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Phylogeny, phylogeography, and evolution in the Mediterranean region: News from a freshwater mussel (*Potomida*, Unionida)

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ABSTRACT

The *Potomida* genus (Bivalvia, Unionida) has a Circum-Mediterranean distribution and like other freshwater mussel species, its populations have suffered dramatic declines. Although this genus is currently considered as monotypic, it has a long history of taxonomic revisions and presently many aspects of its systematics and evolutionary history are unclear. We sampled a total of 323 individuals from 39 different sites across the *Potomida* genus distribution, and sequenced two mitochondrial (16S rDNA and Cytochrome c Oxidase Subunit I) and one nuclear (28S rDNA) genes to clarify its phylogeny and phylogeographic history. Our results show that the genus includes two well-supported clades, one comprising solely the western Mediterranean species *Potomida littoralis*, and the other including two eastern Mediterranean species, the Greek endemic *P. acarnanica* and the Anatolian and Middle Eastern *P. semirugata*. We suggest that *Potomida* started radiating during the upper Miocene, and that both vicariance and dispersal events shaped the diversification and distribution of the genus along the Mediterranean region. *P. littoralis* is further divided in two mitochondrial lineages, one restricted to Europe and the other occurring mostly in North Africa. Moreover, some European basins present both lineages in sympatry. The conservation status of the three recognized species should be reevaluated, particularly *P. acarnanica*, since it is restricted to two Greek river basins presenting a high risk of extinction. Overall, our results clarify some important gaps in knowledge concerning the phylogeny, phylogeography and evolution of the *Potomida* genus in the Mediterranean region with important taxonomical, ecological and conservational implications.

1. Introduction

The Mediterranean region has long been recognized for its exceptional species richness and high level of endemism, being classified as a hotspot of biodiversity (Myers et al., 2000). This

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region is one of the most complex, diverse, and heterogeneous areas of the planet and includes a unique combination of geographic, climatic and human histories that resulted in a remarkable evolutionary radiation of plants and animals colonizing a myriad of aquatic and terrestrial ecosystems (Blondel et al., 2010). The Mediterranean region is particularly rich in endemic plant species (Médail and Diadema, 2009) but a high proportion of animal species are also unique including 66% of amphibians, 50% of crabs and crayfish, 48% of reptiles, 25% of mammals and 14% of dragonflies, among others (Cuttelod et al., 2008). This high biodiversity is mainly found in two distinct areas, one in the West (especially in the Iberian Peninsula and Northwest Africa) and the other in the East (especially in the Balkans and Anatolia) (Schmitt, 2007). These areas are also considered temperate refugia where populations of plants and animals were often isolated during unfavorable climatic periods, resulting in strong genetic imprints (e.g. Hewitt, 1999; Taberlet et al., 1998). The existence of fragmented distributions within Mediterranean refugia, together with the dispersion processes and gene flow occurring during range expansions in the interglacial periods, has resulted in complex patterns of population genetic structure (e.g. Feliner, 2011; Schmitt, 2007).

The complex geography of the Mediterranean region, including land discontinuities and topography, has produced a multitude of different sub-centers responsible for present-day complex genetic differentiation patterns and uneven distributions of regional diversities. For example, the Strait of Gibraltar contributed to the diversification of Iberian and North African terrestrial and freshwater organisms, with several biogeographical studies describing different clades on both sides (e.g. Paulo et al., 2008 and references herein). The Messinian Salinity Crisis (MSC) occurred around 5.6 million years ago (Mya), at the end of the Miocene, causing the separation of the Mediterranean Sea from the Atlantic Ocean and temporarily desiccating the Mediterranean in a series of events (for a review see Rouchy and Caruso, 2006). This allowed interchange between the present mainland discontinuities followed by the re-establishment of the marine connection at 5.3 Mya (Krijgsman et al., 1999) fragmenting the ranges of the taxa involved.

Numerous studies have shown distinct patterns ranging from strong genetic splits (e.g. Fonseca et al., 2009; García-París et al., 2003; Miraldo et al., 2011; Steinfartz et al., 2000) to low mitochondrial differentiation across both sides of the Strait of Gibraltar (e.g. Carranza et al., 2006; Fromhage et al., 2004; Schmitt et al., 2006). The Eastern Mediterranean region, located at the margin of the Eurasian and African plates, has another complex geological history with multiple events of land divisions and connections during the late Tertiary (Bianco, 1990; Blondel et al., 2010; Schmitt, 2007). These also resulted in distinct phylogeographic patterns, from endemicity (e.g. Bohlen et al., 2006; Sotiropoulos et al., 2007), to widespread lineages of variable genetic variation (e.g. Veith et al., 2003; Stöck et al., 2012).

In this context, and given the heightened scientific interest in glacial refugia and postglacial colonization routes in the Mediterranean region, it is unexpected that the diversity and phylogeographic patterns of freshwater mussels (Bivalvia, Unionida) remain poorly understood. Aquatic freshwater species, such as freshwater mussels, are excellent candidates to study the extent of lineage diversification in this region due to their low dispersal ability. However, little attention has been given to their genetic diversity, especially when compared with other faunal groups or even with freshwater mussels from other continents (e.g. North America; Elderkin et al., 2008; Mock et al., 2013; Zanatta and Harris, 2013). In fact, published studies on these taxa evaluated genetic diversity based mainly of partial distributions (e.g. *Anodonta* sp. Geist et al., 2010; Nagel et al., 1996; and Lopes-Lima et al., 2016a; *Margaritifera margaritifera* (Linnaeus, 1758), Machordom et al., 2003; and *Unio* sp. Araujo et al., 2005, 2009; Prié and Puillandre, 2014). Detailed phylogeographic data covering entire species ranges is still insufficient (but see Froufe et al., 2014; Froufe et al., 2016a, for two rare examples).

Potomida Swainson, 1840 is a freshwater mussel genus with a Circum-Mediterranean distribution (Lopes-Lima et al., 2014). Although this genus is currently considered monotypic, it has a long history of taxonomic revisions, and presently its taxonomic status, and many aspects of its evolutionary history, remain unclear. The traditional conchological characters, of limited use for identifying taxonomic units due to the high phenotypic plasticity of unionoid mussels, are responsible for more than 90 synonyms described for *P. littoralis* (Cuvier, 1798) before the middle of the twentieth century (Graf, 2010). Haas (1969) has recognized this caveat, synonymizing many of these synonyms, and integrating all into eight *P. littoralis* subspecies (Fig. 1). These taxa were never validated with molecular studies, and some subspecies have been recently re-evaluated. The Iberian *P. l. umbonata* (Rossmässler, 1844), was synonymized with *P. l. littoralis* (Araujo et al., 2009). Khalloufi et al. (2011) have shown that *P. l. fellmanni* (Deshayes, 1848) populations present in Tunisia have considerable genetic differentiation of 3.16% (COI) from the Iberian *P. l. littoralis* populations. However, the remaining distribution of the subspecies, i.e., Morocco and Algeria, was not included in the study. Therefore, to confirm the validity of *P. l. fellmanni* these data need to be included. Finally, based on a comprehensive comparative morphological and anatomical study, *P. l. homsensis* (Lea, 1865) was properly re-assigned to other family (Margaritiferidae) and is now *Margaritifera homsensis* (Lea, 1865) (Smith, 2001). As for the remaining four subspecies, no recent data is available, and so the taxonomic status of all these subspecies is still unclear. This is worrisome since *Potomida* populations (Barea-Azcón et al., 2008; Pérez-Quintero, 2007; Sousa et al., 2008) and other freshwater mussels (e.g. Lopes-Lima et al., 2016b; Prié et al., 2014; Sousa et al., 2015, 2016), have suffered dramatic declines. For this reason, *P. littoralis* has been recently listed as Endangered in the IUCN Red List (Lopes-Lima et al., 2014).

Given the above uncertainties, in tandem with the conservation status of the *Potomida* genus, there is an urgent need for a comprehensive phylogeographic study to unravel its evolutionary history and taxonomy. In this study we used information from two mitochondrial (16S rDNA and Cytochrome c Oxidase Subunit I) and one nuclear (28S rDNA) genes from 323 individuals in 39 sites sampled across the *Potomida* distribution. Sequences for these three genes were combined and analyzed to: (i) clarify and establish the phylogeny and taxonomy of the genus; (ii) propose historical biogeographic and demographic scenarios to accommodate the genetic variation observed; and (iii) discuss the conservation implications of the obtained results. We also aim to test the role of the MSC as a vicariant event as well as the "oriental origin" hypothesis in the Balkans, using a calibrated phylogeny.

2. Materials and methods

2.1. Sample collection

Potomida specimens were collected from 39 sites across its reported distribution, in Morocco, Iberia, France, Greece and Turkey (Fig. 2 and Table S1). A small sample from the foot was collected in the field (following Naimo et al., 1998) and placed in 99% ethanol, returning the mussel to the substrate immediately afterwards. Genomic DNA was extracted from the tissue samples, using a standard high-salt protocol (Sambrook et al., 1989). Voucher specimens from France are deposited in Muséum National d'Histoire Naturelle (Paris, France) and voucher specimens from

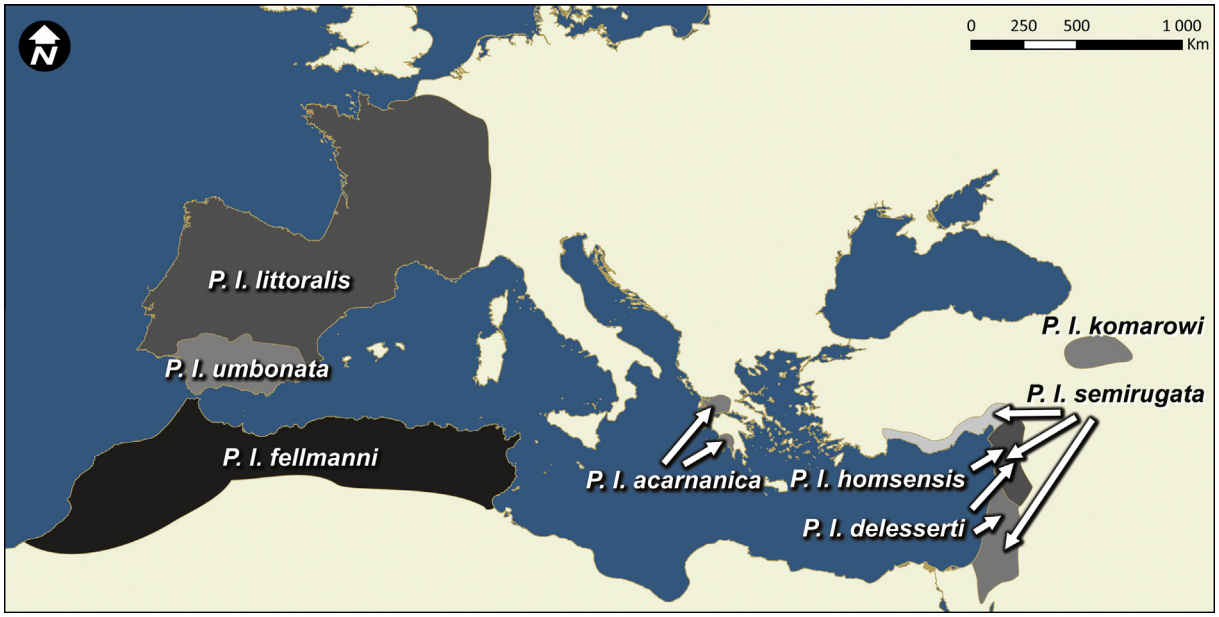

Fig. 1. Distribution map of the eight *Potomida littoralis* subspecies recognized by Haas (1969).

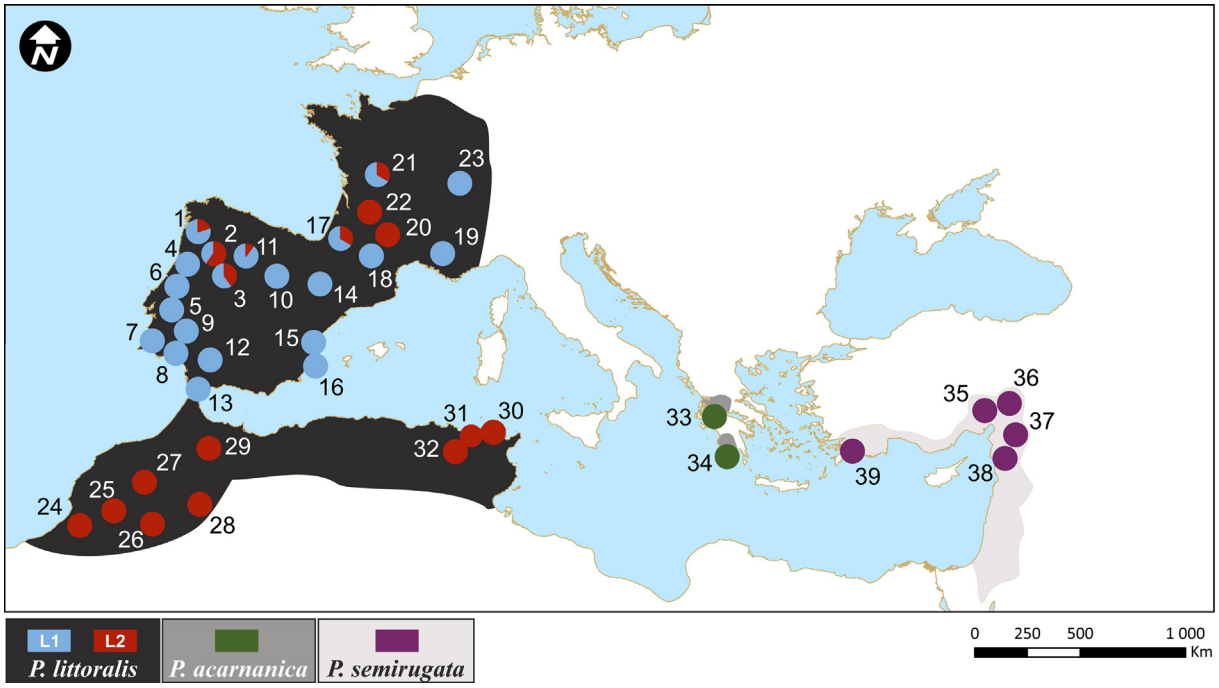

Fig. 2. Map showing the location of the sampled *Potomida* populations. The distribution of the three recognized species and the geographic distribution of mtDNA Lineages are represented in pie charts.

Morocco, Iberia, Greece and Turkey have been deposited in Museu Nacional de História Natural e da Ciência (Lisboa, Portugal).

2.2. Sequencing, alignment, phylogenetic analyses

A total of 323 *Potomida* specimens and four outgroup species: *Pronodularia japanensis* (Lea, 1859), *Lamprotula leai* (Griffith & Pidgeon, 1833), *Leguminaia wheatleyi* (Lea, 1862) and *Microcondylaea bonellii* (A. Ferussac 1827), were amplified for mtDNA 16Sr DNA (16S rRNA; ca. 500 bp fragment), with 16SL and 16SH primers (Palumbi et al., 1991); the F-type mtDNA cytochrome oxidase subunit 1 gene (COI; ca. 700 bp fragment), with LCO_22me and HCO_700dy primers (Walker et al., 2006, 2007); and for the nuclear 28Sr DNA (ca. 800 bp fragment) with 28S-RD1.3f and 28S-rD4b primers (Whiting, 2002). PCR conditions are described in Froufe et al. (2014) with annealing temperatures ranging from 49 °C (16S) to 55 °C (COI and 28S). Sequences were obtained using the BigDye sequencing protocol (Applied Biosystems 3730xl) by Macrogen Inc., Korea. Forward and reverse sequences were edited and assembled using ChromasPro 1.7.4 (Technelysium, Tewantin, Australia).

Alignments were built with ClustalW, in Bioedit 7.2.5 (Hall, 1999), including sequences from GenBank (France, Tunisia and Turkey; Table S1). Unionida exhibit a peculiar mode of mitochondrial DNA transmission, known as doubly uniparental inheritance (DUI), of mitochondrial DNA (Zouros et al., 1994a,b; Hoeh et al., 1996). As a result, the nucleotide divergence between female and male mitochondrial genomes of *P. littoralis* is at least 30% (Froufe et al., 2016b). In the present study, this value was used as a reference to assure that the obtained COI sequences were indeed all F-type.

Topological differences among single-marker phylogenies were assessed according to Mason-Gamer and Kellogg (1996) and a final concatenated alignment, including outgroups, was then analyzed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. The best-fit model of nucleotide substitution under corrected Akaike Information Criterion was selected using JModelTest 2.1.7 (Darriba et al., 2012) for each gene partition. ML trees were built in PhyML (Guindon and Gascuel, 2003) with 1000 bootstrap replicates. Phylogenetic BI was performed on MrBayes version 3.2.6 (Ronquist et al., 2012) with sequences also partitioned according to genes. Analyses started with program generated trees, with four heated Markov chains with default incremental heating; two independent runs 24×10^6 generations long were sampled at intervals of 1000 generations producing a total of 24,000 trees. Burnin was determined upon convergence of log likelihood and parameter estimation values using Tracer 1.6 (Rambaut et al., 2014). Haplotypes, for each gene, were retrieved using DNASp v5.1.0.1 (Rozas and Rozas, 1995). All new sequences have been deposited in GenBank (Table S1).

2.3. Divergence time estimates

At present, no internally calibrated molecular clock is available for *P. littoralis* (using the fossil record). Therefore, divergence times among lineages were estimated from COI sequences using Beast v1.8.2 (Drummond and Rambaut, 2007), and the substitution rate of 0.265 ± 0.06% per million years recently estimated for *Unio* (Froufe et al., 2016a) was applied with normal distribution prior. Individual haplotypes were used to reduce computational load and optimize the analysis. The dataset was run under a TIM3 substitution model according to jModelTest results. An uncorrelated lognormal relaxed clock (Drummond et al., 2006) was used, and the tree prior was set to yule speciation process (Gernhard, 2008; Yule, 1925) given the nodes of interest are at an interspecific level. Other parameters used default settings. The random seed was 1434916511075. The analysis ran for 10^7 generations, sampling every 1000 generations. The quality of the runs was assessed through parameter convergence using Tracer 1.6 (Rambaut et al., 2014). The maximum credibility tree of mean heights was constructed using TreeAnnotator and discarding 200 trees as burn-in.

2.4. Diversity, differentiation and demographic analyses

For each gene, sequences were joined in unrooted networks using the fixed connection limit of 30 steps criterion implemented in TCS 1.21 (Clement et al., 2000). Genetic distances (*p*-uncorrected) within and between lineages were calculated with MEGA 6.0 (Tamura et al., 2013) and the diversity for each gene fragment (i.e. the number of haplotypes (h), haplotype diversity (Hd) (Nei and Tajima, 1981) and nucleotide diversity (π) (Nei, 1987) were calculated using the software DnaSP v5.1.0.1. An analysis of molecular variance (AMOVA) was performed using ARLEQUIN v.3.5.1.3 (Excoffier and Lischer, 2010) to estimate the hierarchical distribution of mtDNA genetic differentiation (COI), within and among populations, using ΦST (from the absolute number of nucleotide differences) and with 10,000 permutations.

In order to test for molecular signatures of demographic expansion of each of the major clades obtained in the phylogeny and hypothesizing post-Pleistocene range expansions, a pairwise mismatch-distribution analysis was carried out (Rogers and Harpending, 1992) and Fu's Fs (Fu, 1997), Tajima's D (Tajima, 1989) and Ramos-Onsins and Rozas' R2 (Ramos-Onsins and Rozas, 2002) statistics were calculated.

3. Results

3.1. Phylogenetic relationships

All mtDNA COI sequences obtained were considered F-type, since no sequences similar to the M-type were detected. There was no major length variation within each gene (lack of indels in the COI alignment and both 16S and 28S presenting four 1 bp-gaps each) and no stop codons were observed after translating all sequences to amino acids. Following the methodology of Mason-Gamer and Kellogg (1996) there was no significant topological differences between estimates of phylogenies based on the individual gene trees. Thus, the three fragments were concatenated and analyzed in a combined approach. Model TIM3 was chosen for COI, HKY + G for 16S and GTG + G for 28S. The final combined data set included 1790 aligned positions for 323 individuals, comprising 608 bp of COI, 491 bp of 16S, and 691 bp of 28S.

The concatenated tree topologies resulting from the single tree recovered from the ML and BI approaches were congruent and produced topologically identical trees. The results of the Bayesian phylogenetic analysis are shown in Fig. 3. The results show a well-resolved phylogeny that includes two well-supported sister clades: one comprising solely the Western Mediterranean species *P. littoralis* (Cuvier, 1798), and the other including two Eastern Mediterranean species, the Greek endemic *P. acarnanica* (Kobelt, 1879) and the relatively widespread Anatolian and Middle East *P. semirugata* (Lamarck, 1819). *P. littoralis* is divided in two major highly supported lineages (L1 and L2 in the phylogeny). L1 (blue[1]) is very shallow and includes the majority of the individuals collected in Iberian and French basins (Figs. 2 and 3). L2 (red) includes all the individuals collected in Morocco, the ones retrieved from GenBank from Tunisia and some individuals from North-West Iberia and French Atlantic basins, collected in sympatry with the individuals depicted in L1 (Table S1; Figs. 2 and 3). *P. acarnanica* individuals (from Greece) cluster together and join with high support with *P. semirugata* individuals collected in Turkey.

Measures of genetic diversity for the mitochondrial COI gene are summarized in Table 1. Mean genetic distance (*p*-uncorrected) ranged from 3.0% between *P. acarnanica* and *P. semirugata* and 4.2% between *P. littoralis* and *P. semirugata* (Table 1). Levels of haplotype diversity were higher in *P. semirugata* when compared to the remaining species (Table 1). The divergence between the two *P. littoralis* lineages (i.e. L1 and L2 in the phylogeny, Fig. 3) was 2.4%, with L2 showing higher levels of genetic variation (Table 1).

3.2. Divergence time

All ESS values assessed in Tracer v1.6 were above 200. The average estimated crown ages for the four lineages was between 1.38 and 2.88 Mya (Fig. 4), with the MCS far predating any of the 95% confidence intervals. Moreover, this analysis determined the age

[1] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

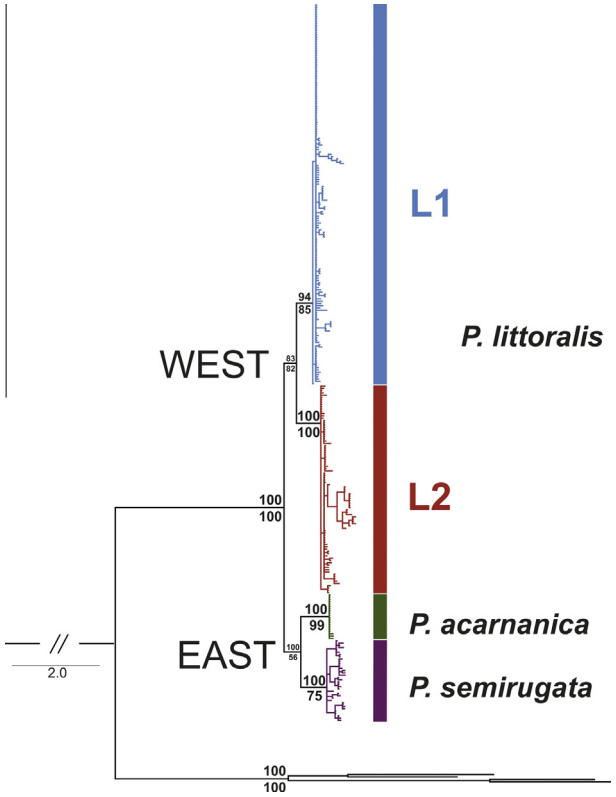

Fig. 3. Phylogenetic tree obtained by Bayesian Inference analysis of *Potomida* individuals (COI + 16S + 28S). For the major nodes support values (%) are given as Bayesian posterior probability above nodes and Bootstrap support (ML) below nodes.

of the most recent common ancestor (MRCA) of the *Potomida* genus as 8.1 Mya (Fig. 4).

3.3. Genetic diversity, differentiation and historical demography

Overall diversity estimates for each population and for each fragment are shown in Table 2. Haplotype networks based on mtDNA and nuclear sequences are presented in Fig. 5. As expected, very similar networks were retrieved for both mitochondrial genes, clearly identifying the same four lineages as the phylogenetic tree. *P. littoralis* L1 (blue) exhibits a star-like topology, with one high frequency central haplotype and several additional haplotypes connected by few-step mutations. In this haplogroup, the most common haplotype represents 50.8% (COI) and 62.2% (16S) of the individuals sampled from all localities in Iberia and France (Figs. 2 and 5, and Table S1). The haplogroup corresponding to *P. littoralis* L2 (red) in the phylogeny is linked by 10 steps to L1 (blue) and has several frequent haplotypes that are widely distributed. Most of these haplotypes were found in Morocco. However, 31 European individuals (marked with ∗ in the both mtDNA networks) cluster together with others from North Africa (Fig. 5). The Tunisian individuals retrieved from GenBank have 5 COI haplotypes (90–94) that cluster together at 5 mutations from the closest Morocco haplotype. *P. acarnanica* (green) is the species with the fewest haplotypes found (4 in COI and 2 in 16S; Fig. 5). On the other hand, from the 46 *P. semirugata* individuals, 27 haplotypes were retrieved (purple, Fig. 5) being 21 only found in a single individual (COI; Table 2). The 28S nuclear network was generally compatible with mtDNA in the diagnosis of the shared haplotypes between Europe and North Africa. *P. acarnanica* is represented by a single haplotype (green) and *P. semirugata* presents four unique haplotypes (purple; Fig. 5).

The results from AMOVA showed that differences between the two major Clades retrieved in the phylogeny, i.e., western and eastern Mediterranean (Fig. 3), accounted for 63.4% of overall variation in *Potomida*, whereas only 6% of total mitochondrial haplotype variation occurred within populations, with all structured levels presenting highly significant genetic differences (data not shown).

Tests of demographic history yielded significantly negative results for D (Tajima's) and Fs (Fu's) statistics for both *P. littoralis* and *P. acarnanica* (COI, Table 1) suggesting genuine rapid demographic expansion for these species. However, as *P. littoralis* is further divided in two additional lineages, the results of these tests were also carried on for each of these lineages. Only *P. littoralis* L1 yielded significantly negative results for the neutrality test (Table 1). Mismatch distributions showed similar patterns, supporting scenarios of demographic expansion in both *P. littoralis* L1 and *P. acarnanica* (Fig. 6), while for *P. littoralis* L2 and *P. semirugata* it revealed multimodal distributions (Fig. 6).

4. Discussion

4.1. Phylogeny and systematics of the genus Potomida

Results revealed a well-resolved phylogeny divided in two major allopatric clades, western and eastern Mediterranean. Reciprocal monophyly in mtDNA and diagnostic exclusivity of nuclear markers, in addition to allopatric distributions, allowed us to distinguish three species within the *Potomida* genus: *P. littoralis* (Cuvier, 1798) in the western clade and *P. acarnanica* (Kobelt, 1879) and *P. semirugata* (Lamarck, 1819) clustering in the eastern clade. The subspecies *P. l. fellmani* (Deshayes, 1848) and *P. l. umbonata* (Rossmässler, 1844) are here synonymized with *P. l. littoralis* (Cuvier, 1798) (L1 and L2). As for *P. l. acarnanica* (Kobelt, 1879) it is here recognized as *P. acarnanica* (Kobelt, 1879). The name *P. acarnanica* is valid for being the oldest description from the known distribution (i.e. Greece). Finally, both Middle East and Turkey subspecies are here synonymized and considered as *P. semirugata* (Lamarck, 1819). Among the various names that could apply to this *Potomida* species, e.g. *P. delesserti* (Bourguignat,

Table 1
Mean genetic divergence for the COI data set, between the three *Potomida* species (above) and within the two *Potomida littoralis* lineages (below) depicted in the phylogeny (Fig 3). *N* = Number of individuals; *h* = haplotypes; Hd ± SD = haplotype diversity; and π ± SD = nucleotide diversity. Tests of population growth within each *Potomida* species (above) and within the two *Potomida littoralis* lineages (below), i.e., the results of Tajima's *D* and Fu's Fs neutrality tests are also shown. Statistically significant values are followed by an asterisk ($p < 0.05$).

	P. littoralis	*P. acarnanica*	*P. semirugata*	*N*	*h*	Hd	π	Fu's FS	*D*
P. littoralis				257	54	0.745 ± 0.029	0.01242 ± 0.00062	−2.504*	−0.998
P. acarnanica	0.037			20	4	0.284 ± 0.128	0.00082 ± 0.00041	−24.184*	−1.975*
P. semirugata	0.042	0.030		46	24	0.908 ± 0.035	0.00749 ± 0.00069	−12.935	−1.166
P. littoralis		Lineage 1	Lineage 2						
	Lineage 1			166	29	0.423 ± 0.050	0.00139 ± 0.00024	−41.832*	−2.52401*
	Lineage 2	0.024		91	25	0.894 ± 0.017	0.00752 ± 0.00062	−6.794	−0.91894

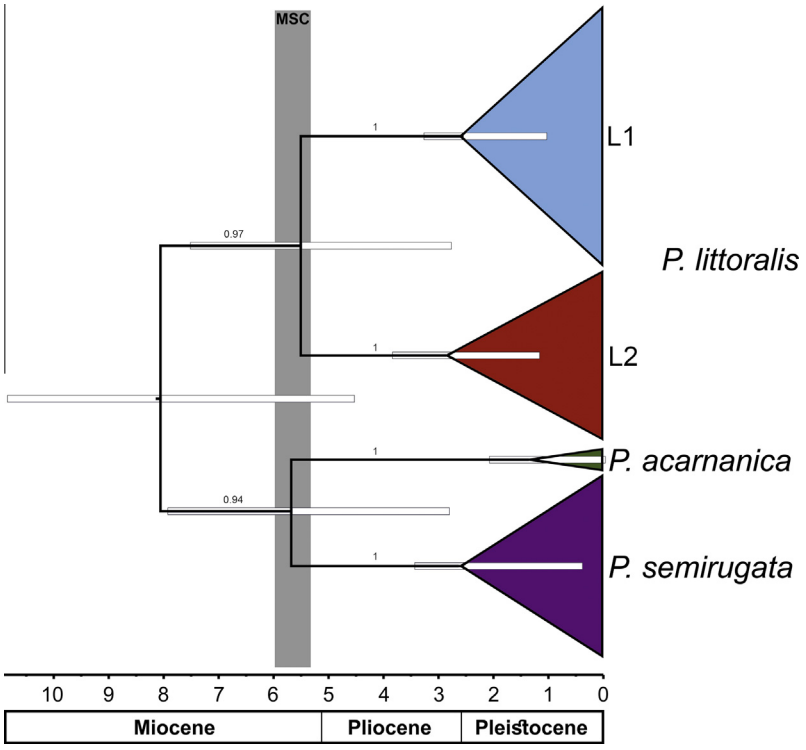

Fig. 4. BEAST maximum clade credibility tree for *Potomida*. Time scale is in million years. The grey horizontal bars indicate the height 95% HPD interval for the crown-age estimates. The size of the triangles is proportional to the number of haplotypes.

Table 2
Summary of the indices of genetic diversity estimated from the COI, 16S and 28S sequencing data for each sampled population (populations with $N < 5$ not shown). N = sample size; h = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity. Population numbers follow Fig. 2 and Table S1.

Population	Species	COI				16S				28S			
		N	*h*	Hd	π	*N*	*h*	Hd	π	*N*	*h*	Hd	π
P1	*P. littoralis*	10	4	0.644	0.00826	10	4	0.644	0.00709	10	2	0.200	0.00029
P2	*P. littoralis*	10	5	0.822	0.01268	10	6	0.844	0.01276	10	3	0.511	0.00110
P3	*P. littoralis*	10	5	0.844	0.01096	10	2	0.533	0.00878	10	2	0.200	0.00029
P4	*P. littoralis*	10	5	0.844	0.00610	10	2	0.200	0.00041	10	2	0.200	0.00029
P5	*P. littoralis*	10	3	0.378	0.00066	10	2	0.200	0.00082	10	2	0.200	0.00029
P6	*P. littoralis*	10	3	0.511	0.00150	10	4	0.644	0.00155	10	3	0.644	0.00107
P7	*P. littoralis*	10	3	0.6	0.00110	10	2	0.200	0.00041	10	3	0.644	0.00268
P8	*P. littoralis*	10	5	0.667	0.00132	10	1	–	–	10	3	0.378	0.00058
P9	*P. littoralis*	10	7	0.911	0.00256	10	4	0.711	0.00197	10	2	0.200	0.00029
P10	*P. littoralis*	9	5	0.806	0.00411	9	1	–	–	9	2	0.222	0.00032
P11	*P. littoralis*	10	5	0.667	0.00519	10	4	0.533	0.00412	10	1	–	–
P12	*P. littoralis*	10	4	0.644	0.00150	10	2	0.200	0.00041	10	2	0.356	0.00052
P13	*P. littoralis*	10	5	0.756	0.00216	10	2	0.200	0.0041	10	3	0.511	0.00110
P14	*P. littoralis*	10	1	–	–	10	1	–	–	10	3	0.600	0.00165
P15	*P. littoralis*	10	3	0.378	0.00267	10	3	0.378	0.00114	10	2	0.200	0.00058
P16	*P. littoralis*	10	1	–	–	10	1	–	–	10	3	0.644	0.00191
P17	*P. littoralis*	9	4	0.806	0.01106	9	3	0.750	0.00926	9	2	0.389	0.00057
P21	*P. littoralis*	12	6	0.758	0.01268	12	2	0.485	0.00798	10	1	–	–
P22	*P. littoralis*	10	3	0.378	0.00066	10	3	0.378	0.00114	10	3	0.689	0.00171
P25	*P. littoralis*	10	1	–	–	10	1	–	–	10	2	0.200	0.00029
P26	*P. littoralis*	10	1	–	–	10	1	–	–	10	2	0.200	0.00029
P27	*P. littoralis*	10	4	0.778	0.00548	10	4	0.733	0.00238	10	4	0.733	0.00178
P28	*P. littoralis*	10	1	–	–	10	1	–	–	10	2	0.467	0.00136
P29	*P. littoralis*	10	5	0.756	0.00208	10	1	–	–	10	2	0.467	0.00136
P30	*P. littoralis*	5	3	0.700	0.00329	5	3	0.800	0.00206	–	–	–	–
P33	*P. acarnanica*	10	4	0.533	0.00164	10	2	0.200	0.00041	10	1	–	–
P34	*P. acarnanica*	10	1	–	–	10	1	–	–	10	1	–	–
P35	*P. semirugata*	5	3	0.700	0.00165	5	3	0.700	0.00206	5	1	–	–
P36	*P. semirugata*	10	7	0.911	0.00581	10	3	0.378	0.00206	10	1	–	–
P37	*P. semirugata*	11	8	0.927	0.00847	10	4	0.711	0.00544	11	1	–	–
P38	*P. semirugata*	10	9	0.978	0.00662	10	6	0.844	0.00713	10	1	–	–
P39	*P. semirugata*	10	1	–	–	10	2	0.200	0.00041	10	2	0.200	0.00029

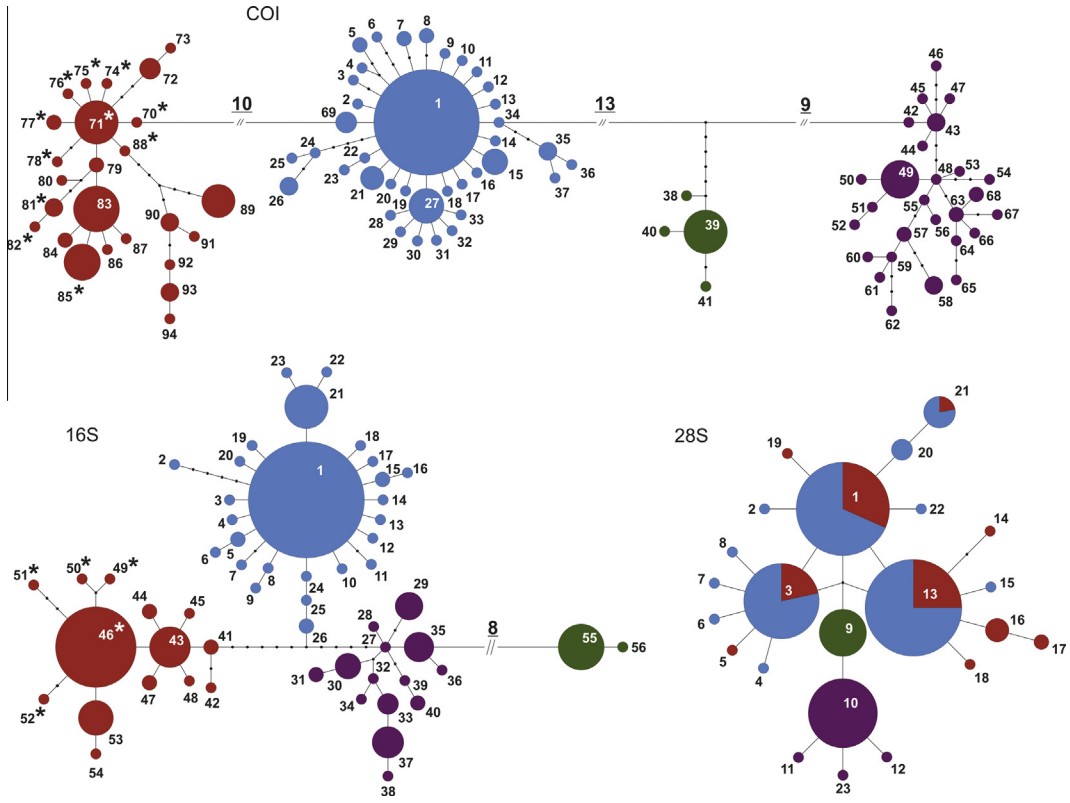

Fig. 5. Haplotype (TCS) networks showing the relationships of *Potomida* individuals sequenced (Table S1). **(Top)** COI; **(Left below)** 16S; **(Right below)** 28S. Circle size is proportional to the observed haplotype frequencies and black points represent unobserved haplotypes and potential intermediates. Colors represent the four lineages detected in the obtained phylogeny; *Potomida littoralis* L1 (blue); *P. littoralis* L2 (red); *Potomida acarnanica* (green); and *Potomida semirugata* (purple). Underlined numbers correspond to the number of steps among the lineages; the presence of European individuals in L2 are marked with *. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1852) and *P. tracheae* (Kobelt, 1895), *P. semirugata* (Lamarck, 1819) is the oldest. Hence, following the priority principle, the name *P. semirugata* is here applied.

According to the Bayesian analysis, *Potomida* probably started radiating during the upper Miocene (8.1 Mya) in which the two major *Potomida* clades (i.e. western and eastern Mediterranean) emerged. Subsequently, two diversification events occurred more or less contemporaneously devising the current four major lineages. These events occurred at the Miocene-Pliocene transition, closely overlapping with the MSC (5.9–5.3 Mya) (Krijgsman et al., 1999), which indicates the range shifts occurring at the time, might be responsible for these divergences. Such deep divergences also suggest the existence of distinct species, rejecting the monotypic status of the genus.

4.2. Genetic differences across the Mediterranean geographic discontinuities

4.2.1. Western Mediterranean

The western Mediterranean clade recovered in the phylogeny, and corresponding to *P. littoralis*, is further divided in two lineages (L1 and L2, Fig. 3). Opposite patterns of within-lineage demographic changes were observed. L1 revealed to be very shallow with no clear phylogeographic pattern being observed in Iberia and in France. Both mtDNA gene networks show star-like patterns of connectivity between haplotypes providing support for a recent population expansion in this lineage (Fig. 5). The mismatch analysis and neutrality tests also support a demographic expansion of L1 in Southwest Europe (Table 1; Fig. 6). Therefore, the origin of this lineage was very likely Iberia followed by a population bottleneck event and a posterior expansion. These results are in line with the southern refugia hypothesis followed by post-glacial expansion (Hewitt, 1999).

In contrast, no genetic signal of demographic expansion was observed for L2 (Table 1; Fig. 6). This suggests that in this lineage a series of small relict populations might have existed surviving the successive Quaternary glaciations in isolated refugia, without a detectable subsequent expansion. L2 encompasses all the North African haplotypes but is also present in North-Western Iberia and the Atlantic French river basins. In fact, in the populations from the Douro and Loire basins, each containing both mitochondrial lineages in sympatry, 60% of the individuals fall into L1 and 40% into L2. Notwithstanding, the results suggest a possible relatively recent South-North expansion of L2, as it has higher levels of genetic variation in North Africa populations than in Southwestern Europe (Tables 1 and 2). Under this scenario, we would expect that North Africa was the center of origin of this lineage.

We propose and discuss three hypotheses to explain the unexpected phylogeographic pattern described above. In the first, L2 might have existed throughout the entire species distribution, i.e. Southwestern Europe and North Africa, but due to the Pliocene climate changes, it became extirpated in most of its European range. Thus, the shrinking and fluctuating population sizes (Fig. 6) resulted in a loss of ancestral diversity via population bottlenecks and drift. This pattern of incomplete lineage sorting between the two *P. littoralis* lineages is in our view the most likely to explain the recent break of gene flow between the European and North African populations.

In an alternative scenario, if L2 had reached Iberia from North Africa during the MSC, a substantial genetic divergence would be expected in both sides of the Western Mediterranean, resulting from a vicariance event dating back to the reopening of the Strait.

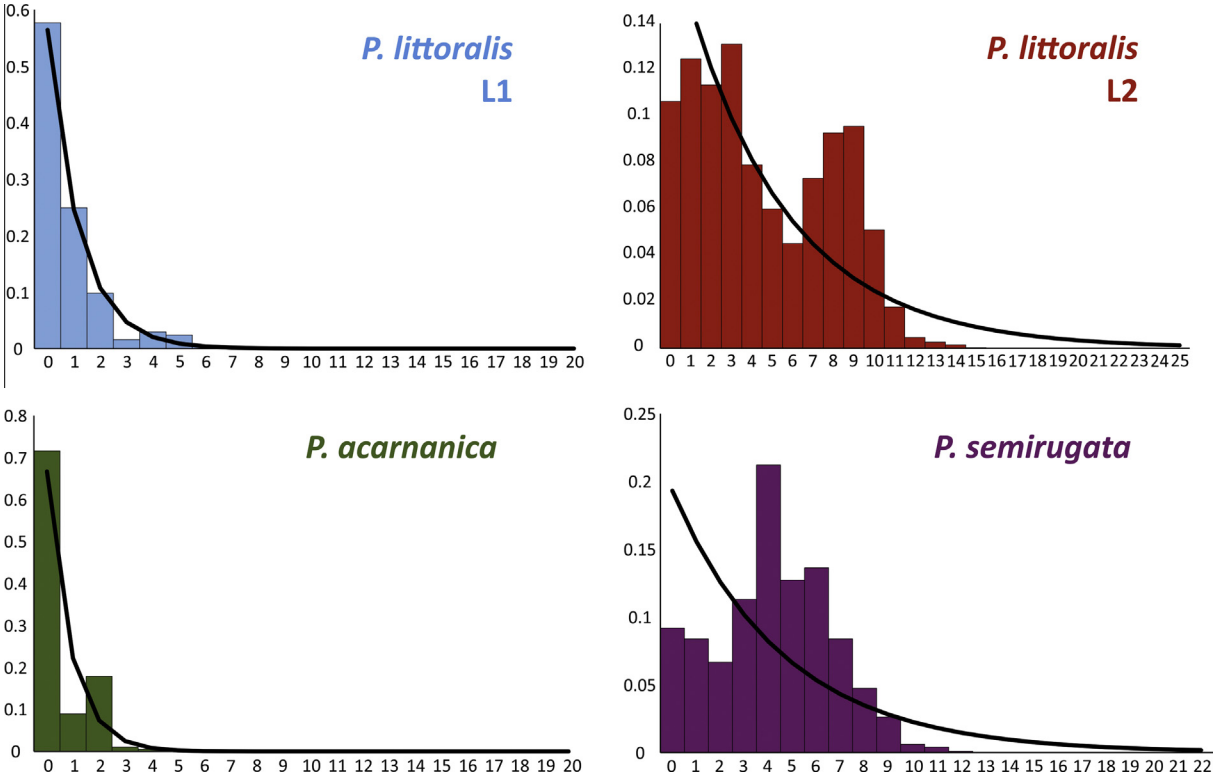

Fig. 6. Mismatch-distributions for each *Potomida* species/lineage. Black curves show the expected distribution of mutations according to the null hypothesis of demographic expansion. The number of pairwise differences and their frequencies is shown on the horizontal and vertical axes, respectively. Colors represent the four lineages detected in the obtained phylogeny; *Potomida littoralis* L1 (blue); *P. littoralis* L2 (red); *Potomida acarnanica* (green); and *Potomida semirugata* (purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

However, the unexpectedly similarity of both mtDNA and nuclear sequence haplotypes in North African and European populations, and the lack of variation in the European populations in contrast to the North African ones as a whole, suggests that colonization was recent. In addition, L1 and L2 average divergence dates are contemporaneous with the land bridge established during the MSC and this situation raises the hypothesis that the separation of Iberia and North Africa might have caused a vicariance event in this species. Using our calibration framework, the age of the diversification between both lineages is the same as the land bridge. As for the diversification within each of the four main lineages, at least for the extant diversity, it most likely took place well after the end of the MSC.

Finally, and although unlikely, the unexpected geographic pattern of L2 in Europe, i.e., apparently being restricted to Northern Iberia and Atlantic French River basins, can also be explained by the failure of this study in sampling haplotypes that would fall inside L2 in the Southern Iberian basins. Regrettably, the Endangered *P. littoralis* has been declining in recent decades in all Iberian populations (Barea-Azcón et al., 2008; Pérez-Quintero, 2007) and has suffered a major range contraction in France, being extirpated from the north of its former distribution (Prié et al., 2014), which restricts the collection of more specimens.

Within North Africa, the Tunisian haplotypes (in L2) appear in the tip of the COI network (HCOI 90–94, Fig. 5) with a minimum of six mutations to a central haplotype (HCOI 71, Fig. 5) and to the Morocco haplotype (HCOI 71, Fig. 5) found in the individuals from the only sampled endorheic basin (River Ziz – P28, Figs. 2 and 5; Table S1). Interestingly, the mean COI genetic distance between the newly sequenced Moroccan individuals and the previously published Tunisian ones (Khalloufi et al., 2011) is 1.5%, while 2.4% corresponds to the mean COI genetic distance between the entire L2 and the European L1. These results further add to the evidence of other studies (e.g. amphibians: Recuero et al., 2007; and freshwater mussels, *Unio* sp.: Khalloufi et al., 2011) suggesting the presence of an important biogeographical barrier between eastern and western Maghreb.

4.2.2. Eastern Mediterranean

The east Mediterranean clade contains *P. acarnanica* and *P. semirugata*, which diverge 3%. In Western Greece, *P. acarnanica* is restricted to the southern part of the Ionian Ecoregion, an area well known for its higher endemicity in the Hellenic peninsula (e.g. freshwater fish; Economou et al., 2007). The results from the demographic changes (Table 1, Fig. 6) revealed a recent bottleneck. This could be due to a contraction in the mussel's distribution as the species inhabits relatively small river basins whose lowland river valley habitats were decreased by sea level rise after the last glacial maximum (Perissoratis and Conispoliatis, 2003). On the contrary, its sister species, *P. semirugata* has a wider range through southern Anatolia and the Levant. In addition, the results have shown that this species has the higher haplotype diversity, and both tests of demographic history and mismatch distributions support a scenario of more stable populations. Furthermore, these populations occur in areas with a long history of intra-basin isolation (Heller, 2007) that might help to explain their higher genetic diversity.

The divergence between mtDNA lineages of these species seems likely contemporaneous with the MSC period (5.9–5.3 Mya), while diversification within each lineage took place much after, particularly for *P. acarnanica*. It is possible that the low diversity observed in this species could be due to recent population reductions and lineage extirpation. This pattern is not seen in *P. semirugata*. This could be due to the more geographically isolated and fragmented

riverine habitats in Peninsular Greece (i.e. being more vulnerable to climate-driven extirpations) but it could also mean that Greece was colonized (or re-colonized) from Anatolia during the MSC, thus leading to a more extended evolutionary lag time.

This pattern of "oriental origin" has been observed in freshwater fishes in the Balkans. However, the timing is still controversial, as are processes that have led to the key assemblage components of each biogeographic region (Bănărescu, 2004; Gaubert et al., 2009). The biogeographical boundary of the mid-Aegean trench separating Anatolia from Southern Greece is long established (Poulakakis et al., 2015; Zogaris et al., 2009), but dispersal of freshwater organisms may have occurred either during the MSC or even at an earlier land contact event. Tsigenopoulos et al. (2003) hypothesised that during the MSC a network of interconnected rivers facilitated dispersal of the cyprinid genus *Luciobarbus* across the dried Mediterranean. Indeed, *Luciobarbus* exists in Southern Turkey, Eastern Greece and Western Greece, and different species on both sides of the Aegean are genetically very close (Geiger et al., 2014). Therefore, the MSC is the most likely hypothesis within our dating framework (Fig. 4). The alternative scenario is a colonization during the connection of the Hellenic Peninsula and Anatolia in the upper Miocene, i.e., before the MSC (Economou et al., 2007; Poulakakis et al., 2015). However, this seems unlikely since the age of this event falls outside the confidence interval for the *semirugata-acarnanica* divergence. Even if a common ancestor carried out the colonization, one could expect a higher diversity in Greece.

4.3. Comparison with other Mediterranean freshwater mussels and conservation implications

Classic "refugia within refugia" phylogeographic patterns (Gómez and Lunt, 2007), with a nearly or complete lack of mtDNA admixture among populations, have been described in several taxa for Iberia, Maghreb, Balkans, Anatolia and the Middle East (e.g. Schmitt, 2007). However, examples from freshwater mussels are still scarce in Europe, scarcer in North Africa, and absent for the Eastern Mediterranean region. The lack of genetic differentiation among Southwest European river basins, here obtained for *P. littoralis* L1, has been recently reported for another freshwater mussel species in Iberia (*Unio delphinus* Spengler, 1793; Froufe et al., 2016a). On the other hand, several allopatric distinct lineages were detected for another freshwater mussel in Iberia (*Anodonta anatina* (Linnaeus, 1758); Froufe et al., 2014). In the Maghreb region, while no genetic differentiation was observed for *Unio foucauldianus* Pallary, 1936 populations across its range (Froufe et al., 2016a) in *Potomida* two possible refugia were here detected with 1.5% (COI) genetic divergence between them (i.e. Moroccan and Tunisian populations). Unfortunately, there are no molecular studies using freshwater mussels as target organisms in the Balkans and Turkey, which precludes further considerations regarding the presence of diversity sub-centers for these taxa in the area.

Despite having a Circum-Mediterranean distribution and being one of the most endangered freshwater bivalves in Europe, little was known about intraspecific variation in *Potomida*. The present study recognized Iberia, Maghreb, Southwestern Greece and Southern Turkey as its main four "classic refugia". Thus, they should constitute priority areas of conservation for *Potomida* species. This is especially urgent given the threats posed by the extensive environmental changes occurring in the Mediterranean region. Additionally, since the last IUCN global assessment for *P. littoralis* (Endangered) included the three *Potomida* species now recognized, they should be re-evaluated individually, probably giving each a heightened threat status. *P. littoralis* (L1 + L2) for instance has suffered a strong decline with a 75% population decrease in its European range (Lopes-Lima et al., 2014). The Moroccan populations are also declining due to the dramatic decrease of water quantity and quality in several Moroccan river basins (Sousa et al., 2016). As for *P. acarnanica*, it is classified as highly threatened since it revealed a very low genetic diversity and presents a restricted distribution, known only from two basins (Pamisos and Acheloos). Distinct pressures (e.g. dams, artificial riverbed desiccation, river quarrying, urban, agricultural and industrial pollution, and other anthropogenic pressures; Skoulikidis et al., 2009) affect both basins. Finally, and although we could still detect high genetic diversity patterns within *P. semirugata*, its distribution has been declining since most rivers in Southern Turkey have been dramatically affected by urbanization and agriculture, including the desiccation of entire lakes and wetland areas (e.g. Avlan, Söğüt, Karagöl, Hula Lakes; Ereğli and Amik wetlands; Seddon et al., 2014).

4.4. Conclusions and future directions

This study presents for the first time a geographically comprehensive dataset of *Potomida*, including representatives from most of its known distribution in one multilocus phylogenetic study, updating the knowledge on local biodiversity and providing useful data for taxonomy, ecology, and conservation.

Future research should include specimens from potential missing key biogeographical regions (e.g. the Arax river basin, to verify the phylogenetic and taxonomic status of *P. l. komarowi* (O. Boettger, 1880)) or refugia-within-refugia (e.g. sampling in Algeria, to map where the two North African observed haplogroups meet); the use of faster evolving markers to study present connectivity (e.g. Microsatellite markers already developed for *Potomida littoralis*; Froufe et al., 2013); and assess the male mtDNA genetic structuration for a stronger evolutionary signal.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.04.030.

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