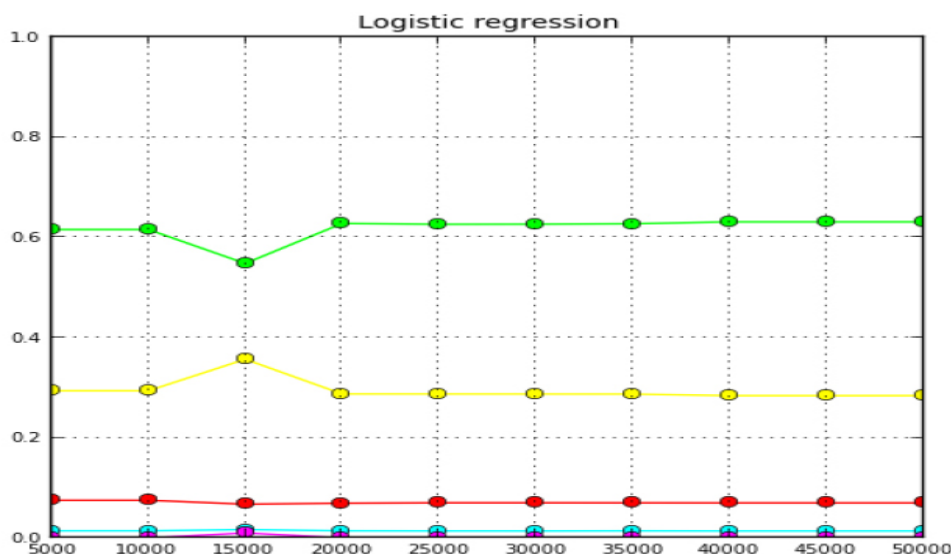

Figure 3. *Regression analysis of the scripts in the diy-abc v.2.1.0 application. as formulated in table 2, where it is demonstrated that scenario 1 (green graph) is more likely to apply with a percentage of 60%. The analysis of the Aplysina Aerophoba population of the Aegean Sea. It is clarified that scenario 5 is represented in yellow, scenario 2 in red, scenario 3 in blue and scenario 4 in purple.*
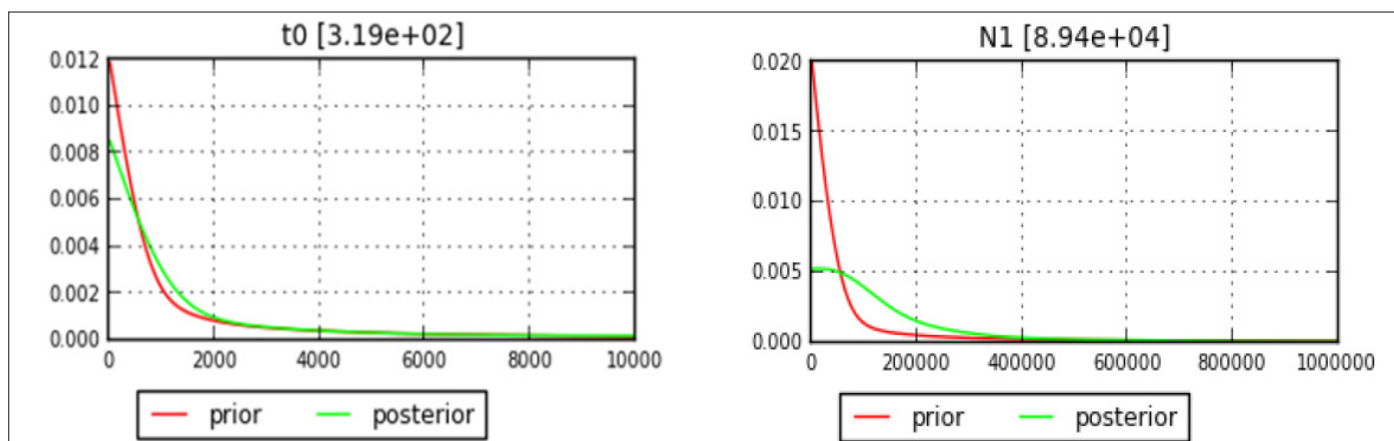
Figure 4. *Analysis of scenario 1 parameters, the distribution of which appears to confirm this scenario, as performed by diy-abc v.2.1.0. An intersection of the plots of both t0 and N1 with the x-axis is observed. The x-axis indicates the number of priors we set when building the models.*

Table 6. *Mode and median statistical parameters, as calculated in the diy-abc v.2.1.0 for t0 and N1 (scenario 1).*

	N1	t0
Median	8,49e+04	3,19+02
Mode	4,57e+04	1,1e+01

4. Discussion – Conclusion

Over time, the Mediterranean Sea has been a field of radical geomorphological and climatic changes (Antonioli et al., 2007; Lionello et al., 2014). Therefore, the strong environmental pressures received by the permanent populations of the basin led to variations in their population dynamics (McKelvey et al., 1983), while evolutionary forces sometimes worked in favor of the merging of populations and sometimes inhibited it (Cox & Moore, 1993). As for the sponge populations, they were more vulnerable due to their established form, but simultaneously they constitute one of the most primitive metazoan phyla in the Mediterranean (Voultsiadou, 2007). Therefore, it is concluded that the evolution of sponges could shed light on various aspects of the evolution of metazoans, in particular the sedentary phyla, without, of course, yet being clarified whether the last common ancestor of metazoans is spongiform, as the monophyletic theory says (Wörheide et al., 2012).

From the analysis of molecular variance (AMOVA) it appears that there is a high genetic differentiation of the order of 50% both within the EAST and WEST populations, as well as between them. This high genetic differentiation between populations is also evidenced by the calculation of the fixation index (F_{ST}). The high genetic diversity within each *A. Aerophoba* population contrasts with the high endemism index generally observed in Mediterranean sponge populations (Coll et al., 2010). This pattern of population differentiation between the eastern and western basin (Feidantsis et al., 2022; Pansini et al., 2003;), is also observed in other sponges such as *Petrosia ficiformis* (Riesgo et al., 2019), while in others such as the commercial *Spongia officinalis* shows minimal genetic flow (Nm) (Dallianis et al., 2011).

The eastern Mediterranean Sea is deeper and more oligotrophic than the western one, while it is characterized by different physicochemical characteristics (Donavaro et al., 1999). If disruption of gene flow is taken into account, populations will tend to evolve distinctly, which is demonstrated in many studies of sponge populations based on some molecular marker (Riesgo et al., 2016; Portela et al., 2015). Regarding *A. Aerophoba*, the EAST population is not only characterized by the existence of five haplotypes, but at the same time no population change seems to have taken place in it to date. This conclusion is also supported by the fact that a high genetic diversity was observed within the population, which would contradict any drastic population decline.

In summary, regarding the demographic and evolutionary history of the sponge *A. Aerophoba*, it appears to be an endemic species of the Mediterranean originating from the Tithys Sea (Schmitt et al., 2005). Then, its populations were subjected to two strong environmental pressures, one during the Messinian Salinity Crisis and one during the last glacial period of the Pleistocene, which led to rapid geomorphological

changes in the basin, uniting land areas and interrupting communication between *A. Aerophoba* populations.

These changes led to differentiation of the sponge populations between the eastern and western basins, suggesting a possible barrier to gene flow in the Sicilian Straits, as also suggested by the pattern of other sponges. As the last glacial period receded, populations continued to evolve independently, possibly because parenchymules are short-lived and therefore unable to be transported long distances. Finally, despite the strong environmental pressures, it seems that in the population of the eastern Mediterranean there was no reduction in the active population size and therefore no reduction in genetic diversity – heterozygosity in evolutionary time. This increased genetic diversity leads to an increase in the fitness of the sponge (Reed et al., 2003; Booy et al., 2000), which now makes it one of the most abundant sponge species of the Mediterranean.

Finally, it is worth emphasizing that the ever-intensifying anthropogenic activity has led in recent decades to a drastic reduction in the populations of many species of Mediterranean sponges (Tal Idan et al., 2018; Lionello et al., 2014), which probably leads to reducing the genetic diversity and reproduction fitness of species. In addition, the continuous increase in temperature due to climate change and the introduction of alien species from the Suez Canal (Galil et al., 2018), are two parameters that directly affect not only the effective population size, but also the biological circle of many species, but especially of the benthic one. Therefore, the question arises, to what extent will the populations of *A. Aerophoba* in the Mediterranean Sea be affected under these constantly changing conditions. Further research effort is required to answer this question and to examine in more detail the biology and ecology of one of the most representative sponges of the Mediterranean fauna.

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