Insights on *Pinna nobilis* genetic connectivity in the Eastern Mediterranean Sea (#80443)

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Insights on *Pinna nobilis* genetic connectivity in the Eastern Mediterranean Sea

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The fan mussel *Pinna nobilis* Linnaeus, 1758 is an endemic species of the Mediterranean Sea, protected by international agreements (Annex IV of the Habitat Directive, Annex II of the Barcelona Convention). It is one of the largest bivalves in the world, playing an important role in the benthic communities; yet it has been recently characterized as “Critically Endangered” by the IUCN, due to a Mass Mortality Event. In this context, the assessment of the genetic variation of the remaining *P. nobilis* populations and the evaluation of connectivity among them are crucial elements for the conservation of the species. For this purpose, samples were collected from six regions of the Eastern Mediterranean Sea; the Islands of Karpathos, Lesvos and Crete; the Chalkidiki and Attica Peninsulas; and the Amvrakikos Gulf. The sampling was performed either by collecting tissue from the individuals or by using a non-invasive method, i.e. by scraping the inside of their shells aiming to collect their mucus and thus avoiding stress induction to them. Conventional molecular techniques (DNA extraction, PCR amplification, Sanger Sequencing) with the use of the COI and 16S rRNA genetic markers were selected for the depiction of the intra-population genetic variability. The analyses included 105 samples from the present study and publicly available sequences of the species across the Mediterranean Sea. The results of this work a) suggest the use of eDNA as an efficient sampling method for protected bivalves and b) shed light to the population connectivity of *P. nobilis* in the Eastern Mediterranean, knowledge that might prove to be fundamental for the species conservation and hence the ecosystem resilience. The haplotype analyses reinforced the evidence that there is a certain degree of connectivity among the distinct regions of the Mediterranean; yet there is evidence of population distinction within the basin. The combination of both genetic markers in the same analysis produced more robust results, revealing a group of haplotypes being present only in the Eastern

Mediterranean and providing insights for the species' most suitable management.

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Short Title:
Pinna nobilis population genetics

Keywords:
Pinna nobilis, population genetics, Mediterranean, COI, 16S rRNA, eDNA, critically endangered, connectivity, haplotype networks

Abstract

The fan mussel *Pinna nobilis* Linnaeus, 1758 is an endemic species of the Mediterranean Sea, protected by international agreements (Annex IV of the Habitat Directive, Annex II of the Barcelona Convention). It is one of the largest bivalves in the world, playing an important role in the benthic communities; yet it has been recently characterized as "Critically Endangered" by the IUCN, due to a Mass Mortality Event. In this context, the assessment of the genetic variation of the remaining *P. nobilis* populations and the evaluation of connectivity among them are crucial elements for the conservation of the species. For this purpose, samples were collected from six regions of the Eastern Mediterranean Sea; the Islands of Karpathos, Lesvos and Crete; the Chalkidiki and Attica Peninsulas; and the Amvrakikos Gulf. Sampling was performed either by collecting tissue from the individuals or by using a non-invasive method, i.e., by scraping the inside of their shells aiming to collect their mucus and thus avoiding stress induction to them. Conventional molecular techniques (DNA extraction, PCR amplification, Sanger Sequencing) with the use of the COI and 16S rRNA genetic markers were selected for the depiction of the intra-population genetic variability. The analyses included 104 samples from the present study and publicly available sequences of the species across the Mediterranean Sea. The results of this work a) suggest the use of eDNA as an efficient sampling method for protected bivalves and b) shed light to the population connectivity of *P. nobilis* in the Eastern Mediterranean, knowledge that might prove to be fundamental for the species conservation and hence the ecosystem resilience. The haplotype analyses reinforced the evidence that there is a certain degree of connectivity among the distinct regions of the Mediterranean; yet there is evidence of population distinction within the basin. The combination of both genetic markers in the same analysis produced more robust results, revealing a group of haplotypes being present only in the Eastern Mediterranean and providing insights for the species' most suitable management.

Introduction

In the autumn of 2016 a phenomenon of massive mortality was observed on the Western Mediterranean populations of *Pinna nobilis*, the largest endemic bivalve of the Mediterranean Sea (Darriba, 2017). The mass mortality events (MME) reached quickly the Eastern Mediterranean Sea (Katsanevakis et al., 2019). Although several pathogens have been proposed

as the MME agents (Catanese et al., 2018; Carella et al., 2019; Panarese et al., 2019), the most likely one is the protozoan *Haplosporidium pinnae*, which is considered to affect the digestive gland of the animal, resulting in stress, starvation and in a general dysfunction and finally death of the organism (Box, Sureda & Deudero, 2009; Grau et al., 2022). Based on all the above, the species status in the IUCN red list changed into that of Critically Endangered (CR) (Kersting et al., 2019).

The decline of the pen shell's population was known several years before the MME (Centoducati et al., 2007) due to threats such as the coastal construction activity, the degradation of its habitat, the anchoring -especially at touristic hotspots- the wave action, the byssus exploitation for production of sea silk and the illegal trawling activity (Hendriks et al., 2013; Basso et al., 2015). This led to a series of regulations aiming to protect the species and ensure its survival. National legislation and international conventions have been in force for the past decades, such as the Barcelona Convention for the Protection of the Marine Environment and the Coastal Region of the Mediterranean and the Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora (Annex IV). Nevertheless, the effectiveness of those measures was argued, since *P. nobilis* was still subject to illegal fishing for personal or massive consumption or for decorative purposes (Katsanevakis et al., 2011).

Undoubtedly, *P. nobilis* is a beneficial species for the benthic communities for a number of reasons and for various ecosystem services. As a filter feeder, it filters large amounts of water contributing to the seawater clarity (Basso et al., 2015), a process that benefits the meadows of the cohabitant species *P. oceanica* (Trigos et al., 2014). Its large valves provide a hard substrate within a sandy area for many sedentary organisms, so it is fairly considered as an ecosystem engineer (Rabaoui et al., 2015). It sometimes also cohabits with the crustaceans *Pontonia pinnophylax* and *Nepinnotheres pinnotheres* (Hassine, Zouari & Rabaoui, 2008; Akyol & Ulaş, 2015), thus increasing even more the complexity and species richness of the community it lives in. Recently, due to the attention it has attracted, *P. nobilis* has been characterized as a flagship species (Scarpa et al., 2020). Without a doubt, this could prove important not only for the conservation of the species itself and the ecosystem it is associated with, but also for raising public awareness for marine environmental issues in general (Polgar & Jaafar, 2018).

For this reason, *P. nobilis* has been the subject of several molecular studies during the past decade conducted in the Aegean Sea (Katsares et al., 2008), Tunisian coasts (Rabaoui et al.,

2011), at a larger area of the Central Mediterranean (Sanna et al., 2013), while in the Adriatic Sea, Ankon (2017) investigated the population genetics of *P. nobilis* in marine parks of Croatia. Microsatellite markers were used for the first time at Catalonian, Balearic and French coasts (González-Wangüemert et al., 2015; Wesselmann et al., 2018; Peyran et al., 2021), reinforcing the existing knowledge about the genetic structure and variability of the populations of the species. In this context, the mitochondrial DNA (mtDNA) has proved to be a very useful marker for population genetics studies. Due to its high variability and evolutionary rate (Sunnucks, 2000) it can depict differences that nuclear DNA cannot (Brown, George & Wilson, 1979). Therefore, a significant differentiation among and within populations is revealed, including animals (Parker et al., 1998), with bivalves not being an exception (Baldwin et al., 1996; Matsumoto, 2003; Wood et al., 2007; Feng et al., 2011). It should be noted though, that mtDNA in certain bivalves, such as *Donax trunculus* (Theologidis et al., 2008) and *Mytilus* spp. (Zouros, 2013), has a biparental inheritance which, undoubtedly, affects population diversity estimates based on it.

The aim of the present study was to a) investigate the genetic diversity of the *P. nobilis* populations at the Eastern Mediterranean Sea, an area that has not been well studied in this regard, and b) compare it with similar studies from the Western and Central Mediterranean in an attempt to c) provide further insights into population structuring of this critically endangered species, which will offer a good estimation on the fitness and diversity of the Greek populations.

Material and Methods

Sampling area

For the purpose of the study 105 samples were analyzed, after being collected within the period of August 2018 – April 2021. The samples were collected from six locations of the Eastern Mediterranean Sea and particularly from the Islands of Karpathos, Lesvos and Crete, the Chalkidiki and Attica Peninsulas, and the Amvrakikos Gulf (Fig. 1, Table 1).

eDNA sampling

The sampling method for Karpathos' samples was non–lethal, non-invasive and low impact aiming at the minimization of the disturbance towards the bivalves, since the tissue removal may provoke stress and make the animal more susceptible to diseases. Initially, a rod of 0.5 cm diameter was placed at the opening of the valves of each animal by the SCUBA divers, taking into account the fragility of the shell's outermost part. Consequently, a sampling brush, resembling a buccal swab was used (Supplementary Fig. 1) to scrape the tissue remnants and mucus from the interior of the valves. The sampling brushes (one for each individual) were placed in small zip bags and stored at -20 °C until further processing. Additionally, the shells' width and length were recorded by the divers.

Tissue sampling

All the other samples were collected under research permits, for the initial aim of the sampling which was the investigation of the infection of *P. nobilis* from the parasite *H. pinnae*. Specifically, 50-100 mg of different tissues of each individual were removed, preserved in absolute ethanol and stored at 4 °C until further processing. As previously, the shells' width and length were recorded by the divers.

DNA extraction

DNA was extracted according to the protocol of Sambrook, Fritsch & Maniatis (1989), both from the brushes as well as from the tissues. In the case of the latter, small pieces of the collected

tissues were chopped with sterile scissors; triplicate extractions were performed for each tissue, in order to minimize biases. Each replicate sample was washed with 800 ul of sterile distilled water for 15 min, following centrifugation at 13000 g for 2 min, as in Darriba (2017). The supernatant was removed and the wash was repeated. Afterwards, each sample was washed with 600 µl of lysis buffer (0.5 M Tris, 0.1 M EDTA, 2% SDS, ph 8.8) for 15 min, following centrifugation at 13000 g for 2 min and removal of the supernatant. The washes with the lysis buffer were repeated twice. The pellet was mixed with 600 µl of lysis buffer and 6 µl of proteinase K (20 mg/ml) and incubated at 55ºC overnight. DNA was extracted by precipitation with isopropanol and ammonium acetate (5 M) (Sambrook, Fritsch & Maniatis, 1989). In the final step of the DNA extraction protocol; i.e. the elution of the DNA pellet, replicate samples were pooled and their concentration was measured in a NanoDrop 1000 spectrophotometer.

PCR amplifications

For the PCR amplification of the tissue samples, no specific tissue was chosen but rather a mixture of all the extracted DNAs in similar concentrations. Initially PCR amplifications were performed for the COI and 16S rRNA genes with previously used primers and conditions (Folmer et al., 1994; Sanna et al., 2013, 2014; Leray et al., 2013); however the amplifications were not successful. Therefore, new primers were designed (Table 2) based on the available *P. nobilis* sequences in GenBank (Sayers et al., 2023).

Each PCR contained 2 µl of DNA template (about 20 ng/µl), 4 µl of 5X KAPA HiFi Fidelity Buffer, 1 ul of each primer (10 um), 0.8 ul of dNTPs (10 mM each), 1 µl of KAPA HiFi HotStart DNA Polymerase (1 U/uL) at a total volume of 20 µl. Amplifications were performed at a BioRad T100 thermal cycler. The PCR protocol was the same for the two genes; namely a denaturation step at 95 °C for 5 min followed by 35 cycles of 98 °C for 20 sec, 53 °C for 30 sec, 72 °C for 30 sec and a final extension step at 72°C for 5 min.

Amplification of the 16S rRNA yielded in some cases a double PCR product; in this case, purification of both the PCR products was carried out from a 2% agarose gel using the NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL). For the COI amplicons, a sodium acetate-absolute ethanol cleanup protocol was conducted. All purified PCR products were sequenced in an automated sequencer ABI 3730.

Analyses

The ABI chromatograms were checked and corrected by eye using the BioEdit Sequencing Alignment Editor software (Hall, 2011) and MEGA X (Kumar et al., 2018) sequence analysis software. 16S rRNA sequences, COI sequences and concatenated 16S rRNA-COI sequences from the present study were aligned with the Clustal W package (Thompson, Higgins & Gibson, 1994) embedded in BioEdit and MEGA X. In addition, publicly available sequences of the corresponding genes of *P. nobilis*, for which sample location information was available, were also downloaded from GenBank and added to the aforementioned alignments (Supplementary Table 1). DnaSP software (Rozas et al., 2017) was used to estimate the following variables: number of haplotypes (h), haplotype diversity (Hd), number of polymorphic loci (Ps), nucleotidic diversity (Pi) and Fst values. With the use of DnaSP .nex archives (nexus format) median joining haplotype networks were generated in PopART (Leigh & Bryant, 2015). The Arlequin 3.5.2.2 software (Excoffier, Laval & Schneider, 2005) was used for the AMOVA (Analysis of molecular variance). The sampling sites map was generated with the QGIS software. Raw sequences from the present study are available from the European Nucleotide Archive (ENA) (Burgin et al., 2023) using the urls http://www.ebi.ac.uk/ena/data/view/OX406989-OX407068 (16S rRNA) and http://www.ebi.ac.uk/ena/data/view/OX407172-OX407248 (COI) .

Results

From the 60 eDNA samples, amplification was successful in 36 for the 16S rRNA and 33 samples for the COI gene. All the tissue samples had successful amplifications for both genes. For the purpose of the study eight different datasets of sequences were analyzed. Datasets of COI, 16S rRNA and their concatenation from the Eastern Mediterranean Sea and from the whole Mediterranean basin were analyzed providing eight sets of results (Table 3). The first dataset included 294 sequences (N) of 714 bp from the Central (Sardinia, Sicily, Corsica, Venice, Elba) and the Eastern Mediterranean Sea with the concatenation of COI and 16SrRNA genes. It revealed the highest number of haplotypes (104) and polymorphic sites (72) of all the datasets. The haplotypic diversity (Hd: 0.961±0.005) was high and the nucleotide diversity was moderately high (Pi: 0.00511±0.00019). In the Eastern Mediterranean Sea (dataset 5), the 100

sequences of 982 bp (concatenation of both genes) revealed the highest number of haplotypes (34) and polymorphic sites (45). The haplotypic diversity was also the highest (Hd: 0.91±0.017) while the nucleotide diversity was low (Pi: 0.00304±0.00029). These two datasets were the most informative and hence they were chosen for further analyses.

Eastern Mediterranean

In the Eastern Mediterranean the concatenated 16S rRNA-COI dataset depicted a star-like haplotype network with two central haplotypes from which all the other haplotypes derive (Fig. 2). The regions of Epanomi and Aggelochori from the North Aegean Sea along with Chios Island and Korinthiakos Gulf formed a distinct group compared to all the other regions. A similar indication of differentiation appeared also in the South Aegean with samples mainly from Karpathos being distinct from the other ones. The AMOVA maximized the variation among the following groups; Group 1: Aggelochori, Epanomi, Chios and Korinthiakos and Group 2: Attica, Karpathos, Crete. This analysis showed a 20.8% of variation among the groups (P-value<0.05) (Table 4). The AMOVA among North (Aggelochori, Epanomi, Chios, Lesvos, Vourvourou) and South (Attica, Karpathos, Crete) Aegean and Ionian regions (Amvrakikos and Korinthiakos Gulfs) did not show any differentiation.

Central - Eastern Mediterranean

The COI-16S rRNA haplotype analysis from the Central and Eastern Mediterranean depicted a complex network with clear differentiation among 3 subregions; Adriatic Sea (Venice), Central Mediterranean (Sardinia, Corsica, Elba Island, Sicily) and Eastern Mediterranean Sea (Fig. 3). The percentage of variation among these groups in AMOVA was the highest; 30,38% and it was statistically significant (P-value<0.01) (Table 5). A few central, highly frequent haplotypes from the Central Mediterranean Sea split into many closely related unique haplotypes in a star-like scheme. The same structure was observed in the haplotypes that occurred in the Eastern Mediterranean regions although there were a few that were closer to the Central Mediterranean ones. The Venice samples, although distinct, showed a higher relatedness to the Central Mediterranean samples than the Eastern ones.

Discussion

Population genetic structure

Eastern Mediterranean

This is the first population genetics study that includes sequences from several known *P. nobilis* populations from the Eastern Mediterranean Sea (North Aegean, South Aegean, Ionian). The results of this study indicate that within the Eastern Mediterranean Sea there is no differentiation among the different geographic regions that were sampled implying a high connectivity among them, i.e. the isolation by distance of the populations of North and South Aegean Sea, as well as of Ionian and Aegean Sea is not supported. Similar results have been found for the horse mussel (*Modiolus barbatus*), a fact which was attributed to the very long (up to 6 months) pelagic larval stage of the species (Giantsis et al., 2019), which by far exceeds that of *P. nobilis*. Previously, it has been suggested that transplantations may have been responsible for the absence of geographic structure of *Mytilus galloprovincialis* populations in the Aegean Sea (Giantsis, Kravva & Apostolidis, 2012). This might have been the case also for *P. nobilis*, as transplantations had been proposed as a conservation action for the protection of the species (Katsanevakis, 2016; Acarli, 2021). A small population differentiation is observed in both the regions of the North and South Aegean Sea (Fig. 2) which could be attributed to the fact that the island of Karpathos is part of a Marine Protected Area (MPA) or it could be due to the higher number of samples compared to the other regions, leading to a higher haplotypic diversity in this case. Although the design of MPAs is generally not based on genetic and genomic data (Sandström et al., 2016; Xuereb et al., 2020), in certain cases it has been shown that they succeed in capturing most of the genetic diversity of their keystone species (Miller & Ayre, 2008), and combined with the protection measures for those species, they might end up preserving a higher number of haplotypes.

In the haplotype network of the Eastern Mediterranean (Fig. 2) the haplotypes of North Aegean (Epanomi, Aggelochori, Chios) formed a subgroup shown in blue coloring; yet the Korinthiakos Gulf (Ionian Sea) also shares them. These haplotypes were described by Katsares et al (2008) and were grouped with the ones from the Tunisian coasts in the research of Sanna et al (2013), reinforcing the hypothesis of the high connectivity within the Eastern Mediterranean basin. On

the other hand, the populations that were sampled within the present study (sampled in the period 2018-2021) did not share the above mentioned haplotypes with the AMOVA test confirming this distinction (Table 4, scenario C). The intervening period between the studies coincided with the outbreak of the MME, thus raising questions on the association of the populations genetic structuring and the massive mortality events the populations of the species underwent.

Central-Eastern Mediterranean

The findings of this study support the distinction of the *P. nobilis* individuals into three populations in the Mediterranean Sea. The case of the Adriatic Sea is explained in detail in Sanna et al. (2013); it is a semi-enclosed sea where the genetic flow from the rest of the Mediterranean Sea is not that high. The other two basins of the Mediterranean Sea are distinct for a number of other species (Zitari-Chatti et al., 2009; Mejri et al., 2009; Gharbi & Said, 2011; Deli, Said & Chatti, 2015), including *P. nobilis* (Sanna et al., 2013). The present study analyzed a high number of samples from the Eastern Mediterranean Sea in order to confirm this pattern. The concatenation of the COI and 16S rRNA genes that was used in the present study has also proved useful and more informative in other genetic studies of bivalves (Yuan, He & Huang, 2009; Feng et al., 2011; Slynko et al., 2018), and shows that there is a certain level of differentiation between the *P. nobilis* populations in the two basins. This finding suggests that the already known oceanographic barriers at the Sicily Strait and at the Otranto Strait might be limiting the dispersal of the species and minimizing the gene flow (Čekovská et al., 2020). Due to its pelagic larval duration stage, *P. nobilis* is a species which is considered to be weakly affected by currents and fronts but, at the same time, it has a weak recovery to gene flow from other locations (Pascual et al., 2017) and exhibits strong population structuring (Ye, Wu & Li, 2015).

eDNA and mtDNA marker sequencing

eDNA has been used widely for biodiversity assessments (Pereira et al., 2021) and for the detection of cryptic, threatened (Hunter et al., 2018) and invasive species (Ardura et al., 2015). This study was the first, to our knowledge, to use eDNA collected separately from each individual for population genetics assessment on a critically endangered species, although its potential has been advocated for in the literature (Barnes & Turner, 2016; Adams et al., 2019).

Our results suggest that the approach can be replicated to other organisms where minimal disturbance and non-invasive methods are in order. In addition, it can be employed in the few remaining populations of *P. nobilis* around the Mediterranean, such as the ones in Ebro Delta (Prado et al., 2020), Occitan coast (Peyran et al., 2022) and the one in Amvrakikos Gulf. Successful amplification for our chosen markers was possible for about half of the samples, which was still a number considered adequate for the estimation of population genetics indices. Another advantage of this approach is the certainty that each sample of genetic material corresponds to a specific individual which would not have been possible if the eDNA matrix was e.g. water or sediment collected from the study sites; however, there have been studies on population-level inferences from eDNA water samples mostly regarding large populations of fish (Sigsgaard et al., 2020).

The results of the present study are based on the sequencing of two mtDNA genes and there is the possibility that they would be different if another approach was used instead or as complement to ours, such as sequencing of microsatellites markers (Meenakshi, Remya & Sanil, 2010; Vanhaecke et al., 2012) or ddRAD sequencing (Darschnik et al., 2019; Ortiz et al., 2021) or even the addition of more mtDNA markers (e.g. D-loop) (Pourkazemi, Skibinski & A.Beardmore, 1999; Parmaksiz, 2019). However, as mentioned previously, *P. nobilis* is a critically endangered species and the amount of available samples for deciphering population genetic structure is quite limited; it is challenging to detect the remaining populations of the species and obtain the appropriate number of samples, with a subsequent high DNA quality, while ensuring the well-being of the organisms.

Conclusions

The present study is the first one including such a high number of *P. nobilis* specimens from different areas of the Eastern Mediterranean basin. Therefore it significantly contributes to the knowledge of the genetic variability of the pen shell's populations. In light of the MME, coordinated studies on the genetic diversity of *P. nobilis* throughout the Mediterranean Sea should be performed towards the aim of the conservation and management of the remaining populations of the species. An orchestrated attempt of a pan-mediterranean investigation appears to be indispensable. Scientific cooperation and use of common standards should be implemented in order to obtain more FAIR data and therefore lead more efficiently to knowledge (Wilkinson

et al., 2016). In future conservational plans on a national level, the Eastern Mediterranean basin should be considered as homogenous, based on the findings herein. It is obvious that more samples from the Southern-Eastern Mediterranean (Turkey, Syria, Lebanon, Israel, Egypt, Libya) Sea would shed more light on the population genetics status of the species. Furthermore, more detailed methodologies should be employed to unravel the genetic structure of *P. nobilis* throughout the Mediterranean.

Acknowledgements

We would like to thank the Management Agency of the Dodecanese Protected Areas (MADPA) and especially Mr Dinos Protopapas and Mr Giorgos Prearis (captain of the R/V Saria) for providing assistance during our sampling campaign in Karpathos island. The authors would like to also thank the scuba divers for their help in sampling, namely M. Salomidi, V. Gerakaris and Hippocampus Bali dive center. D. Karagiannis of the National Reference Laboratory for Mollusc Diseases (Greek Government) provided the samples MYT1-MYT9 from Lesvos, which were collected under a permit from local authorities (MEE/GDDDP89926/1117). Samples TS1-TS6 from Lesvos were collected under a permit by the Department of Agriculture and Fisheries, Decentralized Administration of the Aegean (No. 52321/6-9-2018). Samples from Crete, Chalkidiki and Attica Peninsulas were collected under a relevant research permit (175828/2195 of 14/11/2018) issued by the Greek Ministry of Environment and Energy, General Directorate for the Forests & Forest's Environment, Department of Wildlife and Hunting Management. We would also like to thank Dr Katerina Vasileiadou (ORCID: 0000-0002-5057-6417) and Ms Xenia Sarropoulou (ORCID: 0000-0003-3671-9693) for their invaluable contribution to the manuscript and the statistical analyses.

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Figure 1

Map of the sampling locations.

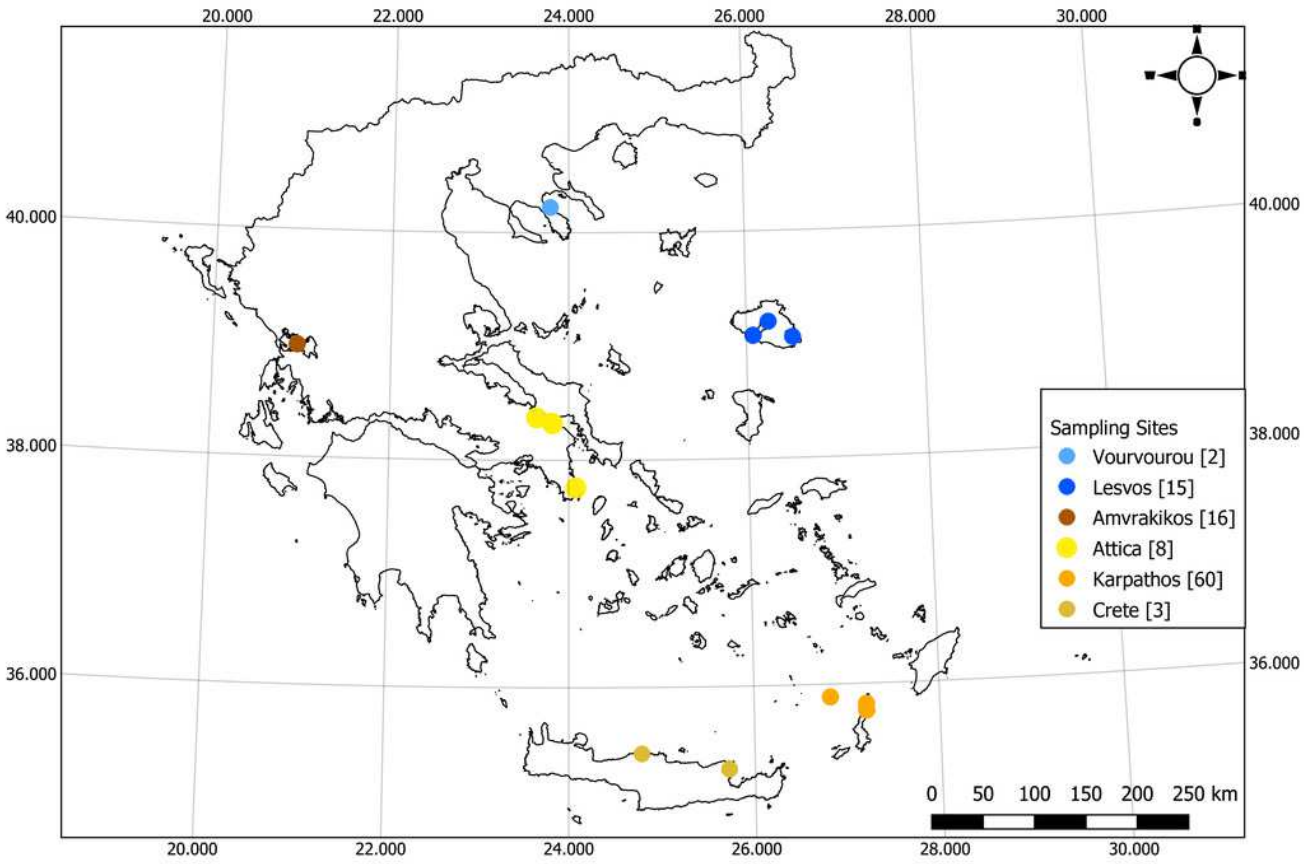

Figure 2

Haplotype network for the Eastern Mediterranean of 16S rRNA-COI dataset. The circle size depicts the haplotype frequency (10 regions).

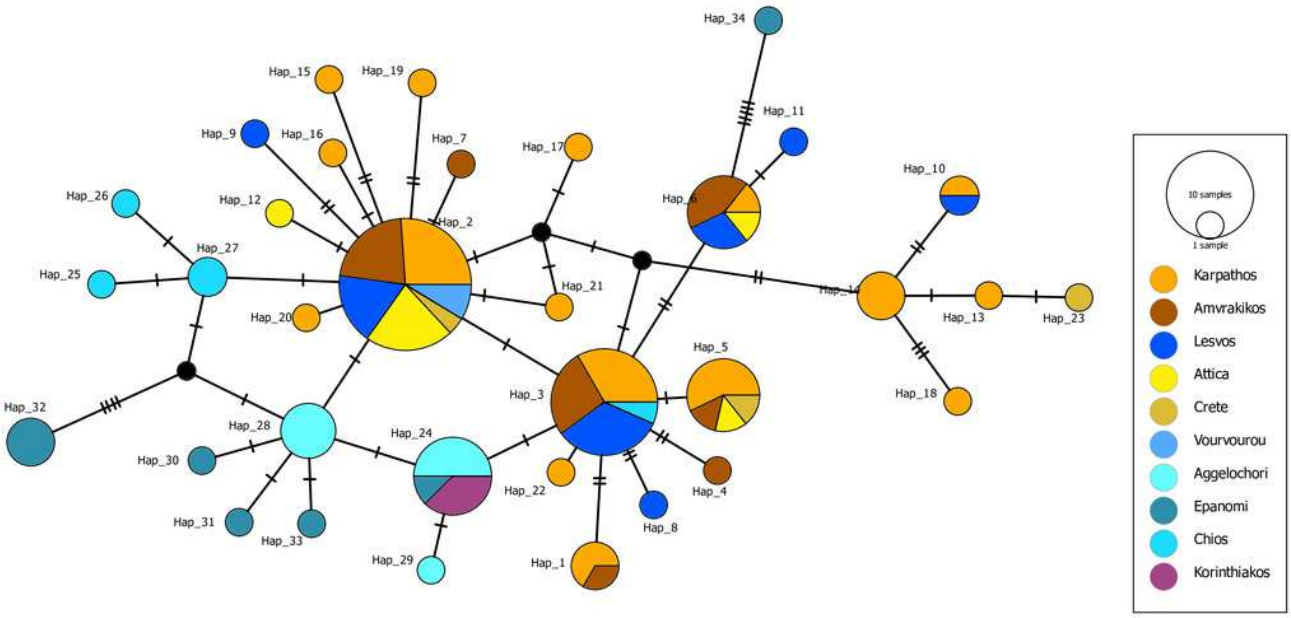

Figure 3

Haplotype network for the whole Mediterranean of 16S rRNA-COI dataset. The circle size depicts the haplotype frequency (7 regions).

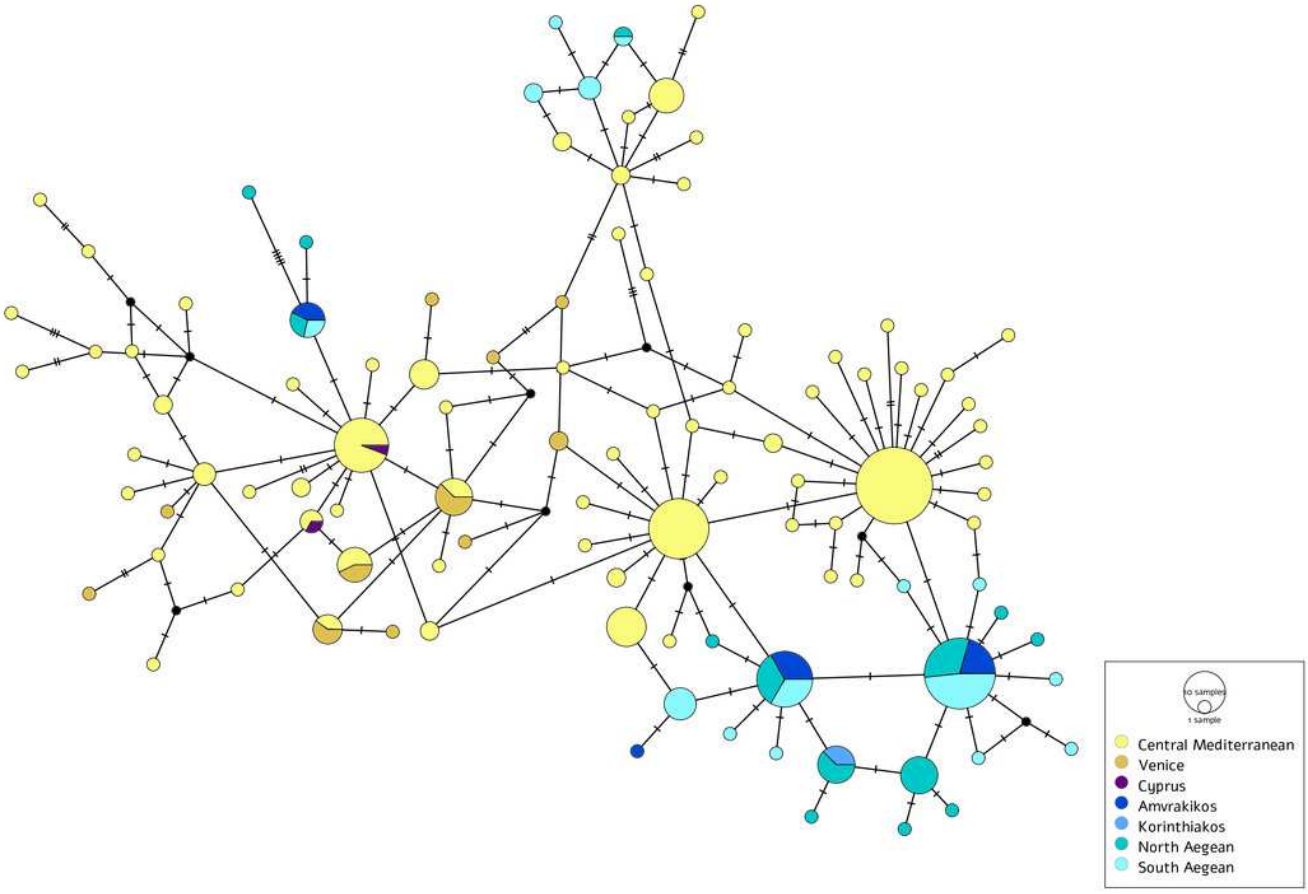

Table 1(on next page)

Details and metadata of the samples. TH: Total height, HS: Height above sediment, HD: Height inside the sediment, W: Greater width.

Table 1: Details and metadata of the samples. TH: Total height, HS: Height above sediment, HD: Height inside the sediment, W: Greater width.

Sample code	Location	Latitude/Longitude	Collection date	Sampling method	16S rRNA accession number	COI accession number	Depth (m)	TH (cm)	HS (cm)	HD (cm)	W (cm)
EL01	Elounda (Crete)	35.272369/25.723469	12/2/2019	tissue	OX406991	OX407172	3	38.55	22.8	15.75	16.1
EL02	Elounda (Crete)	35.272389/25.723472	12/2/2019	tissue	OX406992	OX407173	3	36.19	22.4	13.79	16.45
BAL01	Bali (Crete)	35.416267/24.785967	6/3/2019	tissue	OX406993	OX407174	5	28.4	15.15	13.25	13.85
AV01	Avlida (Attica)	38.373219/23.640273	3/3/2019	tissue	OX406994	OX407175	1.5	46	29.7	16.3	17
AV02	Avlida (Attica)	38.373219/23.640273	3/3/2019	tissue	OX406995	OX407176	1.5	36	21.2	14.8	16
OR01	Oropos (Attica)	38.328049/23.807744	8/3/2019	tissue	OX406996	OX407177	3.4	29	16.6	12.4	14
OR02	Oropos (Attica)	38.328049/23.807744	8/3/2019	tissue	OX406997	OX407178	4.3	26.5	15.1	11.4	13
OR03	Oropos (Attica)	38.320699/23.820207	8/3/2019	tissue	OX406998	OX407179	3.8	54	37.2	16.8	21
OR04	Oropos (Attica)	38.320699/23.820207	8/3/2019	tissue	OX406999	OX407180	3.6	49	33.1	15.9	19

VOUR01	Vourvourou (Chalkidiki)	40.221874/23.788816	28/4/2019	tissue	OX407000	OX407181	7	61	34	27	24
VOUR02	Vourvourou (Chalkidiki)	40.221874/23.788816	28/4/2019	tissue	OX407001	OX407182	7	62	46	16	24
A1	Astakida (Karpathos)	35.886497/26.824323	10/7/2018	eDNA	OX407002	OX407183	10	19.3	9.6	9.7	8.9
A2	Astakida (Karpathos)	35.886497/26.824323	10/7/2018	eDNA	--	--	10	19.8	11.7	8.1	10.2
A3	Astakida (Karpathos)	35.886497/26.824323	10/7/2018	eDNA	OX407003	OX407184	10	21.5	13.8	7.7	11.2
A4	Astakida (Karpathos)	35.886497/26.824323	10/7/2018	eDNA	OX407004	OX407185	10	14.5	6.7	7.8	7.2
A5	Astakida (Karpathos)	35.886497/26.824323	10/7/2018	eDNA	--	--	10	10.7	5.6	5.1	4.7
A6	Astakida (Karpathos)	35.886497/26.824323	10/7/2018	eDNA	OX407005	OX407186	10	13	7.2	5.8	7.1
A7	Astakida (Karpathos)	35.886497/26.824323	10/7/2018	eDNA	OX407006	OX407187	10	14.1	6.7	7.4	7.3
AMV1	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407016	OX407196	--	--	--	--	--
AMV2	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407017	OX407197	--	--	--	--	--
AMV3	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407018	OX407198	--	--	--	--	--
AMV4	Amvrakikos	38.9856717451461/20.94545	17/4/202	tissue	OX4070	OX4071	--	--	--	--	--

	Gulf	047741468	1		19	99					
AMV5	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407020	OX407200	--	--	--	--	--
AMV6	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407021	OX407201	--	--	--	--	--
AMV7	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407022	OX407202	--	--	--	--	--
AMV8	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407023	OX407203	--	--	--	--	--
AMV9	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407024	OX407204	--	--	--	--	--
AMV10	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407025	OX407205	--	--	--	--	--
AMV11	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407026	OX407206	--	--	--	--	--
AMV12	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407027	OX407207	--	--	--	--	--
AMV13	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407028	OX407208	--	--	--	--	--
AMV14	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407029	OX407209	--	--	--	--	--
AMV15	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407030	OX407210	--	--	--	--	--
AMV16	Amvrakikos Gulf	38.9856717451461/20.94545047741468	17/4/2021	tissue	OX407031	OX407211	--	--	--	--	--

MYT1	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407032	OX407212	5-6	25.8	11.2	14.6	3.5
MYT2	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407033	OX407213	5-6	30.5	13.1	17.4	4.2
MYT3	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407034	OX407214	5-6	34.5	13.6	20.9	4.4
MYT4	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407035	OX407215	5-6	39	14	25	4.5
MYT5	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407036	OX407216	5-6	27.8	12.9	14.9	4
MYT6	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407037	OX407217	5-6	30.1	12.6	17.5	4.2
MYT7	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407038	OX407218	5-6	34.2	12.8	21.4	4.3
MYT8	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407039	OX407219	5-6	30.5	12.4	18.1	3.8
MYT9	Kalloni (Lesvos)	39.081244/26.07566	19/1/2019	tissue	OX407040	OX407220	5-6	29.3	11.4	17.9	3.5
TS4	Kalloni (Lesvos)	39.20174957104768/26.24919936396623	12/2018	tissue	OX407044	OX407224	1–1.5	--	--	--	--
TS5	Kalloni (Lesvos)	39.20174957104768/26.24919936396623	12/2018	tissue	OX407045	OX407225	1–1.5	--	--	--	--
TS6	Kalloni (Lesvos)	39.20174957104768/26.24919936396623	12/2018	tissue	OX407046	OX407226	1–1.5	--	--	--	--
TS1	Gera (Lesvos)	39.062908364108715/26.519	12/2018	tissue	OX4070	OX4072	2–5	--	--	--	--

		669291535266			41	21					
TS2	Gera (Lesvos)	39.062908364108715/26.519669291535266	12/2018	tissue	OX407042	OX407222	2–5	--	--	--	--
TS3	Gera (Lesvos)	39.062908364108715/26.519669291535266	12/2018	tissue	OX407043	OX407223	2–5	--	--	--	--
D1	Diafani (Karpathos)	35.762570/27.211337	11/7/2018	eDNA	OX407047	OX407227	13	26.5	19.7	6.8	13.6
D2	Diafani (Karpathos)	35.762570/27.211337	11/7/2018	eDNA	OX407048	OX407228	13	26.3	17.1	9.2	15.6
D3	Diafani (Karpathos)	35.762570/27.211337	11/7/2018	eDNA	OX407049	OX407229	13	32.1	23.4	8.7	15.1
D4	Diafani (Karpathos)	35.762570/27.211337	11/7/2018	eDNA	--	--	13	34.3	18.6	15.7	14.2
GD1	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407050	OX407230	13	29.9	19.7	10.2	14.9
GD2	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407051	OX407231	13	23.2	15.4	7.8	12.3
GD3	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407052	OX407232	13	26.4	19.1	7.3	13.2
GD4	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407053	OX407233	13	23.6	15.2	8.4	11.3
GD5	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	--	--	13	38.5	27.1	11.4	15.7
GD6	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407054	OX407234	13	45.1	30.2	14.9	19.3

GD7	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407055	--	13	26.9	18.2	8.7	14.7
GD8	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407056	OX407235	13	33.6	23.4	10.2	13.5
GD9	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	--	--	13	29.8	20	9.8	14.6
ID1	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407060	OX407239	13	26	18	8	16
ID2	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	--	--	13	26	16	10	13
ID3	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	--	--	13	43	26	17	20
ID4	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407061	OX407240	13	18	11	7	10
ID5	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407062	OX407241	13	18	12	6	10
ID6	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	--	--	13	33	18	15	17
ID7	Diafani (Karpathos)	35.762570/27.211337	14/7/2018	eDNA	OX407063	OX407242	13	39	26	13	18
X1	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407007	OX407188	6	39.5	24.4	15.1	17.7
X2	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407008	OX407189	6	62.5	50.2	12.3	27.6
X3	Tristomo	35.820845/27.211023	8/7/2018	eDNA	OX4070	OX4071	6	49.5	29	20.5	21

	(Karpathos)				09	90					
X4	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407010	--	6	53.1	30.2	22.9	22.7
X5	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	--	--	6	42.4	19.2	23.2	18.1
X6	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407011	OX407191	6	51.6	27.2	24.4	23.9
X7	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407012	OX407192	6	47.3	20.9	26.4	21.2
X8	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407013	OX407193	6	23.9	13.1	10.8	14.6
X9	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407014	OX407194	6	27.6	20.4	7.2	15.4
X10	Tristomo (Karpathos)	35.820845/27.211023	8/7/2018	eDNA	OX407015	OX407195	6	43.2	19.4	23.8	20.2
GT1	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407057	OX407236	6	53.9	24.2	29.7	20.4
GT2	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	40.5	20.8	19.7	17.6
GT3	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407058	OX407237	6	36	20.4	15.6	17.3
GT4	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	21.5	11.2	10.3	13.1
GT5	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	29.1	16.7	12.4	16.8

GT6	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	37.4	20.7	16.7	18.7
GT7	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407059	OX407238	6	31.8	20.6	11.2	17.8
GT8	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	8	4.4	3.6	3.3
GT9	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	39.2	21.9	17.3	16.7
IT1	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	18	6	12	11.5
IT2	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407064	OX407243	6	33	21	12	18
IT3	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	34	18	16	16.5
IT4	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	39	18.5	20.5	21.5
IT5	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	27.5	11	16.5	15.5
IT6	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	30.5	16	14.5	17.5
IT7	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	36.5	18	18.5	19.5
IT8	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407065	OX407244	6	53	26	27	23
IT9	Tristomo	35.820845/27.211023	13/7/201	eDNA	--	--	6	40	23	17	15.5

	(Karpathos)		8								
IT10	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	42	23	19	21
IT11	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407066	--	6	49.5	27.5	22	22
IT12	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407067	OX407245	6	48	24.5	23.5	17
IT13	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	OX407068	OX407246	6	13	13	0	11
IT14	Tristomo (Karpathos)	35.820845/27.211023	13/7/2018	eDNA	--	--	6	43	23	20	17.5
LAV01	Lavrio (Attica)	37.757668/24.077698	3/8/2018	tissue	OX406989	OX407247	12	33	17	16	16.7
LAV02	Lavrio (Attica)	37.757373/24.077915	3/8/2018	tissue	OX406990	OX407248	14	53.8	34.6	19.2	21.2

Table 2(on next page)

Primers used in the present study.

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Table 2: Primers used in the present study.

Target gene	Forward primer (5′- 3′)	Reverse primer (5′- 3′)	Amplification length (bp)	Reference
COI	5’-CAGCTTTTGTAGAGGGCG-3'	5’-CCAAATTACACCAGTCAGCC-3’	722	this study
	5’-GATCCGGGATAGTAGGTAC-3'	5’-CMGGATGACCAAARAACC-3’	645	this study
	5’-ATGGCYGTCGATTTAGC-3'	5’-CMGGATGACCAAARAACC-3’	298	this study
COI	LCO 1490	HCO 2198	710	Folmer et al, 1994
	mlCOIintF	jgHCO2198	313	Leray et al, 2013
	5’-GGTTGAACTATHTATCCNCC-3’	5’-GAAATCATYCCAAAAGC-3’	338	Sanna et al 2013
16S rRNA	5’-GGTAGCGAAATTCCTAGCC-3’	5’-AAKGGTSGAACAGACCC-3’	408	this study
16S rRNA	5’-TGCTCAATGCCCAAGGGGTAAAT-3’	5’-AACTCAGATCACGTAGGG-3’	450	Sanna et al 2013
nad3	5’-CCTTATGARTGYGGBTTT-3’	5’-TCHATAAGYTCATARTAYARCCC-3’	203	Sanna et al 2014

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Table 3(on next page)

Genetic diversity estimates: N: number of sequences, Bp: Base pairs, h: number of haplotypes, Hd: Haplotype diversity, Ps: Polymorphic sites, Pi: Nucleotide diversity.

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Table 3: Genetic diversity estimates: N: number of sequences, Bp: Base pairs, h: number of haplotypes, Hd: Haplotype diversity, Ps: Polymorphic sites, Pi: Nucleotide diversity.

	Dataset	N	Bp	h	Hd	Ps	Pi
1	16S rRNA-COI whole Mediterranean	294	714	104	0.961 ± 0.005	72	0.00511 ± 0.00019
2	COI Italy Tunisia Greece	392	338	71	0.915 ± 0.006	48	0.00768 ± 0.00024
3	COI whole Mediterranean	450	243	48	0.652 ± 0.024	36	0.00475 ± 0.00028
4	16S whole Mediterranean	341	376	38	0.601 ± 0.03	31	0.00243 ± 0.00018
5	16S rRNA-COI Eastern Mediterranean	100	982	34	0.91 ± 0.017	45	0.00304 ± 0.00029
6	COI Tunisia Greece	149	606	33	0.793 ± 0.024	39	0.00311 ± 0.0003
7	COI Eastern Mediterranean	100	606	27	0.84 ± 0.026	36	0.00388 ± 0.00041
8	16S Eastern Mediterranean	105	376	10	0.507 ± 0.051	9	0.00161 ± 0.00021

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Table 4(on next page)

AMOVA table using genetic distances based on haplotype frequencies (FST) of the Eastern Mediterranean populations.

Table 4: AMOVA table using genetic distances based on haplotype frequencies (FST) of the Eastern Mediterranean populations.

Scenarios	Source of variation	Degrees of freedom	Sum of squares	Var. components	Percentage of variation	Fixation Indices	P-value
A. Group 1 (Agg, Epa, Vou) Group 2 (Att, Kar, Cre) Group 3 (Kor, Amv) Group 4 (Les, Chi)	Among groups	3	18,933	0,07939	5,12	FCT : 0.05120	0.19550± 0.01411
	Among populations within groups	6	16,825	0,22789	14,7	FSC : 0.15490	0.00098±0.00098
	Within populations	90	111,902	1,24336	80,18	FST : 0.19817	0.00000±0.00000
B. Group 1 (Agg, Epa, Vou, Les, Chi) Group 2 (Kor, Amv), Group 3 (Att, Kar, Cre)	Among groups	2	9,257	-0,06751	-4,43	FCT : -0.04431	0.55621±0.01366
	Among populations within groups	7	26,501	0,34759	22,82	FSC : 0.21848	0.00000±0.00000
	Within populations	90	111,902	1,24336	81,61	FST : 0.18385	0.00000±0.00000
C. Group 1 (Agg, Epa, Kor, Chi) Group 2 (Vou, Les, Amv, Att, Kar, Cre)	Among groups	1	16,18	0,36139	20,8	FCT : 0.20800	0.01173±0.00363
	Among populations within groups	8	19,578	0,13269	7,64	FSC : 0.09643	0.00098±0.00098
	Within populations	99	111,902	1,24336	71,56	FST : 0.28437	0.00000±0.00000
D. Group 1 (Agg, Epa, Chi, Vou,	Among groups	1	1.711	-15.269	-10.58	FCT : -	0.98631±0.0

Les, Att, Kar, Cre) Group 2 (Amv, Kor)						0.10580	0367
	Among populations within groups	8	34.047	35.254	24.43	FSC : 0.22090	0.00000±0.00000
	Within populations	90	111.902	124.336	86.15	FST : 0.13848	0.00000±0.00000

Table 5(on next page)

AMOVA table using genetic distances based on haplotype frequencies (FST) of the whole Mediterranean.

Table 5: AMOVA table using genetic distances based on haplotype frequencies (FST) of the whole Mediterranean.

Scenarios	Source of variation	Degrees of freedom	Sum of squares	Var. components	Percentage of variation	Fixation Indices	P-value
A. Group 1 (Ven, Elb, Sic, Cor, Sar) Group 2 (Vou, Agg, Epa, Les, Chi) Group 3 (Att, Kar, Cre, Cyp) Group 4 (Amv, Kor)	Among groups	3	87.613	0,44877	21,72	FCT : 0.21718	0.00098± 0.00098
	Among populations within groups	12	57.626	0,21771	10,54	FSC : 0.13459	0.00000± 0.00000
	Within populations	278	389.162	139.986	67,75	FST : 0.32254	0.00000± 0.00000
B. Group 1 (Elb, Sic, Cor, Sar) Group 2 (Vou, Agg, Epa, Les, Chi) Group 3 (Att, Kar, Cre, Cyp) Group 4 (Amv, Kor) Group 5 (Ven)	Among groups	4	111.851	0,53997	26,34	FCT : 0.26338	0.00000± 0.00000
	Among populations within groups	11	33.388	0,11034	5,38	FSC : 0.07307	0.00000± 0.00000
	Within populations	278	389.162	139.986	68,28	FST : 0.31720	0.00000± 0.00000
C. Group 1 (Elb, Sic, Cor, Sar, Cyp) Group 2 (Vou, Agg, Epa, Les, Chi, Att, Kar, Cre) Group 3 (Amv, Kor) Group 4 (Ven)	Among groups	3	109.576	0,59015	28,17	FCT : 0.28171	0.00000±0.00000
	Among populations within groups	12	35.663	0,10488	5,01	FSC : 0.06970	0.00000± 0.00000
	Within populations	278	389.162	139.986	66,82	FST : 0.33178	0.00000± 0.00000

D. Group 1 (Elb, Sic, Cor, Sar, Cyp) Group 2 (Vou, Agg, Epa, Les, Chi, Att, Kar, Cre, Amv, Kor) Group 3 (Ven)	Among groups	2	108.201	0,64274	30,04	FCT : 0.30042	0.00000± 0.00000
	Among populations within groups	13	37.039	0,09685	4,53	FSC : 0.06471	0.00000± 0.00000
	Within populations	278	389.162	139.986	65,43	FST : 0.34569	0.00000± 0.00000