First insights towards the population genetic structure and the phylogeographic status of the horse mussel (*Modiolus barbatus*) from the eastern Mediterranean

Ioannis A. Giantsis[1], Athanasios Exadactylos[2], Konstantinos Feidantsis[3] and Basile Michaelidis[3]

[1]Department of Animal Science, Technological Educational Institute of Western Macedonia, 53100, Florina, Greece; [2]Department of Ichthyology and Aquatic Environment, University of Thessaly, 38446, Volos, Greece and [3]Laboratory of Animal Physiology, Department of Zoology, Faculty of Science, School of Biology, University of Thessaloniki, GR-54124 Thessaloniki, Greece

Abstract

The horse mussel *Modiolus barbatus* is a marine benthic bivalve, distributed mainly in the Mediterranean basin, that constitutes a fishery product of high economic importance and a promising candidate for aquaculture. The current study provides the first insights regarding the genetic profile of *M. barbatus* populations from the eastern Mediterranean, by analysis of a partial segment of the mitochondrial *COI* gene in individuals collected from five sampling localities within the Aegean Sea. To the best of our knowledge, the derived haplotypes represent the first DNA barcodes of *M. barbatus* from the entire Mediterranean region. Maximum likelihood phylogenetic analysis revealed that *M. barbatus* from the North Sea and *M. barbatus* from the eastern Mediterranean may not belong to the same species and as a consequence, there might be three species of the genus *Modiolus* in Europe. On the other hand, eastern Mediterranean *M. barbatus* haplotypes were found to be more closely related to the Asian-Pacific *Modiolus* species. All geographic populations analysed displayed high levels of genetic diversity, in terms of haplotype and nucleotide diversity and a considerable number of unique alleles. Divergence among populations was found at generally low levels, corresponding with the majority of pairwise F_{st} values not being significant. These findings suggest no population structure and high levels of gene flow, a common feature observed in marine bivalves with long pelagic larval phases.

Introduction

The bearded horse mussel *Modiolus barbatus* (Linnaeus, 1758) is a benthic marine bivalve, mainly distributed in the Mediterranean Sea and Moroccan and Iberian coasts of the eastern Atlantic Ocean (Poppe & Goto, 2000). On account of their physiological features, such as survival and growth rates, in combination with the existence of market demand, *M. barbatus* mussels constitute a promising candidate species for aquaculture (Peharda *et al.*, 2013). Horse mussel populations are indigenous in most of the Aegean Sea and are also products of high economic value for the Greek fisheries.

Evaluation of the genetic structure of an organism intended for farming is considered one of the major essential tools prior to the development of a new form of aquaculture. The capability of aquatic organisms to adapt towards environmental changes mainly depends on the intraspecific genetic variability of their populations. In order to prevent potentially harmful effects of inbreeding depression in the cultured populations, management strategies should take into consideration the preservation of genetic variability (Ye *et al.*, 2012). Furthermore, historical evolutionary events may influence the genetic variability of distinct geographic populations by bottleneck effects, effective population size alterations or by creating barriers to gene flow (Geraghty *et al.*, 2013). In this sense, phylogeographic relationships and comparison of estimated genetic variation among populations are key factors that provide necessary information for the evaluation of the evolutionary history of a species, with an eventual focus on the proper design of population management strategies.

Analysis of mitochondrial DNA (mtDNA) has been utilized in a wide range of aquatic taxa for the evaluation of molecular phylogeny, phylogeography and genetic diversity within and among geographic populations (Giantsis *et al.*, 2014*a*, 2015; Ye *et al.*, 2015; Ladoukakis & Zouros, 2017). Widely accepted key factors for the utility of mtDNA markers include the relative lack of recombination, the selective neutrality, the fact that animal mitochondrial genes lack introns, the elevated mutation rate compared with nuclear DNA and the maternal inheritance in the majority of marine animal species (Ladoukakis & Zouros, 2017). Particularly, the analysis of a standardized fragment of cytochrome oxidase subunit I (*COI*) gene has been proven highly informative for providing the first genomic insights with regard to animal species for which there is limited information available concerning their genetic structure.

This ~650 bp fragment serves as a DNA barcode for identifying animal species (Hebert *et al.*, 2003). As a consequence, it has been described for more animal species than any other DNA segment, being available in public databases, e.g. GenBank and BOLD (Barcode of Life Data Systems, http://www.boldsystems.org), and hence supporting an integrated phylogenetic reconstruction of the examined species. Although in most animal species mtDNA is inherited strictly through maternal lineage (Hassan *et al.*, 2009), this feature meets some interesting idiomorphic exceptions in several bivalve molluscs (Ladoukakis & Zouros, 2017), including the horse mussels that belong to the species *Modiolus modiolus* (Robicheau *et al.*, 2017). Briefly, according to this unusual pattern of inheritance, namely doubly uniparental inheritance (DUI), maternal mtDNA is transmitted to offspring of both sexes, whereas paternal mtDNA is transmitted exclusively to males (Ladoukakis & Zouros, 2017). As a result, two types of differentially evolved mtDNA co-occur, a fact that should receive special attention in population genetic studies of marine bivalves (Giantsis *et al.*, 2014a).

Larvae of marine molluscan species come up against a passive dispersal through sea currents, resulting in the absence of genetic differentiation among distant geographic populations (Larsson *et al.*, 2016). Instead, oceanographic, temporal or environmental factors rather than geographic ones may form barriers to gene flow with an eventual effect on population sub-structure (Wu *et al.*, 2016; Banci *et al.*, 2017; Gutierrez *et al.*, 2017). Particularly in the Aegean Sea, following the intense water mass circulation (Olson *et al.*, 2007) that may favour gene flow among molluscan bivalve populations, absence of or small genetic differentiation has been revealed in the Mediterranean mussel populations through different genetic markers (Giantsis *et al.*, 2014a, 2014b). Regarding horse mussels, phylogeographic and population genetic relationships have been studied from various sampling localities throughout the distribution of *M. modiolus* (Halanych *et al.*, 2013), as well as by the inclusion of *M. modiolus* and *M. barbatus* COI haplotypes in a large phylogenetic analysis of molluscs (Barco *et al.*, 2016). Nevertheless, the genetic composition of horse mussels (*M. barbatus*) from any marine area in the Mediterranean is inadequately elucidated. In the context of this lack of knowledge, the main goal of the present study was the characterization of the population genetic structure of the horse mussels (*M. barbatus*) from the Aegean Sea, due to their ecological importance constituting an exported fishery product of high economic value. Moreover, due to their promising potential for aquaculture, our scope was also to compare genetic diversity indices among different geographic populations. Finally, the systematic status of *M. barbatus* from the eastern Mediterranean was re-stated, by constructing phylogeny upon previously published COI haplotypes of closely related species.

Materials and methods

Horse mussel collection and sampling sites

Adult *Modiolus barbatus* individuals were collected by professional scuba divers from five marine areas in the Aegean Sea, between April and September 2017. Approximately 40–50 specimens were collected from each locality, all of which originated from low depths (<15 m). All specimens were stored shelled in the freezer (−20°C) until further molecular analysis. Three out of the five sampling sites were located in Thermaikos Gulf, whereas the remaining two were located on the coast of Porto Lagos and in the western marine area of the island of Mytilene. All sampling sites were representative of the different microhabitats that favour high abundance (Figure 1).

Thermaikos Gulf is a semi-closed basin in northern Greece, plentiful of organic material due to the outflow of four rivers and therefore hosting various filter-feeding bivalve species. The three sampling sites in Thermaikos Gulf were representative of the different microhabitats of the gulf. Particularly, samples from Aggelochori were collected from the outer part of Thermaikos Gulf, which is affected by the higher salinity and clean waters of the north Aegean Sea (Krestenitis *et al.*, 2012). The second sampling site, Peraia, is located in the inner Thermaikos Gulf, where the lower depths allow faster water temperature fluctuations, while the topography reduces the open sea impact (Krestenitis *et al.*, 2012). Paliomana is located in inner Thessaloniki Bay, which is the second largest harbour and Thessaloniki is the second largest city in Greece, with ~1 million inhabitants. It should also be noted that the main Greek aquaculture area for the Mediterranean mussels (*Mytilus galloprovincialis*) is located in the western part of the Thermaikos Gulf. Fishery and trade of horse mussels originating from Thermaikos Gulf has been occasionally prohibited during the past years based on bio-accumulation of heavy metals in specific tissues (Katsikatsou *et al.*, 2011).

The marine area of Porto Lagos is located in the region of Thrace (north-eastern Greece, Figure 1), extended between two big cities, Xanthi and Komotini. Similarly to Thermaikos Gulf, Porto Lagos is affected by freshwater outflows due to the connection with the Vistonis Lake system, forming excellent conditions for marine bivalve populations.

The island of Mytilene is located in the Eastern Aegean Sea, only a few kilometres away from the coast of Turkey. Fisheries around the coasts of Mytilene are very well developed. Specifically, it should be noted that horse mussels exported from Greece, mainly originate from the island of Mytilene.

DNA extraction, PCR and sequencing

Approximately 20–30 mg of mantle tissue was cut with UV sterilized tools from each individual and subjected to DNA extraction. Genomic DNA was extracted using NucleoSpin Extraction Kit (Macherey-Nagel, Germany), following the manufacturer's protocol. The quantity and quality of the extracted DNA were estimated on a Biospec-nano spectrophotometer (Shimadzu, Japan).

A standardized fragment of the COI gene was amplified using the universal primers HCO2198 and LCO1490 (Folmer *et al.*, 1994). Amplifications were performed in total volumes of 25 μl, containing 0.6 μl of each primer (10 pmol μl^{-1}), 12.5 μl MyTaqTM ready-to-use 2× mix (Bioline, UK), 50 ng of template DNA and ultrapure water up to 25 μl. Initially, 10 successfully amplified samples were purified using the NucleoSpin PCR Clean-up Kit (Macherey-Nagel, Germany) and were then sequenced in both directions using the PCR primers in an ABI Prism 3730XL capillary sequencer in Eurofins Genomics (Austria).

Male mitochondrial genome (M mitotype) evolves faster than the female (F) mitotype, whereas extreme sequence divergence has been noted between the two mitotypes in *Modiolus modiolus* (Robicheau *et al.*, 2017). Keeping this in mind, in the present study the COI gene exclusively of the F mitotype was analysed since it is present in both males and females. Therefore, based on the 10 derived sequences as well as on six *Modiolus* haplotypes of both sexes obtained from the GenBank database (accession numbers: GQ480314, KC119352, KC119353, KC119349, AB076912, KX452400), a new internal primer was designed (5′-TATTGAGTTAGGTCGGCCCGGGAG-3′), specific for the female mitochondrial genome. A non-conserved region of high nucleotide divergence between the two mitotypes in *M. modiolus* was selected for the design of the primer, in order to exclude the
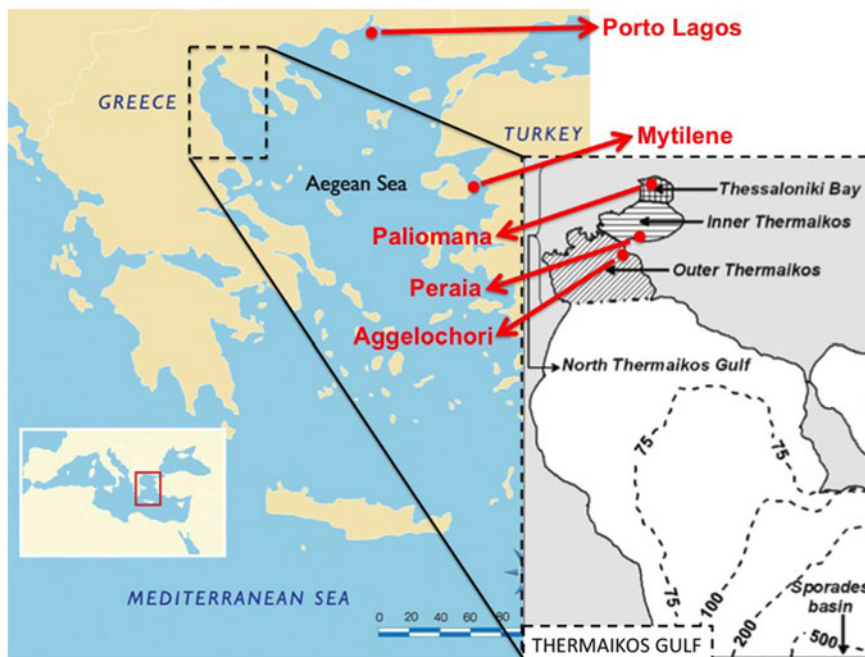
Fig. 1. Sampling sites of the five collected *Modiolus barbatus* populations in the Aegean Sea. Sub-division of Thermaikos Gulf (40.2893°N 23.0250°E) areas as defined by Krestenitis *et al.* (2012).

possibility of hybridizing to the M mitotype. Our designed primer was utilized for sequencing of all the remaining amplified specimens using the purification kit and instruments described above.

Statistical analysis

Sequences were edited and aligned using the MUSCLE algorithm in the software MEGA version 6.05 (Tamura *et al.*, 2013). Representative *COI* sequences of all available *Modiolus* species in the GenBank database including also a *M. modiolus* male haplotype, were utilized for the construction of the haplotype phylogeny dataset and one *Mytilus galloprovincialis* haplotype was also included to be defined as an outgroup in the phylogenetic analysis. Poorly aligned regions were excluded using the GBlocks program (Castresana, 2000) applying the default parameters. To examine potential saturation in any codon position, the uncorrected distances (p-distances) were plotted against corrected model distances (Maximum Likelihood) in the sense that a curved or plateau relationship would indicate evidence for saturation.

Phylogenetic relationships of the derived haplotypes against congeneric species were investigated by the Bayesian approach using the software BEAST 1.7.5 (Drummond *et al.*, 2012) and the Maximum Parsimony (MP) and the Maximum Likelihood (ML) approaches embedded in the software MEGA, applying 1000 bootstrap iterations. As evaluated by MEGA, the best-fit evolution model was the HKY + G + I substitution model, which was therefore used for the ML analysis. Since all Greek haplotypes were grouped together, only a bunch is presented in the constructed ML dendrogram, in an effort to provide a better depiction of the interspecific genetic relationships. An approximate Bayesian computation (ABC), in the software DIYABC (Cornuet *et al.*, 2008), was used to evaluate the possible scenarios of the horse mussel's introduction in the Eastern Mediterranean. For simplicity, apart from the Eastern Mediterranean haplotypes, congeneric haplotypes from north Europe and Asia-Pacific were included in the ABC and were considered as three populations. Relatedness among haplotypes was further evaluated by a median joining network generated in the software PopART (Leigh & Bryant, 2015), using default goodness to fit parameters.

Genetic diversity indices such as the number of segregating sites, haplotype and nucleotide diversity were estimated considering all individuals as one population, as well as for each geographic population separately, using the software DnaSP version 6.10.04 (Rozas *et al.*, 2017). Selection neutrality was examined by Tajima's *D* index (Tajima, 1989) for each population separately and at the species level in the software MEGA. The software Arlequin version 3.11 (Excoffier *et al.*, 2005) was finally used to perform pairwise comparisons for genetic differentiation (F_{st}) and to estimate the levels of significance of these comparisons.

Results

In total 94 partial sequences of the *COI* gene were obtained from the F mitotype of the horse mussel (*M. barbatus*) from the sampled geographic populations within the research area (Figure 1). The final alignment dataset was 660 bp in length, with no insertions or deletions (indels) detected. From the 85 observed variable sites, 46 were parsimoniously informative, generating 49 different haplotypes, the distribution of which in the sampled populations and their accession numbers after deposition in GenBank are shown in Table S1. The relationship between corrected and model distances was linear (Figure 1S), indicating no evidence for saturation in any codon position.

Using the most shared haplotypes, a dendrogram was constructed depicting the phylogenetic relationships of *M. barbatus* from eastern Mediterranean with congeneric haplotypes from other marine areas of the world (Figure 2). The topology of the dendrograms of all three methods used (MP, ML and Bayesian) was identical and the bootstrap values and Bayesian posterior probabilities are indicated on each clade of the tree. Similarly to *M. modiolus* female haplotypes that were grouped together with high levels of genetic distance from the male mitotype, all novel *M. barbatus* haplotypes were monophyletic, providing evidence that all generated sequences belong to the female mitotype. With the exception of Eastern Mediterranean *M. barbatus* haplotypes that were unexpectedly not closely related with *M. barbatus* haplotypes from the North Sea, all the remaining groups of conspecific haplotypes were clustered together, supported by high bootstrap scores. The sequence JF496770 of *Modiolula phaseolina* (Philippi, 1844) originating from the Adriatic Sea was the most
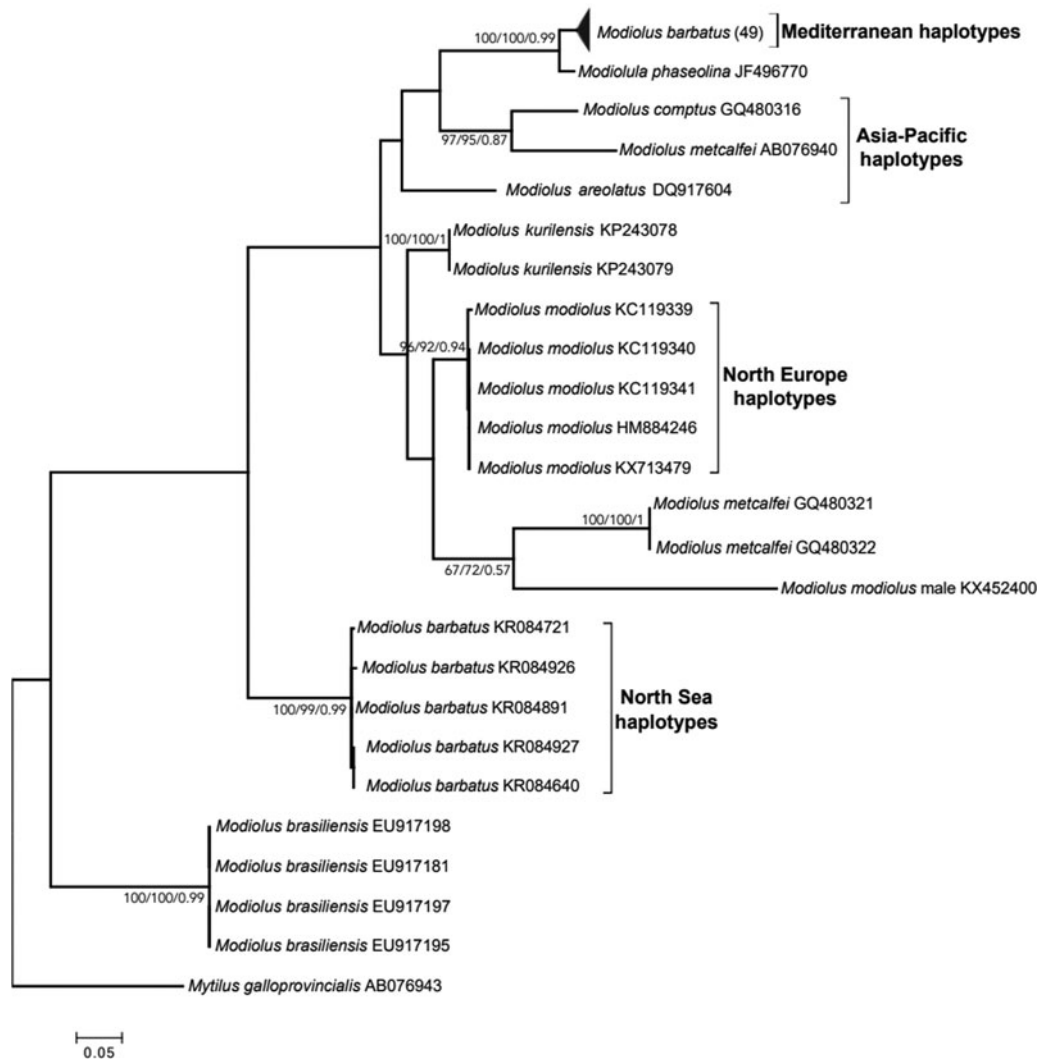
Fig. 2. Phylogeny of *COI* haplotypes originating from different *Modiolus* species based on three methods (Maximum Likelihood, Maximum Parsimony and Bayesian). Bootstrap values and posterior probabilities greater than 50% as analysed by 1000 iterations are shown for the main clades as follows: ML/MP/Bayesian.

closely related haplotype to the *M. barbatus* haplotypes from the Aegean Sea. Based on these observations we evaluated the origin of horse mussels and their historical route into the Mediterranean. According to the ABC analysis, the most likely scenario of colonization suggests the introduction of *M. barbatus* from Asia-Pacific into the Eastern Mediterranean Sea (Figure 3).

The genetic relationships and the mutational steps among the haplotypes are depicted in the network of Figure 4. The most widely distributed haplotype, which was found in individuals from all populations, is connected with several private haplotypes by one or two steps. This star-like pattern indicates that the Mb04 haplotype, holding a relatively central position, is probably the most ancestral one. Several pairs of haplotypes were separated by multiple mutational steps in line with their high nucleotide diversity (Table 1). Furthermore, no exclusive cluster was observed for a specific geographic region.

Genetic diversity indices (haplotype and nucleotide diversity) for each population separately as well as at the species level are listed in Table 1. Haplotype diversity was at high levels in all examined populations, with the population from Aggelochori presenting the lowest genetic diversity. Although negative neutrality test values were found in a few populations, they were not significant (Table 1). These results, in combination with high haplotype diversities, indicate no departures from the assumption of neutrality, and absence of sudden demographic alterations such as

population decreases or expansions after potential bottlenecks. Furthermore, generally low levels of population differentiation were observed, with F_{st} values ranging from negative to 0.1812 (Table 2). Apart from differentiation of the population pairs Porto Lagos–Mytilene and Mytilene–Peraia, all comparisons were statistically non-significant.

Discussion

The present study provides the first insights towards the genetic relationships of eastern Mediterranean *Modiolus barbatus* populations, using sequence analysis of a *COI* mitochondrial gene segment. While DNA barcoding has evolved as a valuable tool for marine species identification, Barco *et al.* (2016) reported that more than 90% of estimated living marine species are molecularly undocumented in public databases such as GenBank. To the best of our knowledge, the newly described sequences deposited in the GenBank database under the accession numbers MH277125–MH277173, represent the first DNA barcodes of *M. barbatus* from the Mediterranean Sea biogeographic basin.

Phylogeography of the eastern Mediterranean horse mussel

Although *M. barbatus* is known to be distributed in the Mediterranean and neighbouring marine areas, such as the
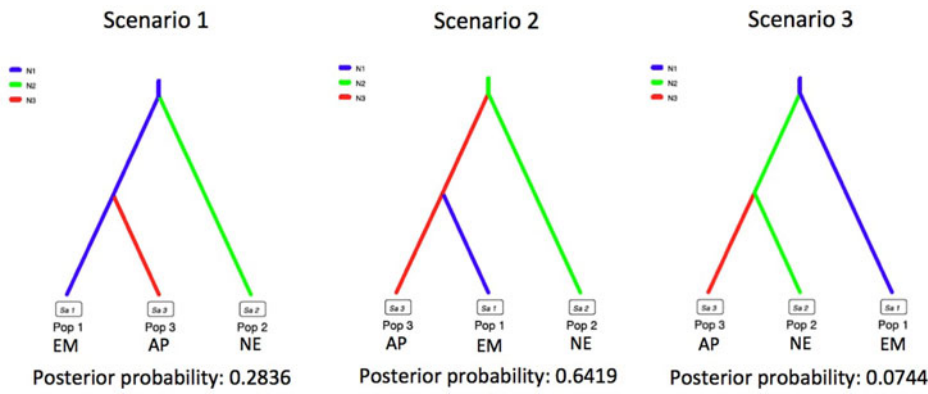
Fig. 3. Approximate Bayesian computation of potential colonization scenarios of horse mussels into the eastern Mediterranean basin. Scenario 1 indicates an independent introduction, whereas scenario 2 and 3 suggest introduction from Asian-Pacific and North Europe, respectively. The most probable scenario is scenario 2. EM, Eastern Mediterranean; AP, Asia-Pacific; NE, North Europe.
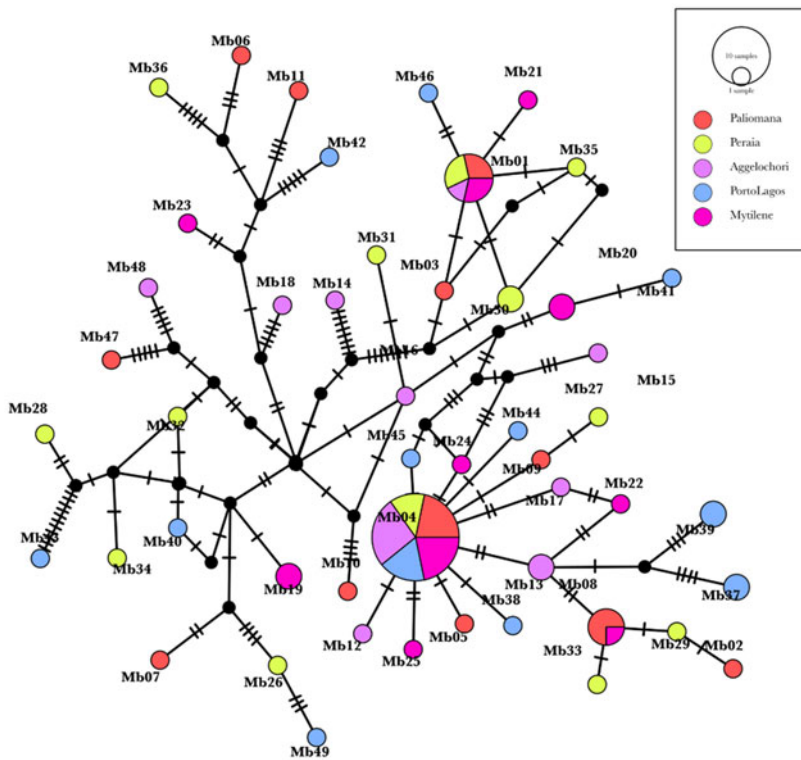

Fig. 4. Median-Joining network of *M. barbatus* haplotypes. Circle sizes are proportional of the number of times each haplotype occurred. Different colours indicate the different sampling locations. Hatch marks correspond to the number of mutational steps between two haplotypes. Black dots indicate potential missing haplotypes, not sampled in the present study.

Table 1. Genetic diversity parameters and Tajima's neutrality index for each population as well as at species level

	Number of haplotypes	Polymorphic sites	Nucleotide diversity	Haplotype diversity	Tajima's *D*
Paliomana	11	39	0.018	0.925	−0.2876
Peraia	12	38	0.019	0.967	−0.5504
Aggelochori	15	37	0.012	0.876	−1.2745
Porto Lagos	11	50	0.019	0.961	−1.0327
Mytilene	10	35	0.017	0.981	0.3536
Species level	49	85	0.017	0.947	−1.1493

All Tajima's *D* estimates were non-significant at level *P* > 0.05.

Black Sea and the Moroccan and Iberian Atlantic coasts, it has also been reported in west Ireland and the southern and western coasts of Great Britain (Lok *et al.*, 2006). European populations of the congeneric bivalve *M. modiolus* are believed to span their range throughout the coastline of Europe, except from the Mediterranean and the Black Sea (Halanych *et al.*, 2013). In the current phylogenetic analysis, while all *M. modiolus* haplotypes are clustered in the same clade, *M. barbatus* haplotypes are separated by higher levels of genetic distance than those found for congeneric species. The first branch includes all Aegean haplotypes collected in the current sampling, whereas the second branch includes all previously described *M. barbatus* haplotypes

Table 2. Pairwise genetic differentiation (F_{st}) among the sampled populations

	Paliomana	Peraia	Aggelochori	Porto lagos	Mytilene
Paliomana	–				
Peraia	0.0055	–			
Aggelochori	0.0151	0.0367	–		
Porto Lagos	0.0109	0.0425	−0.0317	–	
Mytilene	0.0316	0.1812*	0.0497	0.1048*	–

Significant values ($P < 0.05$) are shown with an asterisk (*).

originating from the North Sea (Figure 2). It is also worth noting that other *Modiolus* species from the Asian-Pacific region such as *M. metcalfei*, *M. comptus* and *M. areolatus* were found to be phylogenetically more closely related to the eastern Mediterranean haplotypes than to *M. barbatus* from the North Sea. These inferences indicate that the horse mussel populations from the eastern Mediterranean may not belong to the same species as those from the North Sea. A possible explanation for this puzzling situation could be the potential erroneous nomination of European horse mussel species. Examination of the distribution of *M. modiolus* as shown in the map of figure 1 in Halanych *et al.* (2013), as well as the reported distribution of *M. barbatus* in Lok *et al.* (2006) and the geographic origin of both *M. modiolus* and *M. barbatus* haplotypes in the study of Barco *et al.* (2016), reveals that both species are sympatric in some marine areas of the North Sea. In this case there may be three *Modiolus* species in Europe, two of which co-occur in northern European coasts and the third in the eastern Mediterranean Sea. This third species, which inhabits the Eastern Mediterranean basin, seems to be phylogenetically more closely related to *Modiolula phaseolina* from the Adriatic. Alternatively, a potential explanation could be related with the unusual mitochondrial DNA inheritance of marine bivalves. Particularly, in marine bivalves with two mitochondrial genomes, such as *Modiolus* species (Robicheau *et al.*, 2017), phylogeny reconstruction without distinguishing male from female mitochondrial haplotypes could lead to controversial inferences. Nevertheless, the inclusion of a male mitotype that was grouped with neither *M. modiolus* nor *M. barbatus* in the dendrogram of Figure 2 clearly rejects this hypothesis. Altogether, although more molecular data are probably needed to resolve the horse mussel's systematic status, *COI* results clearly indicate that their taxonomic status should be reconstructed.

Based on the depicted results of our phylogenetic analysis (Figure 2), as well as the comparisons of the newly described haplotypes as query sequences in the Basic Local Alignment Search Tool (BLAST) on the NCBI website, the horse mussel from the Aegean Sea is more closely related to the Asian-Pacific *Modiolus* species than to North European ones and very closely related to *Modiolula phaseolina*. Based on our results, *M. phaseolina* seems most likely to belong in the genus *Modiolus* and to have been misidentified as a different genus. In accordance with phylogenetic analysis, colonization analysis suggested that horse mussels were most probably introduced into the eastern Mediterranean from the Asian-Pacific coasts. However, these inferences may need further verification including more sampling locations, before conclusions can be drawn. A similar pattern has been observed in the southern hemisphere where *Mytilus* haplotypes were found closely related to the Mediterranean mussel *M. galloprovincialis* (Gérard *et al.*, 2008), supporting an analogous lineage origination. Alternatively, the close relationship of the Mediterranean haplotypes with those from the Indo-Pacific, could be a consequence of migration through the Suez Canal that has to be examined in future studies by analysis of samples from the Red Sea.

However, questions still remain regarding the phylogeographic positioning of western Mediterranean horse mussel populations that would probably clarify the phylogeny and taxonomy of European *Modiolus* species. On account of the very few available genomic analyses of European horse mussel populations there is a basic lack of information regarding the potential existence of the Siculo-Tunisian genetic break, causing distinction between western and eastern Mediterranean populations in several marine species (Kaouèche *et al.*, 2011; Sanna *et al.*, 2013; Giantsis *et al.*, 2014a). This lack of genomic data may be related to the difficulties in horse mussel sampling, which is only feasible by professional experienced divers and restricted to marine areas that can be reached by them. Thus, future studies should rely on comprehensive samplings from any possible marine area in the western Mediterranean and the Black Sea, in order to convincingly resolve taxonomic aspects of European *Modiolus* species and reconstruct their phylogeny.

Genetic diversity and population structure

The high levels of intraspecies and intrapopulation genetic diversity observed in *M. barbatus* from the Aegean Sea, in terms of haplotype and nucleotide diversity (Table 1), is a common feature of wild mytilid populations (Koehn *et al.*, 1976; Koehn, 1978; Giantsis *et al.*, 2014a). It is also worth mentioning the great number of 49 haplotypes found in the relatively restricted geographic marine area of the Aegean Sea, in comparison to the 52 derived haplotypes from the wider geographic marine area in the study of Halanych *et al.* (2013), which included samples from North Europe, the Pacific and the Atlantic. These inferences in combination with the high number of private haplotypes in all examined populations, suggest that the eastern Mediterranean marine area is likely the origin of *M. barbatus*. The high genetic diversity could also be attributed to the large effective population size (Ne), which although in mtDNA is one-quarter of the autosomal (Ladoukakis & Zouros, 2017), is still large in species of the family Mytilidae (Lallias *et al.*, 2009) and may even reach Ne > 30,000 (Kartavtsev & Nikiforov, 1993).

Despite the fact that farming of *M. barbatus* has not been developed yet in the Aegean, it is a promising candidate species for suspended aquaculture, similar to the long-line system of *M. galloprovincialis* (Lok *et al.*, 2006; Peharda *et al.*, 2013). Aquaculture activities may occasionally cause harmful effects in farmed bivalve populations, owing to phenomena related to genetic drift or inbreeding (Ye *et al.*, 2012). Nevertheless, due to the combination of (a) the lack of artificial breeding in suspended mussels, coupled with (b) the high genetic diversity observed, we do not foresee any negative impact of potential aquaculture activities on the genetic composition of horse mussel populations in the Aegean Sea.

Examination of the population structure of Aegean horse mussel populations revealed low levels of interpopulation genetic differentiation, most of which was statistically non-significant. The generally low levels of population structuring in eastern Mediterranean horse mussels are in accordance with previous studies of *Modiolus* species (De la Rosa-Velez *et al.*, 2000) and are mostly attributed to the long pelagic larval stage that can reach up to 6 months (Halanych *et al.*, 2013). During this period, larval translocation depends on waving and sea currents, promoting extensive gene flow. Thus, in contrast to the strong subdivision of populations often observed in bivalves with short larval stages (Ye *et al.*, 2015), genetic homogeneity is an expected phenomenon for horse mussel populations.

Despite the non-significant results, values of genetic differentiation were comparable with, or occasionally even greater than those estimated between pairs of more distant North European populations (Halanych *et al.*, 2013). Although there was no Single Nucleotide Polymorphism (SNP) to determine a particular geographic location, the high numbers of unique haplotypes may provide an indication for designing the development of other types of molecular markers towards resolving traceability issues. The characterization of the first DNA barcodes from the eastern Mediterranean is of considerable importance in the context of traceability of horse mussels from the eastern Mediterranean basin or from other European coasts. Detection of specific geographic origin is of highlighted importance in horse mussels on account of local rules and policy. Particularly, fisheries and trade of horse mussels within the Aegean Sea are permitted when originating from islands such as Mytilene, while prohibited when originating from Thermaikos Gulf (I.A. Giantsis, pers. comm.), due to the high concentrations in heavy metals there (Katsikatsou *et al.*, 2011). In this sense, a ready-to-use molecular tool for tracing horse mussels' origin would be extremely useful. To achieve this goal, sequencing of further genes, such as 16S or ITS ribosomal DNA, RADseq methodology or microsatellites would probably constitute promising molecular markers for such utility in mytilids (Larraín *et al.*, 2014), in which extensive gene flow is usually observed in wild populations.

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