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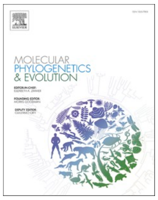

The evolutionary journey of freshwater crabs of the genus *Potamon* (Decapoda: Brachyura: Potamidae)

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ABSTRACT

Aim: Freshwater ecosystems host a rich biodiversity, including freshwater crabs. The family Potamidae is a diverse group of freshwater crabs with a Palearctic and Oriental distribution. Specifically, the genus *Potamon* is found in the Middle East, Southern Europe, and Northern Africa. *Potamon* species are considered true freshwater crabs due to their total independence from the marine environment, which makes them idea organisms to study historical freshwater connectivity dynamics. In this study we aim to elucidate the phylogenetic relationships of the genus *Potamon* and discuss the historical freshwater connectivity in the Mediterranean region.
Methods: Here we use up to eleven genetic markers to reconstruct a robust phylogenetic tree of the group and use Bayesian methods to time-calibrate the tree. We further use historical biogeography methods implemented in a Bayesian framework to assess the ancestral distribution ranges of the group.
Results: In this study we generate the most complete dataset for the group, covering practically the whole distribution range of the genus. We obtained a robust phylogenetic hypothesis and evaluated the times of divergence of the group. The result of the historical biogeography shows the evolution of historical distribution ranges of species of *Potamon*.
Main conclusions.
The Mediterranean Sea is placed in a key intersection for the exchange of fauna and flora. Here we present evidence for the potential of freshwater fauna exchange, through the Mediterranean Sea after a period of desiccation and the loss of contact with the ocean. The origin of *Potamon* genus is found to be in Western Asia, probably Eastern Iran.

1. Introduction

Brachyura (Decapoda) consists of over 7000 described species, of which a surprising one-fifth are freshwater species (Yeo et al. 2008; Tsang et al. 2014; Álvarez et al. 2021). They occur in Afrotropical, Australasia, Nearctic, Neotropical, Oriental and Palearctic biogeographic regions. Most of the genera of freshwater crabs are endemic to a limited region due to their limited dispersal potential (Barry 2001; Yeo et al. 2008). When they do occur, expansion events are generally short distance, local, and limited by several ecological factors. Drylands, deserts, low temperatures, and large bodies of saltwater like oceans are obstacles to the dispersal of most freshwater crabs (Cumberlidge and Ng 2009), limiting these events to rare, documented cases (Daniels 2011; Daniels et al. 2015).

The freshwater Potamidae family, with over 500 species, is found in the Oriental, Palearctic and Afrotropic regions, but its greatest diversity is in the eastern territories (Yeo and Ng 2003; Cumberlidge and Ng 2009). Nearly all the recent studies within the Potamidae focus on the description of new species from Oriental and Eastern Palearctic regions (e.g., Do et al. 2016; Chu et al. 2017, 2018; Huang et al. 2017; Huang 2018; Ng and Mar 2018) and relatively fewer studies focus on the evolutionary history of these species (Shih et al. 2007a, 2007b, 2011; Yeo et al. 2007; Ji et al. 2016; Zhang et al. 2020). The majority of phylogenetic studies on Potamidae focus on genera occurring in Central

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and Eastern Asia. When you consider the genus *Potamon* Savigny, 1816 there is only limited studies on their evolutionary history, with the primary focus being on the western part of their distribution (Jesse et al. 2009, 2011; Parvizi et al. 2018, 2019; Marrone et al. 2020).

The genus *Potamon*, with 22 described species (Horton et al. 2020), is distributed from Nepal in the East to Morocco in the West, passing through the Caucasus and Southern Europe (Cumberlidge and Ng 2009). In Southern Europe, the genus is distributed in Italy and other North-Eastern Mediterranean countries and absent from the Iberian Peninsula. The presence of *Potamon ibericum* (Bieberstein, 1809) in France is probably due to human activity (Nöel and Guinot 2007). In Northern Africa, the genus is distributed in South-Western Mediterranean countries and Northern Atlantic basins of Morocco (our sampling and observation). The westernmost distribution range of the species of genus *Potamon*, is the Mediterranean freshwater basins, in Northern Africa (Marrone et al. 2020). The North-eastern Africa and the South-western Europe is not home to any member of the genus. This pattern of distribution and diversity observed in *Potamon*, and the age of the group, makes it a perfect group to study the biogeography and faunal exchange of North Africa and Southern Europe. The interesting distribution pattern of *Potamon* species in this region, reminds us of the complexity of biogeographical exchanges of the Mediterranean region.

The Mediterranean Sea is surrounded by three continents: Africa, Europe and Asia, making it a major exchange zone for the distribution of fauna and flora. One of the most important events in the history of the Mediterranean Sea for faunal and floral exchange is the Messinian Salinity Crisis (MSC). This event is known for a significant desiccation and salinity crisis (Roveri et al. 2014) of the region which created a land bridge allowing a possible dispersal route for many terrestrial organisms. Relatively little is known about the dispersal of freshwater organisms in this region. The evolutionary history of the genus *Potamon* allow us to bring light on the evolution of freshwater ecosystems, and their faunal and floral exchanges, in the region.

1.1. *The aim of the present work*

Phylogenetic relationships among the species of freshwater crabs of genus *Potamon* have been poorly described. The few studies that focused on this genus have a limited sampling, both in the number of species and the geographical distribution of those (Jesse et al. 2009, 2010; Parvizi et al. 2019; Marrone et al. 2020). In this work, we aim to i) obtain a robust phylogenetic hypothesis for the species of the genus *Potamon*, using different nuclear and mitochondrial genetic markers; ii) obtain a time calibrated phylogenetic hypothesis; and iii) study the historic biogeography of the genus. Based on the obtained results we discuss the systematics and phylogeny of the group, and the freshwater fauna exchange history between Middle East, Southern Europe, the Mediterranean Region, and Northern Africa.

2. Material and methods

2.1. *Taxon sampling*

Our studied area includes 37 localities across seven countries: Albania, Algeria, Bulgaria, Greece, Iran, Morocco, and Tunisia (Fig. 1). Fresh samples were collected from 14 sampling sites between 2009 and 2019, resulting in 49 specimens. In addition, 18 specimens were obtained from the National Museum of Natural Sciences of Madrid (MNCN-CSIC) and 5 from the Netherlands Centre for Biodiversity Naturalis, Leiden (RMNH). This resulted in a total of 72 specimens, see Supplementary Material for full details of individuals included and sampling sites.

In addition, 145 samples with at least two amplified genes in GenBank (Supplementary Material) have been included. These were from Algeria, Bulgaria, Cyprus, France, Greece, Iran, Italy, Malta, Morocco, Tunisia, Turkey, and Turkmenistan (Fig. 1). In total, the final dataset consisted of 17 out of 22 described *Potamon* species. Some of the species not considered in this study were looked for in various field trips without success. We also contacted different museums and collections to include the remaining species available from public institutions but did not obtain DNA-grade material.

Species of other genera of the family Potamidae were included as outgroups (Supplementary Material), based on available phylogenetic studies of the family (Daniels et al. 2006; Shih et al. 2009; Tsang et al. 2014). Two samples identified as *Potamon karpathos* Giavarini, 1934 (C128 and C132) presented inconsistencies and were deleted from the final dataset. The *Potamon potamios* (Oliver, 1804) samples obtained from GenBank are from Turkey. A single specimen (C122) was available from Golan Heights, but it was deleted from the final dataset because of inconsistencies between the data in GenBank and the related publication (Jesse et al. 2011).

2.2. *DNA extraction, amplification and alignment*

All the freshly collected specimens were injected with and preserved in absolute ethanol prior to be deposited in the National Museum of

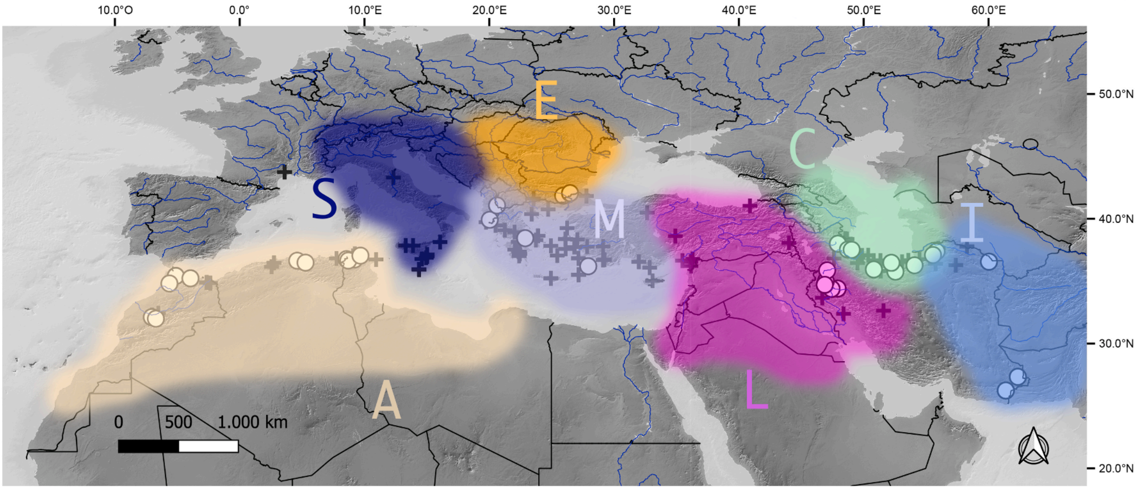

Fig. 1. Sampling sites of the individuals used in this study. Black cross are samples downloaded from GenBank and white circles are samples collected for this study. The colour codes for different biogeographic distributions are the same as in Fig. 4. The letter "A" stands for North Africa, "C" Caspian, "E" South East Europe, "I" South East Iran, "L" Levant, "M" East Mediterranean and "S" South Europe. See Supplementary Material for the geographic coordinates of the sampled sites.

Natural Sciences of Madrid (MNCN-CSIC). For DNA extraction, a ~ 2 mm piece of muscle tissue was removed from the inside of an ambulatory leg. Genomic DNA was isolated using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturers protocol.

A total of 11 genes were targeted for amplification, six of mitochondrial origin (*large subunit ribosomal RNA* (16S)*, small subunit ribosomal RNA* (12S), *NADH dehydrogenase 1* (ND1), *cytochrome c oxidase subunit I* (COI), *cytochrome b* (Cytb), and *Leucine-tRNA* (tRNA-Leu)) and five nuclear markers (*large subunit ribosomal RNA* (28S); *histone three* (H3), phosphopyruvate hydratase (enolase), *sodium–potassium ATPase α-subunit* (NaK), and *phosphoenolpyruvate carboxykinase* (PEPCK)). Each reaction consisted of: 1 µl DNA, 1 µl of each primer, 2.75 µl H_2O and, 6.25 µl of DreamTaq GreenPCR MasterMix, for a total of 35 – 40 cycles, with primer specific annealing temperatures being shown in Table 1. The primers and their annealing temperatures are shown in Table 1. The PCR products were sequenced using an external commercial company (Macrogen, South Korea).

The resulting sequences were trimmed and assembled in Geneious (Geneious® 10.2.6; Biomatters https://www.geneious.com) before aligning with MAFFT as implemented in Geneious. As an additional test to avoid any cross-contamination issue, we constructed simple distance-based neighbour-joining trees for each gene separately and confirmed the correct clustering of the sequences and the phylogenetic position of each cluster in each gene. All the generated sequences for this study have been deposited in GenBank (Supplementary Material).

2.3. Phylogenetic analyses

To estimate the maximum-likelihood (ML) tree, two independent analyses were conducted in IQ-TREE v2.1.2 (Nguyen et al. 2015) and in RAxML v8.2.12 (Stamatakis 2014). To select for the best partitioning scheme and substitution model, we used PartitionFinder2 (Lanfear et al. 2017). The RAxML analysis was performed on CIPRES Science Gateway (Miller et al. 2010), using the result of PartitionFinder2, and implementing a rapid bootstrap (RBS) to corroborate the branch support (Stamatakis et al. 2008). For the IQ-TREE analysis, we used the ModelFinder (Kalyaanamoorthy et al. 2017) with the option MFP + MERGE. The support for each node was evaluated with the SH-like approximate likelihood ratio test (Guindon et al. 2010) and 1000 ultrafast bootstrap (UFBoot2) approximations (Hoang et al. 2018).

Bayesian inference (BI) was carried out in MrBayes v.3.2.2 (Ronquist et al. 2012) as implemented in CIPRES and using the result of PartitionFinder2. Two analyses were run for 10^7 generations simultaneously, each with two parallel runs and four MCMC chains with a sampling frequency of 5000 generations. We reject the first 25 % of generations as burn-in and obtained the 50 % majority rule consensus tree and the posterior probabilities (PP). The convergence of the runs was corroborated using Tracer v1.7.1 (Rambaut et al. 2018).

Table 1
Specific primers used for the amplification of the genes.

Gene	Primers	Annealing Ttmperature (°C)	Fragment size	Reference
12S	12Sai/12Smb	50°	~400 bp	(Kocher et al. 1989)
16S	16Sa/16Sb	50°	~500 bp	(Palumbi et al. 1991)
ND1	NDH5/16L11	48°	~620 bp	(Jesse et al. 2009)
COI	LCO1-1490/ HCO1-2198	42°	~700 bp	(Folmer et al. 1994)
Cytb	R1aScyvF5Scy	51°	~930 bp	This study
28S	28Sa/28Sb – 28L9/28Sb	51°	~730 bp	(Whiting et al. 1997)
Enolase	EA1/ES1	52°	~450 bp	(Tsang et al. 2011)
NaK	Nakforb2/ Nakrev3	51°	~630 bp	(Tsang et al. 2008, 2014)
H3	H3AF/H3AR	47°	~350 bp	(Colgan et al. 1998)
PEPCK	PEPCKfor2/ PEPCKrev3	57°	~670 bp	(Tsang et al. 2008)

2.4. Divergence time analysis

To generate a time-calibrated topology, we used relaxed molecular clocks implemented in the Bayesian software BEAST2 (Bouckaert et al. 2014). The obtained molecular dataset was reduced to include similar number of sequences from each species level cluster identified in the Phylogenetic analyses (Supplementary Material). A separate PartitionFinder2 analysis was performed on the reduced dataset and the data was partitioned correspondingly. The tree priors were linked across all partitions. The clock priors were linked for the mitochondrial genes and unlocked for nuclear genes and a lognormal relaxed clock model (Drummond et al. 2006) were used. Two calibration points were used to infer absolute divergence times: The most recent common ancestor (MRCA) of all *Potamon* and the stem age of *Potamon potamios*. For the first calibration point, the age of the oldest known fossil from this genus, *Potamon quenstedti* (Zittel, 1885) was used as the lower boundary (16.5 Ma) (Klaus and Gross 2010). The upper limit for this point was set to the age of the Potamidae family root (53.87 Ma) (Klaus et al. 2010). A lognormal distribution was used with the M parameter set to 1, the S parameter set to 1.59 and an offset of 16.5. For the second calibration point an uninformative (uniform) distribution was used, with the lower boundary set to the age of the oldest known fossil of *P. potamios* species (0.79 Ma) (Ashkenazi et al. 2005) and the upper boundary set to the MSC (5.96 Ma) age (Krijgsman et al. 1999). The choice of this distribution is a conservative approach allowing for a wide variation range. Two independent analyses of $2x10^7$ generations were performed. Each run was checked for adequate convergence using Tracer, first independently and then together. All parameters' ESS values were confirmed to be higher than 200. The software LogCombiner and TreeAnnotator (both included in the BEAST2 package) were used to merge the tree and log files from the independent runs, and generate a maximum clade credibility (MCC) tree, representing the mean and 95 % HPD interval for all nodal ages. All the trees were visualized using FigTree v1.4.4 software (Rambaut 2016).

2.5. Historical biogeography

The ingroup subtree was extracted from the resulting time-calibrated tree in R using the ape package (Paradis and Schliep 2019). Biogeographic history was inferred using the Dispersal-Extinction-Cladogenesis (DEC) model (Ree et al. 2005; Ree and Smith 2008) implemented in RevBayes (Höhna et al. 2016; Landis et al. 2018). Following Ree and Sanmartín (2018), cladogenetic events were limited to narrow sympatry, peripheral-isolate speciation (subset sympatry) and vicariance (allopatry). We separated the distribution range of the genus into seven biogeographic regions, corresponding to: North Africa (A), Caspian (C), Southeast Europe (E), East Iran (I), Levant (L), Eastern Mediterranean (M) and South Europe (S) as shown in Fig. 1. As the placement of these regions in the last 30 million years has been relatively constant, a unique epoch or time slice was considered for the whole analysis. The dispersal rates between adjacent regions were coded as 1 but the non-adjacent were not given a dispersal probability of zero, but a very low number (10^{-5}) as recommended by the authors of the method in the tutorials. The result of the Biogeography analysis was plotted in R (Tribble et al. 2021).

The alignments, supplementary tables, xml files, scripts and resulting log files, intermediate files and trees are available in supplementary material and our online GitHub repository (https://github.com/Hamidhrg/cangrejitos).

3. Results

3.1. Sampling and sequencing

In total, 73 % of the targeted gene regions were successfully amplified (Table 2). In general, the older museum specimens from the MNCN-CSIC collection (collected between 1932 and 1990) amplified less successfully, likely as a result from either age or fixation method related DNA damage. In particular, some samples were originally fixed in formol (5–10 %) and then changed to alcohol (70–95 %). The final dataset, including the data from GenBank, consisted of 217 samples and had a final overall completeness 40 % (Table 2).

The alignments produced a final dataset of 6537 base pairs (bp): 493 bp corresponding to the *12S* gene, 756 bp to *16S*, 99 bp to *tRNA-Leu*, 452 bp to *ND1*, 923 bp to *COI*, 930 bp to *Cytb*, 763 bp to *28S*, 458 bp to *enolase*, 629 bp to *Nak*, 367 bp to *H3*, and 667 bp to *PEPCK*. Both methods of finding the best partitioning scheme (PartitionFinder and ModelFinder) resulted in similar solution merging the potential 11 partitions into seven (Table 3).

3.2. Molecular phylogenetics and divergence time

The final dataset used for the phylogeny consisted of a concatenated alignment of 11 genes with a total of 6504 bp sites. The phylogenetic analyses resulted in three different trees with very similar well-resolved and robust topologies with well-supported main clades. Therefore, only the tree resulting from the IQ-TREE analysis is shown in Fig. 2, with the support values of the other analysis added. The different methods of evaluating robustness tended to agree in supporting the same nodes. We considered statistically well-supported nodes with ultrafast bootstrap (UFBoot2) values higher than 95 (Hoang et al. 2018), values of SH-aLRT higher or equal to 80 (Guindon et al. 2010), rapid bootstrapping (RBS) values in RAxML higher than 90 (Stamatakis et al. 2008) and PP values of the BI method higher than 0.95. In most cases, the SH-values and UFBoot2 from IQ-TREE performed very similar, with SH-values being slightly higher.

To properly evaluate the monophyly of *Potamon* genus, a bigger dataset with more samples from other genera of the family is needed, especially species belonging to closely related genera, like *Himalayapotamon* for example. The first bifurcation event places populations identified as *Potamon gedrosianum* Alcock, 1909 as the sister species to all the other *Potamon* species. The next branch to diverge, is *Potamon ruttneri* Pretzmann, 1962. Both species are recovered highly supported and monophyletic (Fig. 2).

The rest of the tree divides into two big groups. On one side, the *Potamon hueceste* Pretzmann, 1962 is placed as the sister group to both *Potamon persicum* Pretzmann, 1962, and *Potamon elbursi* Pretzmann, 1976. The support values in this group are unstable, most probably because of the C100 sample which only contains 2 genes (downloaded from GenBank) and "jump" to different positions in different analyses. This sample supposedly corresponds to a *P. persicum* species from Turkey (Jesse et al. 2011).

The other group comprises more than 70 % of the species of the genus studied in this work. Here, the first branching event place the samples identified as *Potamon transcaspicum* Pretzmann, 1962 as the sister group to the rest of the species which form two independent lineages. One clade comprises the North African *Potamon algeriense* Bott, 1967, *Potamon pelops* Jesse, Schubart & Klaus 2010 from Greece, the Southern European *Potamon fluviatile* (Herbst, 1785), and one undescribed species *Potamon aff. algeriense* also from North Africa. All of the mentioned species were monophyletic with high support values. One of our samples identified as *P. algeriense* (the sample C040, collected in 1990) from the MNCN collection, was finally not included in the study as we could only amplify-one gene with an acceptable quality.

Table 3
Partition schemes and evolutionary models recovered in PartitionFinder and ModelFinder.

			Partition Finder	ModelFinder (IQ-Tree)
#	**Gene Markers**	**bp**	**Best Model**	**Best Model**
1	*12S, 16S, tRNA*	1347	GTR + G	TIM + F + I + G4
2	*ND1*	452	HKY + I + G	HKY + F + I + G4
3	*COI, Cytb*	1853	GTR + I + G	TIM2 + F + I + G4
4	*28S*	731	GTR + I + G	TIM3 + F + I + G4
5	*enolase, NaK*	1087	K80 + I + G	TNe + R2
6	*H3*	367	HKY + G	HKY + F + G4
7	*PEPCK*	667	GTR + G	TPM2 + F + G4

The rest of the species are placed in a single clade with generally short internal branches. These are *Potamon setigerum* Rathbun, 1904, *Potamon rhodium* Parisi, 1913, *P. karpathos*, *P. potamios*, and *Potamon hippocrate* Ghigi, 1929 on one side and *Potamon kretaion,* Giavarini, 1934 and *P. ibericum* on the other side. All these species were recovered monophyletic with high support values. Within the genus *Potamon*, in the Aegean archipelago, we do not find insular endemics except *P. kretaion,* Giavarini, 1934 from Crete Island in Greece. The widely distributed *P. ibericum* formed a well-supported sister group to *P. kretaion* clade (Fig. 2).

The result of the divergence time analysis is resumed in the Fig. 3 where the two calibration points are indicated with letters "A" and "B". The crown age for the Potamidae family is estimated to be right at the border of Oligocene and Miocene geologic eras at 23 Mya (31.5–16.7). The first speciation event within the genus *Potamon*, separating *P. gedrosianum* from the rest of the genus, occurred around in Early Miocene, around 18.6 Ma (23. 4–16.5). Most of the speciation events seems to have happened in Pliocene and Pleistocene (Fig. 3).

3.3. Biogeography

The result of the biogeography analysis is shown in the Fig. 4. The MRCA of all *Potamon* species was most probably distributed in East Iran, from where it expanded into the Caspian region. In general, the main colonisation events are range expansions and further extinction in one of the areas. The North Africa seems to be colonized once from East Mediterranean region. The Levant region show a key role in all the major biogeographic exchanges of the genus *Potamon*.

4. Discussion

As seen in Fig. 2, *Potamon gedrosianum* is the sister group to all other species of the genus. The next divergent clade in the phylogeny is *P. ruttneri* which is the sister group to the resting species of *Potamon*. Interestingly these two species, bear very similar morphological characters (Keikhosravi et al. 2016; Farhadi and Harlioglu 2018). All the samples included in this study from these two species are caught in Iran. Whereas *P. ruttneri* has a distribution mainly in north-eastern Iran and north-western Afghanistan, *P. gedrosianum* occurs in Afghanistan, south-

Table 2
Percentage of dataset completeness in the dataset considering only the genetic information obtained in this study, and in the final dataset together with data downloaded from GenBank.

	12S	*16S*	*tRNA-Leu + ND1*	*CO1*	*Cytb*	*28S*	*Enolase*	*H3*	*Nak*	*PEPCK*	Total
This study only	94 %	71 %	99 %	67 %	51 %	90 %	74 %	43 %	67 %	72 %	73 %
Final Dataset	33 %	37 %	83 %	88 %	17 %	43 %	27 %	17 %	25 %	25 %	40 %

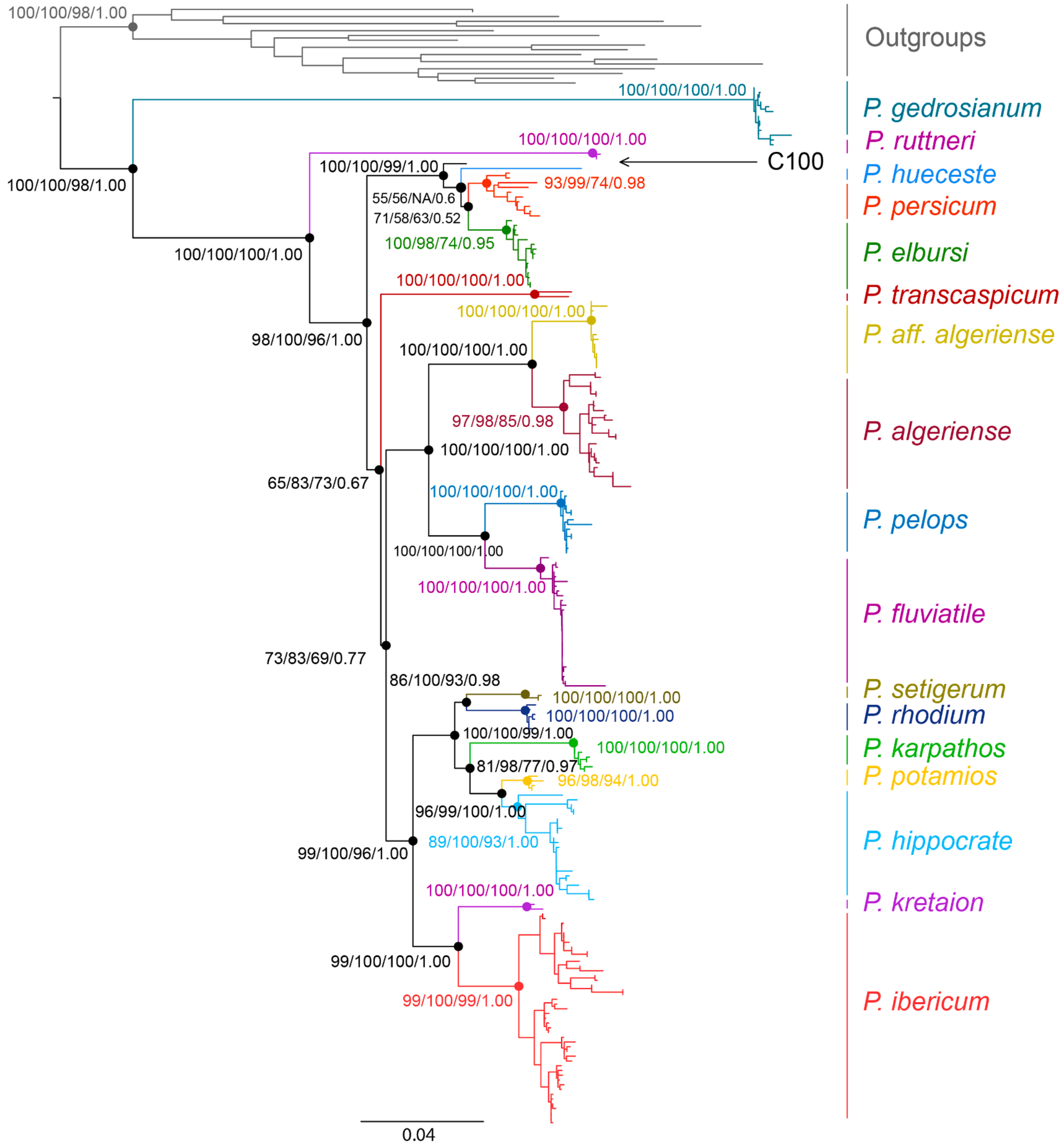

Fig. 2. Consensus tree of the *Potamon* genus as obtained from IQTree. Each colour corresponds to one species. Values at nodes correspond to: UFBoot2/SH-aLRT/RBS/PP. Values of UFBoot2 ≥ 95, SH ≥ 80, RBS ≥ 90, and PP ≥ 0.95 indicate well-supported clades.

eastern Iran, and north-western Pakistan (Cumberlidge 2008a, 2008b; Keikhosravi et al. 2016; Farhadi and Harlioglu 2018). Due to the similarity in the morphological characters of these two species, it is parsimonious to consider that probably the MRCA of all *Potamon* was more like these two species. But to confirm such a conclusion a proper ancestral trait analysis is needed which is out of the scope of this study. Based on the position of these two species, some authors suggested that this family appeared in the far east (Jesse et al. 2011). This hypothesis is strengthened with our historical biogeography results where the root of the tree is recovered in East Iran (Fig. 4).

The group composed by *P. hueceste*, *P. persicum,* and *P. elbursi* formed a well-supported clade. All our samples of *P. persicum* were caught in Iran, but supposedly the species is also distributed in Iraq, Turkey, and Armenia. *P. elbursi* is a native species of the middle and western part of Alborz Mountains, confined to the Caspian Sea and Namak lake and probably only endemic to Iran (Keikhosravi et al. 2015; Farhadi and Harlioglu 2018). These two clades being sister groups is consistent with the results by Keikhosravi and Schubart (2014), where only two genetic markers were used. The land between these two drainage systems (Caspian Sea and Namak basins) most likely does not act as a terrestrial barrier due to their closeness (Keikhosravi and Schubart 2014; Keikhosravi et al. 2015). The speciation event between these two species is calculated to be 2 Mya (3.1–1) and the separation of *P. hueceste* from these two species is 3.3 Mya (5.2–1.5). The MRCA of this clade has most probably been distributed in the Caspian region as recovered in our historical biogeography analysis (Fig. 4).

In this study, we included two samples of *P. transcaspicum*, one from Southern Turkmenistan and one from North-Eastern Iran. The phylogenetic position of this species as the sister group to all mainly western members of this genus is not well supported in our study. This could most probably be due to the small sample size for this species together with very short branch lengths in this part of the tree. Therefore, further studies with more samples from this species are needed to resolve this question. However, based on our results, this species colonised eastward toward East Iran region from an MRCA distributed in Caspian and Levant regions (Fig. 4). Even if a general westward movement is seen in this genus, the biogeographic history remains more complex, and some lineages moved in both directions.

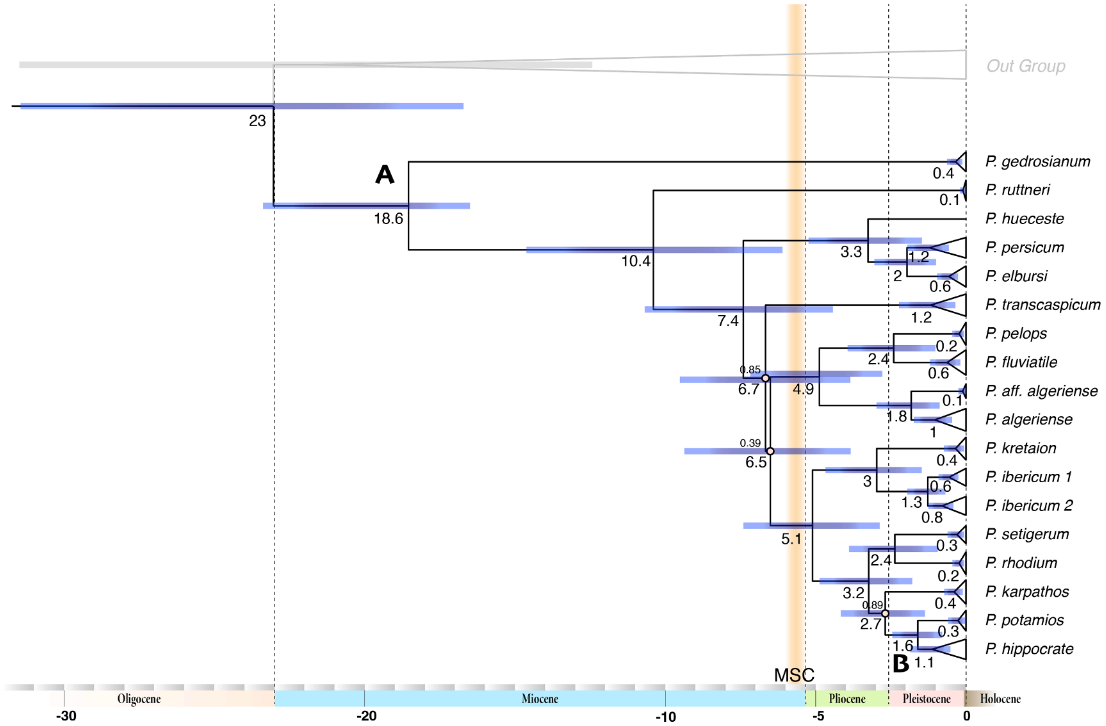

Fig. 3. Time-calibrated tree of the genus *Potamon* with representative species from other genera within the Potamidae family as an outgroup. The number under each node represents the estimated mean age of the node together with the 95 % CI represented using the blue bars. The ages are in million years. The nodes marked with a circle have a PP value < 99. The nodes marked with letters "A" and "B" are the calibration points used in this study. The coloured bar marked with "MSC" delimits the Messinian Salinity Crisis period. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The first ever described freshwater crab, *P. fluviatile*, has a wide and fragmented distribution over different countries and regions forming the north and north-eastern Mediterranean coastline, such as Albania, Greece, Italy, and Balkan countries (Cumberlidge 2008c). This species is placed as the sister clade to the *P. pelops* in our studies (Fig. 2). This result is consistent with the phylogeny published by Marrone et al. (2020). Contrary to *P. fluviatile*, *P. pelops* has a very restricted distribution, it is only found in the Peloponnesus region, a Greek peninsula. Jesse et al. (2009) pointed a Balkans origin (most likely in the south of Greece) of the *P. fluviatile* and then, a plausible expansion to Southern Italy. They discuss the possibility of crossing the Strait of Otranto or migration along the coastal border. A similar distribution occurs in some freshwater fishes such as *Cobitis bilineata* Canestrini, 1865 that inhabits both sides of the Adriatic Sea or *Cottus gobio* Linnaeus, 1758. A connection during the last glacial maximum (LGM), following a marine transgression allowing the exchange of freshwater fish has been claimed to explain this distribution on both sides of the Adriatic (Šlechtová et al. 2004; Perdices et al. 2008). Different scenarios can explain the division of these two sister groups. In Jesse et al. (2010) they considered geographic isolation and then allopatric speciation, due to the formation of the Gulf of Corinth and the paleogeographic isolation of the Peloponnese (3.4–1.8 Mya) (Jesse et al. 2009, 2010). Endemism in the Peloponnesus region, probably occurring due to isolation, has been studied with other organisms such as the cyprinid fishes (Doadrio and Carmona 1998). The timing of the separation between these two species around 2.4 Mya (3.9–1) and their phylogenetic relatedness to the two North African clades seems to support the theory proposed by Jesse et al. (2009). In addition, in our historical biogeography analysis, the MRCA of these two species appear to have been distributed in southeast Europe and east Mediterranean regions both (Fig. 4) which is in line with geographic isolation and allopatric speciation theory.

The two North African clades form a well-supported monophyletic group. The populations identified as *P. algeriense* and its sister group *P. aff. algeriense*, occur in rivers and flows in North Africa that drain into the Mediterranean Sea and Atlantic Ocean. The main distributional difference is that most of our *P. aff. algeriense* samples come from Tunisia, and two of them from Algeria, but not far away from the neighbouring country. We do not have much information about this possible new taxon, which needs a formal taxonomic description. Meanwhile, the distribution of the *P. algeriense* includes Algeria and mostly Morocco. There seems to be an east–west geographical pattern between the two species constituting this North African clade. This result agrees with the disjunction zone identified in the proximities of Kabylia region (Beddek et al. 2018; Marrone et al. 2020). More examples show this pattern of East-West disjunction in the distribution ranges of closely related species. Generally, the species presenting such patterns have low dispersal ability. This can be observed in reptiles (Barata et al. 2008; Perera and James Harris 2010; Metallinou et al. 2015), amphibians (Recuero et al. 2007), scorpions (Sousa et al. 2012), freshwater fishes (Doadrio 1994; Machordom et al. 1998; Touil et al. 2019) and molluscs (Pfenninger et al. 2010). The exact causes of such a historical barrier to the gene flow which caused speciation events in such varied groups are still not well studied. Remarkably, the result of the biogeographic analysis places the MRCA of the African clade and the clade formed by *P. fluviatile* and *P. pelops*, in the east Mediterranean region favouring the colonisation of Africa from east Mediterranean theory over a south European origin.

Currently, there are two hypotheses about the colonization of North Africa by species of potamids. First is the hypothesis of anthropogenic origin for the species of Northern Africa (Marrone et al. 2020). The second hypothesis suggests a natural colonization of North Africa from Europe in the Miocene (Jesse et al. 2009; Marrone et al. 2020). While the first hypothesis can be rejected based on the endemicity and the age of the north African clades, our results seem to (partly) agree with the second hypothesis at least with the origin of the North African clade. The timing of the separation between the *P. fluviatile* and *P. pelops* clade and the two North African clade (4.9 Mya, 7.1–2.7) seems to be more towards after the MSC event and therefore, Pliocene. In the northeast of

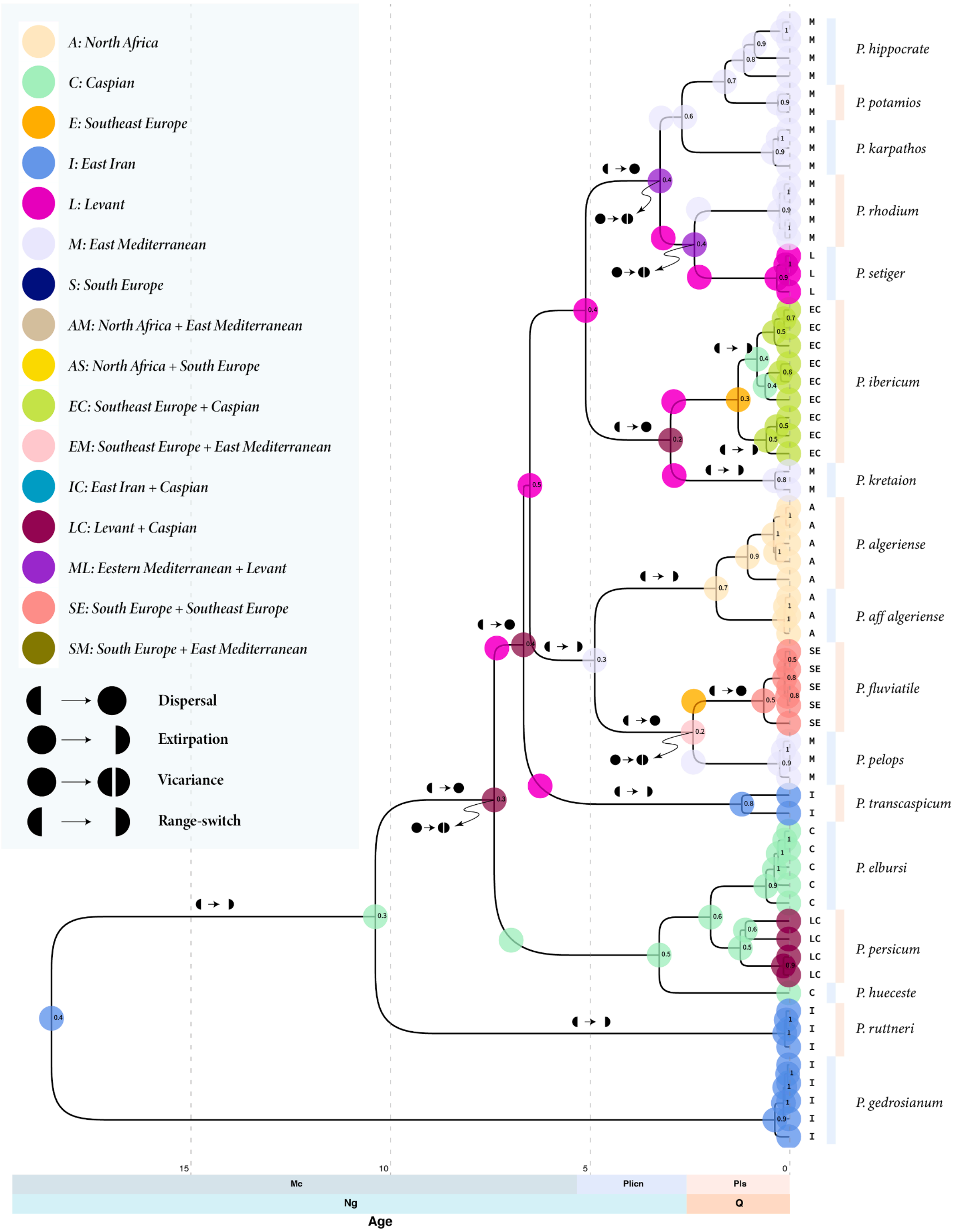

Fig. 4. Biogeography analysis result. The colour code for the single distribution ranges is the same as in Fig. 1. The actual distribution ranges are stated at the tip of the branches. The numbers at each node are the posterior probability of the most probable state. In case of a cladogenetic change at each node, the derived sister ranges are shown. At the bottom of the figure, “Q” stands for Quaternary, “Ng” Neogene, “Pls” Pleistocene, “Plicn” Pliocene and “Mc” Miocene, and the Age is stated in Mya.

the African continent (Libya and Egypt), no freshwater crabs of the genus *Potamon* have been found (Cumberlidge 2008d). Thus, a dispersal from the Middle East to Africa seems unlikely. This might be due to the fact that Nile River’s suitable ecological niches for the *Potamon* freshwater crabs could have been already occupied by a member of the family Potamonautidae, *Potamonautes niloticus* (H. Milne Edwards, 1837). Meanwhile, many species of Southern Europe and the North of Africa are genetically related in other studied taxa. Our results, as well as many other studies, suggest that many dispersal events have happened between the European and African continents through the Mediterranean Sea (Martínez-Solano et al. 2009; García-Alix et al. 2016). We believe this path is the most parsimonious explanation for the origin of the North African potamid crabs. The presence of freshwater organisms in north Africa have been explained by Messinian Salinity Crisis (MSC, 5.97–5.33 Mya) which have had three different stages (Roveri et al. 2014); the two first stages characterized by salinity and desiccation of the Mediterranean Sea (5.97–5.55 Mya) and a third stage (5.55–5.33 Mya) characterized by a low salinity crisis. The end of this last stage is named “Lago Mare” Phase (5.42–5.33 Mya) and has been suggested by some authors as a major freshwater faunal exchange between the North and South of the Mediterranean Sea (Bianco 1990; Durand et al. 2002). Other authors questioned the role of Lago Mare phase as a major dispersion scenario, due to the difference of biodiversity richness between the rivers placed in the north and south of Mediterranean (Levy et al. 2009; Perea et al. 2010, 2016). The general discussion on the effect of Mediterranean desiccation during the MSC on freshwater organisms can be resumed in two predominant models. These two proposed models are a) The isolated Mediterranean, dotted by sabkhas and lakes and, b) The half-full, density-stratified Mediterranean scenarios. Both models have been extensively discussed in Andreetto et al. (2021).

Our data showed connection between North Africa and, south and eastern Europe during MSC. On the other hand, we also show the lack of connection between Iberian Peninsula and Eastern Mediterranean or North Africa. If we consider that presence of *P. fluviatile* in Italy is due to recent dispersion processes (Jesse et al. 2009) we observe a scenario very similar to the one suggested in freshwater fishes. In the case of freshwater ichthyofauna, species of genus *Tropidophoxinellus* from Greece and North Africa are sister groups (Perea et al. 2010). Or for example, *Luciobarbus* species of East Mediterranean, Caspian and Levant are closer to north African species that those of southern Europe (Doadrio et al. 2002; Casal-López and Doadrio 2018). These dispersion events from eastern Mediterranean to North Africa have been interpreted as the existence of intercontinental bridges in the past (Casal-López and Doadrio 2018). The higher dispersion rate of freshwater fauna through Mediterranean Sea in the eastern parts compared to the western parts, could be explained by historically higher rate of freshwater input. This is due to the discharge of freshwater by the Paratethys and north African ancient rivers (Griffin 2002). In addition, Eastern Mediterranean has numerous islands which could develop a land bridge between Southern Europe and North Africa with variations in the sea level. The higher freshwater intake and the numerous islands in the Eastern Mediterranean compared to West, could possibly explain the difference in the dispersion rates of freshwater fauna between them. This theory shows two different evolutionary histories for western and eastern Mediterranean. The estimated timing of the separation between the North African *Potamon* species and the southern European ones in our analysis of 4.9 Mya (7.1–2.7) is slightly younger than the MSC event (Fig. 3).

The Mediterranean Sea has an important archipelago in the modern age, with over 11,000 islands and islets. Most of these islands are in the Aegean region. These islands have had intermittent connection with the mainland, depending on the historical variation of the sea level (Poulakakis et al. 2015). This makes them a perfect natural laboratory to study the impacts of species isolation and island speciation. The Aegean region of the Mediterranean Sea, placed between three continents, Europe, Africa, and Asia, is especially interesting. The rest of the species of *Potamon* in our study are mainly from this region toward the Caucasus Mountains and the Caspian Sea, passing by the Black Sea. The first divergence event within this clade separating *P. ibericum* and *P. kretaion*, from *P. setigerum*, *P. rhodium*, *P. karpathos*, *P. potamios* and *P. hippocrate*, appear to have happened around the end of MSC event, some 5.1 Mya (7.4–2.9). The MRCA of this clade results to have been distributed in Levant region (Fig. 4) from where it colonized westwards to east Mediterranean region and eastwards to the Caspian region. This result support the idea of the eastern Mediterranean being more permeable to freshwater fauna exchanges. Interestingly, *P. ibericum* specimens we have sampled in this study are divided into two subgroups with East-West distribution pattern. It seems like eastern and western population of the species have separated from each other around 1.3 Mya (Fig. 3). Our results show a need for better population level sampling in this species to study the population structure and dispersal history of this wide-spread species.

The species *P. rhodium* is usually found in Rhodes Island, Ikaria, Kos, Samos, but also in Hatay province in Turkey, with *P. setigerum* (Maurakis et al. 2004; Jesse et al. 2011; Harlloğlu et al. 2018). *P. potamios* is distributed in countries that border the Eastern Mediterranean Sea: Greece, Cyprus, Turkey, Syria, Jordan, Palestine, Israel and Lebanon (Cumberlidge 2008e; Jesse et al. 2011; Harlloğlu et al. 2018). Meanwhile, the sister group, *P. hippocrate* has a more limited distribution range: It is found in Turkey (Izmir, Denizli, Aydin), Cyprus, and in the eastern Greece islands like Samos, Naxos, and Cos (Cumberlidge 2008e; Jesse et al. 2011; Harlloğlu et al. 2018). The sister group to *P. potamios* and *P. hippocrate* is formed by populations identified as *P. karpathos*. This species is distributed in Greece and Turkey (Rogers and H. Thorp 2019).

The last clade studied in this work is one formed by populations identified as *P. kretaion* and *P. ibericum*. Each of these species form a well-supported clade. The species *P. kretaion* is only distributed in the Greek island of Crete in the Eastern Mediterranean Sea. The name of *P. ibericum* may imply an occurrence in the Iberian Peninsula but it is not found either in Spain or Portugal. Its name derives from the Iberia kingdom in the Caucasus, on the east coasts of the Black Sea. This species has a wide distribution, in the coastal rivers and lakes near the southern shore of the Caspian Sea (Iran), Black Sea (Bulgaria, Turkey, and Caucasia), and Eastern Mediterranean (Greece and the Northern Aegean region) (Cumberlidge 2008f; Jesse et al. 2011; Harlloğlu et al. 2018; Parvizi et al. 2018, 2019). Interestingly the samples identified as *P. ibericum* in this study clustered in two well distinctive monophyletic clusters with a general East-West distribution pattern. Although this might show two closely related species but specially in widespread species, high degrees of differentiation can be seen specially in the different extremes of their distribution. Therefore, the clear separation in two supported clades might just be an effect of discontinuous sampling effort. Note that two of our samples identified as *P. ibericum* (C136 and C 137) are from France which is more likely due to human introduction (Nöel and Guinot 2007). Although the MRCA of *P. kretaion* and *P. ibericum* has most probably been distributed in the Levant and Caspian regions (Fig. 4), the MRCA of *P. ibericum* was recovered as distributed in the east Europe region from where it colonized the Caspian region, showing again a westward expansion event.

As shown in the phylogenetic tree (Fig. 2), within the samples identified as *P. ibericum*, several well-differentiated groups and subgroups are clustered together. This intraspecific phylogenetic structure suggests many population-level (probably geographical based) structures due to recent alternative expansion and separation events. Especially in the southern shore of the Caspian Sea, where many of our samples come from, the genetic diversity and the phylogenetic clustering, do not correspond to the geographical separation of the different rivers. This pattern of genetic clustering and shared haplotypes in independent rivers is also seen in a freshwater fish species from the same region, *Capoeta razii* Jouladeh-Roudbar, Eagderi, Ghanavi & Doadrio 2017. The high haplotype diversity and this pattern of genetic structure in *C. razii* is explained by the repeated separation and secondary contact of populations of the species. This is due to relatively recent events such as Pleistocene glaciations which raised and lowered the Caspian Sea’s level. These changes in the Sea level separated the river systems in local refugia and connected them into bigger river systems repeatedly (Ghanavi et al. 2016; Jouladeh-Roudbar et al. 2017). The regressions and transgressions of the Caspian Sea coincide with the glacial and interglacial periods, so changes in the range of *P. ibericum* in the southern Caspian Sea could be a consequence of the climatic changes of that period as it is hypothesized in the case of the mentioned *C. razii*. These marine transgressions caused the crab populations to undergo latitudinal range changes (Parvizi et al. 2018). A similar scenario on a larger time scale could explain the complex distribution patterns of the East Mediterranean *Potamon* species in now isolated islands.

In this study, we included 17 species of a total of 22 within the genus *Potamon*. Those that we could not include were *Potamon bileki* Pretzmann, 1971, *Potamon magnum* Pretzmann, 1962, *Potamon ilam* Keikhosravi & Schubart 2014, *Potamon mesopotamicum* Brandis, Storch & Türkay 1998, and *Potamon strouhali* Pretzmann, 1962. In the case of *Potamon bilobatum* Brandis, Storch & Türkay 2000, the genetic data from two specimens were obtained from GenBank which were amplified from the original syntypes of the species (Parvizi et al. 2018). Our phylogenetic results placed them well clustered inside *P. ibericum* clade suggesting a possible synonymy as has already been suggested in other studies (Parvizi et al. 2018; Rezaei Tavabe et al. 2022). All these species are distributed in the Middle East: *P. magnum* is mostly found in eastern Turkey, Northern Syria and Iraq, Armenia, and Western Iran; *P. strouhali* is distributed from the southeast of Iran to the Afghanistan border; *P. mesopotamicum* is found in the border between Syria and Turkey, south of Iraq, and Iran; *P. ilam* is found in the southwest of Iran and *P. bileki* from the south of Turkey (Cumberlidge 2008g, 2008h, 2008i, 2008j, 2008d; Esser and Cumberlidge 2008; Keikhosravi 2013; Farhadi and Harlioglu 2018; Harlloğlu et al. 2018). As mentioned, all the lacking species in this study are from the Middle East region. The freshwater fauna of this region is also one of the most threatened due to many anthropological factors as climate change, water contamination, habitat loss, and water subtraction for human, agricultural and industrial use (Jouladeh-Roudbar et al. 2020). This shows the importance of more studies in the region to develop better conservation policies that could preserve such unique study groups which bring light to important parts of the evolutionary history of organisms on earth which otherwise will get lost forever.

5. Conclusions

Using eleven genes and a wide sampling we obtained the most complete, well-supported and robust phylogeny of the freshwater crab genus *Potamon* to date. The interspecific phylogenetic relationships within the genus are all well resolved. We have corroborated the existence of a new species in North Africa that requires a formal taxonomic description. The Middle East region is an important part of the evolution of this genus which remains relatively poorly studied. Hence, we believe more sampling and more studies are needed in that region.

The historical biogeography of this group shed light on the evolution of freshwater ecosystems in a key region between three different biogeographic realms. This genus is a great example of freshwater faunistic interchange between North Africa and South Europe through the Mediterranean Sea.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Accessibility

All the genetic sequences generated in this study are deposited in GenBank and are publicly available. The data generated in this study, is available in supplementary material and our online GitHub repository (https://github.com/Hamidhrg/cangrejitos/). The physical samples obtained in this study are deposited in the national museum of natural sciences (MNCN-CSIC) of Madrid, Spain, and are available to researchers.

Author Contributions

H.R.G. and I.D. designed the research. H.R.G., I.D., A.J.R. and M.T. carried out the sampling. H.R.G. and P.R. performed the experiments and analysed the data. H.R.G. and P.R. wrote the manuscript with inputs from all other authors. K.R.T., M.T. and A.J.R. helped obtain permits for sampling in Iran. I.D. acquired the funding to perform the study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107690.

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