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Last Interglacial dispersal and genetic diversification, from eastern Mediterranean penultimate glacial refugia, and pre-LGM demographic expansion drive genetic panmixia within the hermit crab *Clibanarius erythropus* (Decapoda: Anomura: Diogenidae) across the East Atlantic and Mediterranean Sea

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Abstract

In this study, we investigate the phylogeography of the hermit crab *Clibanarius erythropus* and reconstruct the evolutionary, biogeographic and demographic histories resulting in the onset of the current patterns of genetic diversity and structure as a continuing effort to unravel and understand the processes underlying the microevolution and genetic diversification within Atlanto-Mediterranean intertidal biota. For this purpose, a total of 149 specimens of the decapod species were collected from fifteen locations, covering almost the entire distribution area, genetically analyzed and compared at the mitochondrial cytochrome oxidase subunit 1 gene. Our results revealed marked macrogeographic genetic homogeneity across the East Atlantic-Mediterranean littoral, with a noticeable lack of phylogeographic structure across postulated biogeographic boundaries within the surveyed geographic spectrum. The recorded pattern of population genetic structure was found to be driven by Last Interglacial (LIG) genetic diversification, coinciding with a dispersal and colonization of the species from putative penultimate glacial refugia in the eastern Mediterranean. Successive demographic expansion of *C. erythropus*, preceding the Last Glacial Maximum (LGM) of the Pleistocene, alongside the detected pattern of regional demographic and spatial expansions, might have played a crucial role in impeding the onset of genetic distinctness. These events could have also been involved in erasing any pre-existing accumulated genetic divergence during the short period since the LIG diversification (resulting from potential founder effects and genetic bottlenecks following colonization episodes). Overall, they might have contributed to the homogenization of gene pool following range expansion, thus intensifying the effect of the recent LIG genetic diversification and leading to the currently observed macrogeographic genetic panmixia in the studied hermit crab species. The resulting lack of phylogeographic structure within *C. erythropus* across its distribution range, reflecting the residual effect of recent evolutionary history, is still likely maintained by the impact of contemporary abiotic and biotic homogenizing processes. These novel insights add evidence to the various and alternative evolutionary strategies causing genetic panmixia within marine biota by unraveling a new genetic diversification process in the studied hermit crab species (interplay between impacts of LIG dispersal from potential eastern Mediterranean refugia and pre-LGM demographic expansion) in comparison to earlier recorded mechanisms in other marine species.

Keywords: Decapoda; mitochondrial DNA; Last Interglacial genetic diversification; dispersal from penultimate glacial refugia; demographic expansion; genetic homogeneity.

Introduction

Identifying the potential factors involved in shaping population genetic structure within marine species is one of the main targets of evolutionary biology. It has been postulated that the combined effect of evolutionary forces acting within populations (i.e., mutation, genetic drift, and selection) or between them (i.e., gene flow driving the mixing of genetic variation among them) determines their genetic structures (Foll & Gaggiotti, 2006). In particular, the intensity of gene flow, regarded as a key contributor to the extent of genetic structuring, may be influenced by the interplay between impacts of abiotic or extrinsic factors (including contemporary [i.e., oceanographic circulation patterns across biogeographic boundaries and habitat discontinuities] and historical [i.e., palaeogeographic and palaeoclimatic shifts] barriers to gene flow; Reuschel *et al.*, 2010; Deli *et al.*, 2016a; 2018a; 2019a; Hui *et al.*, 2016; Catarino *et al.*, 2017; Weiss *et al.*, 2018; Konstantinidis *et al.*, 2022) and biotic or intrinsic features mainly manifested by the life-history traits of the species, such as dispersal potential and reproductive strategies and behaviors (Ma *et al.*, 2018; Shin *et al.*, 2021; Di Crescenzo *et al.*, 2022; Ollé-Vilanova *et al.*, 2022).

Across the north-East Atlantic and Mediterranean Sea littoral, the differential in interaction of species life-history traits with historical and contemporary geological and environmental features of the region has been postulated to shape differently the gene pools and genetic make-up of East Atlantic-Mediterranean marine biota. Such factors give rise to different levels of population genetic connectivity, ranging from panmixia (Lourenço *et al.*, 2017; Deli *et al.*, 2019a) to mild genetic differentiation (Deli *et al.*, 2016b; Fratini *et al.*, 2016; Evangelisti *et al.*, 2017) accentuated, in certain cases, to strong divergence among cryptic evolutionary units that deserve species status (Reuschel *et al.*, 2010; Weiss *et al.*, 2018). Nevertheless, against this background, little is still known about the microevolutionary processes that were initially involved in driving and shaping spatial distribution of population genetic diversity and structure.

The Pleistocene climate and cyclic sea-level fluctuations have been suggested to profoundly affect the coastal environment and influence the evolutionary history of its biota (Avise, 2000). Noteworthy, these historical events might have significantly impacted the distribution and evolutionary dynamics of marine populations following repeated contractions and expansions of their habitats (Hewitt, 1996; 1999), and, therefore, played a crucial role in shaping the contemporary discerned spatial distribution of genetic polymorphism (Hewitt, 2000; Provan & Bennett, 2008). In particular, palaeoclimatic and palaeogeographical shifts, during alternating glacial and interglacial cycles of the Pleistocene, have been shown to affect population dynamics and sculpt contemporary geographic distribution of genetic diversity within species of East Atlantic-Mediterranean distribution (Gysels *et al.*, 2004; Chevolot *et al.*, 2006; Arnaud-Haond *et al.*, 2007; Alberto *et al.*, 2008; Reuschel *et al.*, 2010). Recently, an accumulated body of population genetic investigations has begun to unravel genetic imprints of these historical events and their potential impacts on determining the genetic make-up of Atlantic-Mediterranean marine species (Pérez-Portela *et al.*, 2010; 2017; Deli *et al.*, 2016a; 2018a; 2019a; Pannacciulli *et al.*, 2017; Konstantinidis *et al.*, 2022). Notably, while some investigations unraveled the pattern of genetic divergence driven by long-term persistence in glacial refugia (Tarnowska *et al.*, 2010; Deli *et al.*, 2016a; 2018a; Pannacciulli *et al.*, 2017; Konstantinidis *et al.*, 2022), others highlighted the impact of post-glaciation sea level rise and the associated climate amelioration on erasing short-term accumulated genetic differences in allopatry, resulting in the onset of striking macrogeographic genetic homogeneity following excessive genetic admixture among refugial lineages (Deli *et al.*, 2019a). For both kinds of surveys, the resulting patterns of population genetic structure are likely to be maintained and/or reinforced by the impact of contemporary processes such as the oceanographic circulation and life-history traits (i.e., larval dispersal potential) as well as the ecological requirement of the species.

Despite the bunch of the so far published population genetic studies on marine species across the East Atlantic-Mediterranean littoral, most of these surveys have dealt with assessing levels of gene flow restriction across postulated biogeographic boundaries in the region namely the Gibraltar Strait (Catarino *et al.*, 2017), the Almería-Oran Oceanographic Front (Duran *et al.*, 2004; Reuschel *et al.*, 2010; Sá-Pinto *et al.*, 2012), the Siculo-Tunisian Strait (Deli *et al.*, 2016a; 2017), and the Aegean hydrographic break (Sanna *et al.*, 2013; Pannacciulli *et al.*, 2017; Deli *et al.*, 2018a). Nevertheless, the potential roles played by palaeogeographic and palaeoclimatic shifts, during historical times, in shaping contemporary patterns of genetic polymorphism and levels of population connectivity have been scarcely addressed, leading to a persisting lack of understanding how microevolutionary processes (meditated by glacial-induced divergence and postglacial-induced recolonization) have interacted with contemporary geographic or ecological barriers to gene flow and life history traits of the species resulting in the recorded patterns of population genetic diversity and structure within marine biota. In this context, phylogeographic surveys are considered an appropriate tool and important source of information, not only to delineate patterns of genetic structure and quantify levels of gene flow across biogeographic boundaries but also to reconstruct and understand the potential effect of evolutionary and biogeographic histories in shaping such patterns of phylogeographic structure, particularly within poorly studied intertidal species. Due to the alteration of the shoreline and the reduction of suitable habitats during the Pleistocene glacial periods to refugial zones, intertidal biota are known to be the most affected and vulnerable to palaeoenvironmental shifts (Li *et al.*, 2016).

The hermit crab *Clibanarius erythropus* (Latreille, 1818) represents a good model species to explore these topics. This decapod species has a wide geographical distribution, being common in the Mediterranean and Black seas, and encompassing the eastern Atlantic Ocean from southern Morocco and the Azores to Brittany, the Channel Islands and as far north as the south Cornwall (Zariquiey Alvarez, 1968; Udekem d'Acoz, 1999; González-Gordillo *et al.*, 2001; Patterson *et al.*, 2022). It inhabits intertidal to shallow waters, being found in rock pools and sublittoral waters (Gherardi, 1990). *C. erythropus* develops through four zoea stages and one megalopa (Harms, 1992), suggesting a high dispersal potential within this decapod species. The recent study by Patterson *et al.* (2022) showed that the recent range expansion of *C. erythropus* to the southwest coast of the United Kingdom is likely caused by its long-duration pelagic larvae among other factors. Besides, the species was found to form relatively large aggregations (Gherardi & Benvenuto, 2001). All of these aforementioned eco-biological characteristics may trigger high genetic connectivity among populations. Nevertheless, various population genetics studies focusing on other marine decapod species with quite similar life-history traits, as found within *C. erythropus*, revealed patterns of genetic differentiation and phylogeographic structure across the East-Atlantic Mediterranean littoral (Reuschel *et al.*, 2010; Fratini *et al.*, 2016; Weiss *et al.*, 2018).

In light of these insights, and provided the lack of information on the phylogeography and population genetic structure of *Clibanarius erythropus* across its distribution range, we addressed the following questions. First, are populations of the hermit crab *C. erythropus* genetically structured along their distribution area? In other words, is gene flow restricted among populations of this decapod species across the main postulated biogeographic boundaries spanning the East Atlantic and the Mediterranean Sea (i.e., the Gibraltar Strait, the Almería-Oran Oceanographic Front, the Siculo-Tunisian Strait, and the Peloponnese Hydrographic Break)? Second, how did the alternating range fragmentation and recolonization processes, during the Quaternary glacial and interglacial cycles, respectively, influence the onset of contemporary spatial distribution of genetic diversity and structure within *C. erythropus*? In this context, what potential microevolutionary processes were likely responsible for initially driving and shaping the population genetic structure and variability as currently recorded in this hermit crab species across its distribution range?

In an attempt to answer all these raised questions, we reconstructed the evolutionary, biogeographic and demographic histories of *Clibanarius erythropus* based on the mitochondrial cytochrome c oxidase subunit 1 (Cox1 gene). Mitochondrial DNA has been considered the appropriate molecular marker for reconstructing species evolutionary histories owing to its smaller effective population size, in comparison to nuclear loci markers (Avise, 1994). For instance, mtDNA-based phylogeographies have been continuously clarifying the impact of the Quaternary climate oscillations on the distribution dynamics and the build-up of genetic divergence within marine biota (Deli *et al.*, 2018a; Zhong *et al.*, 2020). In particular, the assessment of Cox1 gene variation in evolutionary and demographic histories has allowed unveiling the potential impacts of Pleistocene glacial and interglacial episodes on forging contemporary patterns and levels of population genetic diversity and structure of marine species (Ibáñez & Poulin, 2014; Barahona *et al.*, 2017; Zelada-Mázmela *et al.*, 2022). Samples of *C. erythropus* were obtained from locations nearly covering its entire distribution range, stretching from the northern East Atlantic coast to the eastern Mediterranean basin. For the newly captured hermit crabs, the mitochondrial Cox1 gene was amplified and sequenced. Noteworthy, the amplified Cox1 fragment, in this study, has already enabled the detection of significant patterns of genetic divergence in previous population genetic surveys of marine decapods (Reuschel *et al.*, 2010; Deli *et al.*, 2018a; b; Weiss *et al.*, 2018). In order to recover the maximum amount of information contained in the relatively small portion of analyzed mtDNA genome, several analytical approaches were implemented at different hierarchical levels, including parsimony and Bayesian inferences of mitochondrial genealogy, assessment and comparison of regional genetic diversification, analysis of molecular variance, calibrated phylogeny, ancestral area reconstruction, inference of genetic diversification by means of approximate Bayesian computation, and demographic history.

Materials and Methods

Hermit crab sampling and genomic DNA isolation

A total of 149 specimens of *Clibanarius erythropus* were collected from 15 sampling sites between 2008 and 2015, covering the whole distribution area of the hermit crab species and stretching from the north-East Atlantic to the eastern Mediterranean basin (Fig. 1, Table 1). Details on the surveyed locations and geographic regions, as well as on the number of captured individuals per sampling locality, are highlighted in Figure 1 and Table 1. In the field, captured specimens were frozen and afterwards preserved in absolute ethanol until genetic analysis. In the laboratory, total genomic DNA was extracted from pereiopod muscle tissue by means of the Puregene kit (Gentra Systems: Minneapolis, MN55447, USA).

Amplification and sequencing of the mitochondrial Cox1 gene

The mitochondrial Cox1 gene (up to 1250 basepairs (bp)) was amplified from the extracted DNAs of *Clibanarius erythropus* using the specifically designed decapod primers COL1b (or COL6a for some samples) and COH16 (Schubart, 2009). PCR reactions were run in a total volume of 25 µL including: ddH$_2$O, dNTPs (0.12 mM each), PCR buffer (1×), MgCl$_2$ (4 mM), both primers (0.24 µM each), Taq polymerase (0.5 U; MBI Fermentas, St. Leon-Rot, Germany) and diluted genomic DNA. The temperature profile of the PCR consisted of an initial denaturation phase of 4 min at 94°C, followed by 40 cycles, each composed of 45 s of denaturation at 94°C, 45 s of annealing at 52°C and 75 s of extension at 72 °C. These cycles were followed by 10 min of final extension at 72 °C. Visualization of PCR products (loaded on 1.5% agarose gel with GelRed staining) was carried out under UV light. The obtained PCR products were outsourced for Sanger sequencing with the forward primer COL1b (or COL6a) to LGC Genomics or EZ-Sequencing of Macrogen Europe. Inspection and editing of the obtained sequences were conducted with Chromas Lite 2.1.1 (Technelysium, 2012). Alignment of the generated Cox1 sequences was carried out with Clustal W, included in BIOEDIT (Hall, 1999).

Statistical analyses

Data analysis procedure

Given the marked difference in the obtained sequences length, we were able to define two kinds of dataset

Fig. 1: Sampling locations of *Clibanarius erythropus* along the northeast Atlantic and Mediterranean Sea. The main phylogeographic breaks are exhibited as dotted lines. G S: Gibraltar Strait; AOF: Almería-Oran Oceanographic Front; S-T S: Siculo-Tunisian Strait; PHB: Peloponnese Hydrographic Break. Construction of the base map was carried out by the software DIVA-GIS 7.5.0 (http://www.diva-gis.org).

Table 1. Sampling information on East Atlantic and Mediterranean specimens of *Clibanarius erythropus* including collection sites, countries, regions, and geographic coordinates. N: the examined number of specimens for each location.

Collection site	Country	Region	Geographic coordinates	N
Devon: Wembury: Church reef	England	East Atlantic	50.3155° N, -4.082861° W	1
Vila Nova de Milfontes: Estuary Rio Mira	Portugal	East Atlantic	37.7223611° N, -8.7822222° W	5
Praia da Luz: Rocha Negra	Portugal	East Atlantic	37.085° N, -8.719027° W	6
Cádiz: Corrales de Rota	Spain	East Atlantic	36.6313889° N, -6.3927777° W	10
Cádiz: La Caleta	Spain	East Atlantic	36.5286111° N, -6.3077777° W	13
Alicante: Moraira: El Portet	Spain	western Mediterranean	38.6863889° N, 0.1475° E	17
Ibiza: Cala Olivera	Spain	western Mediterranean	38.93375° N, 1.502638° E	10
Girona: L’Escala (eulitoral rock rubble)	Spain	western Mediterranean	42.1169444° N, 3.1369444° E	10
Elba: Capoliveri	Italy	western Mediterranean	42.7395833° N, 10.3669166° E	13
Sicilia: Messina: Torre Faro	Italy	western Mediterranean	38.2652778° N, 15.6488888° E	10
Istra: Pula: Valsaline	Croatia	eastern Mediterranean	44.8502778° N, 13.8336111° E	10
Igoumenitsa: Kalami	Greece	eastern Mediterranean	39.473° N, 20.23946° E	10
Chalkidiki: Kassandra	Greece	eastern Mediterranean	40.1216° N, 23.417783° E	10
Çeşme	Türkiye	eastern Mediterranean	38.3286111° N, 26.2956944° E	6
Girne	Cyprus	eastern Mediterranean	35.3426167° N, 33.3010833° E	18

Table 2. Genetic diversity and demographic estimates for the examined hermit crab *Clibanarius erythropus* populations, based on the analysis of 700 base pairs of the mitochondrial Cox1 gene. Values reported for each population as well as for each defined regional dataset (EA-WM: East Atlantic and western Mediterranean; EM: eastern Mediterranean) are: sample size (N), number of polymorphic sites (Nps), number of haplotypes (Nh), haplotype diversity (h), nucleotide diversity (π), mean number of nucleotide differences (K), Tajima's D test, Fu's F_S test and Ramos-Onsins and Rozas's R_2 test. Significant values of the analyzed neutrality tests (D, F_S, and R_2) are indicated in bold.

Population (Region)	N	N**ps**	N**h**	h	π	**K**	D	F_S	R_2
Cádiz (EA)	15	9	5	0.476	0.0017	1.200	**-2.124**	-1.066	0.127
Alicante (WM)	17	11	8	0.728	0.0020	1.397	**-2.117**	**-4.065**	**0.083**
Ibiza (WM)	10	2	3	0.378	0.0006	0.400	-1.400	**-1.163**	0.200
Girona (WM)	10	4	4	0.533	0.0011	0.800	**-1.667**	**-1.344**	0.165
Elba (WM)	13	4	5	0.538	0.0009	0.615	**-1.774**	**-3.059**	**0.115**
Messina (WM)	10	8	6	0.778	0.0023	1.600	**-1.873**	**-2.441**	**0.122**
EA-WM	75	33	24	0.575	0.0015	1.036	**-2.690**	**-29.496**	**0.023**
Pula (EM)	10	9	9	0.978	0.0028	1.956	**-1.687**	**-7.526**	**0.069**
Igoumenitsa (EM)	10	2	3	0.378	0.0006	0.400	-1.400	**-1.163**	0.200
Chalkidiki (EM)	10	3	3	0.378	0.0009	0.600	-1.562	-0.458	0.213
Çeşme (EM)	6	6	5	0.933	0.0029	2.000	-1.367	**-2.111**	**0.136**
Girne (EM)	18	17	12	0.863	0.0027	1.889	**-2.373**	**-8.998**	**0.057**
EM	54	31	27	0.754	0.0020	1.396	**-2.623**	**-28.139**	**0.022**
Total	129	51	47	0.654	0.0017	1.186	**-2.708**	**-29.034**	**0.012**

The population of Cádiz was defined by the combination of specimens of the hermit crab species *Clibanarius erythropus* from the two locations of Corrales de Rota and La Caleta.

alignments. The first alignment resulted in a cropped Cox1 fragment of 316 basepairs (bp) for all 149 generated sequences (Tables 1 and S1, Fig. S1). The second alignment, however, allowed for a longer adjusted Cox1 fragment of 700 bp for an overall dataset of 129 sequences (Table 2). Both Cox1 datasets included almost similar population sequence contents: Alicante (17), Ibiza (10), Girona (10), Elba (13), Messina (10), Pula (10), Igoumenitsa (10), Chalkidiki (10), Çeşme (6), and Girne (18) (Tables S1 and 2). The only difference, residing among the longer and shorter Cox1 datasets, was linked to the East Atlantic specimens from Wembury (1), Portugal (11) and Cádiz (8), for which the generated Cox1 sequences were not long enough to be included in the longer Cox1 alignment. In order to find out which alignment data is suitable and reliable to examine the population genetic structure and reconstruct the evolutionary, biogeographic and demographic histories, we carried out preliminary statistical analyses focusing mainly on genetic diversity, haplotype genealogy and genetic differentiation. The obtained finding (see results) showed that the longer Cox1 sequences consisted of a higher genetic diversity than shorter ones. Besides, the shorter Cox1 dataset showed a lack of population genetic subdivision, while a trend of weak but significant genetic differentiation was discerned within the longer Cox1 dataset (see results). Accordingly, the latter dataset was used to carry out all required statistical analyses for this study, while only referring to the outcomes of genetic diversity, Cox1 genealogy and genetic differentiation corresponding to the shorter alignment dataset in the text for comparison purposes. It has to be mentioned that, in order to increase the sample size per site, Cox1 sequences from geographically proximate sites of Vila Nova de Milfontes and Praia da Luz were pooled together and combined into the newly defined population of Portugal (Table S1). The same criterion was applied for hermit crabs captured in Corrales de Rota and La Caleta. The corresponding sequences were combined into the newly labeled population of Cádiz (Tables S1 and 2). Furthermore, the obtained single Cox1 sequence from Wembury was only used for haplotype genealogy reconstruction of the shorter mitochondrial dataset, and was not included in population genetic diversity and differentiation analyses.

Analyses of sequence diversity and population genetic variability and assessment of regional genetic diversification

Sequence diversity measurements, in terms of the number of variable (parsimony-informative and autapomorphic) nucleotide sites, based on the analysis and comparison of 700 bp of the 129 Cox1 sequences of *Clibanarius erythropus*, were determined with the software MEGA version X (Kumar *et al.*, 2018).

Genetic variability was estimated for each population as well as for the total dataset by means of number of haplotypes (*N*h), number of polymorphic sites (*N*ps), haplotype (*h*) and nucleotide (π) diversities (Tajima, 1983; Nei, 1987), and mean number of nucleotide differences (K). These latter measurements were calculated for both shorter (316 bp) and longer (700 bp) Cox1 alignment datasets with the software DnaSP version 5.10 (Librado & Rozas, 2009). Additional outcomes of these genetic diversity estimates were also reported for two defined regions, East Atlantic-western Mediterranean and eastern Mediterranean, based on the analysis of 700 bp of the mitochondrial Cox1 gene.

Based on the detected pattern of spatial variation in genetic diversity, for the two defined regions, inferred from the analysis of the longer Cox1 alignment dataset (see results), detailed pairwise comparisons of regional genetic diversification were further conducted and assessed among samples from three regions corresponding to the East Atlantic, western Mediterranean and eastern Mediterranean. Six genetic diversity indices (haplotype diversity (*h*), nucleotide diversity (π), mean number of nucleotide differences (K), haplotypic richness (*Hap*r), number of private haplotypes (*N*p), and genetic endemism (*N*p/*Hap*r)) were used for the analyses. Due to the noticeable difference among the sample sizes of the examined regions, a rarefaction procedure was applied in order to discard the possible bias of sample size on inferred diversity. For each sample, a subsample of 15 individuals (corresponding to the size of the smallest dataset of the East Atlantic region) was drawn randomly, and diversity indices were calculated. In order to infer reliable statistics, the procedure was repeated at least 50 times for each sample, and then the mean subsample diversity for each sample was calculated. The unpaired *t*-test, included in the online software T-Tests-Free Statistics and Forecasting Software (Calculators) version 1.2.1 (Holliday, 2017), was used to assess inter-regional difference for each genetic diversity parameter.

Assessment of evolutionary relationships among the recorded Cox1 haplotypes and Bayesian inference of mitochondrial genealogy

Genealogical patterns within the two mitochondrial datasets (based on the two retrieved alignments of 316 bp and 700 bp of the Cox1 gene) of *Clibanarius erythropus* were assessed with the software TCS version 1.21 (Clement *et al.*, 2000). A construction of a haplotype network, by means of the statistical parsimony procedure under the 95 % probability criterion for a parsimonious connection (Hudson, 1990; Templeton *et al.*, 1992), included in TCS, allowed inferring evolutionary relationships among the detected Cox1 haplotypes for each kind of examined dataset.

The Bayesian clustering approach, included in BAPS version 6.0 (Corander *et al.*, 2013), was used to identify potential genetic clusters within the longer and more variable Cox1 dataset of *Clibanarius erythropus*, based on the analysis of 700 bp of the surveyed mitochondrial marker. The Bayesian approach, adopted by BAPS, is characterized by a stochastic optimization algorithm for analyzing models of population structure, which greatly improves the speed of the analysis compared to traditional MCMC-based algorithms (Corander & Marttinen, 2006). This method determines the most likely number of clusters (*K*) within a given set of sequences, by clustering the recorded haplotypes into monophyletic clusters (haplogroups). The only given prior information was the geographic origin of the analyzed Cox1 sequences. The "Clustering with linked loci" function was selected for the BAPS analysis. The upper bound *K* values (i.e., the number of clusters) were set to 14. Five replicates were run for each *K* value (1-14). The likely number of genetic clusters, defined in the examined mitochondrial dataset, was selected by the software according to the log marginal likelihood of optimal partition and the maximum-associated probability (P) of the number of groups in optimal partition.

Quantification of genetic differentiation and assessment of phylogeographic structure

The overall population genetic differentiation, within both shorter and longer Cox1 sequences datasets (based on the analyses of 316 and 700 bp respectively), was assessed by one-level AMOVA (analysis of molecular variance) (Excoffier *et al.*, 1992), using the software ARLEQUIN version 3.1 (Excoffier *et al.*, 2007). Based on the different trends of genetic subdivision, recorded in the two Cox1 datasets, genetic distances among pairs of populations of *Clibanarius erythropus* were only computed for the longer Cox1 dataset by means of ARLEQUIN version 3.1. Measurements corresponding to these two kinds of analyses were inferred from both nucleotide divergence (based on the Tamura distance, corresponding to the T92 substitution model (Tamura, 1992) already determined as the best-fitting model for the analyzed dataset by the software MEGA X, see below for further details) and haplotypic frequencies. Significant genetic distances ($P < 0.05$) were assessed by a randomization procedure with 10,000 permutations. In order to test the hypothesis that isolation by distance (IBD) could be the potential driver of the retrieved weak but significant genetic differentiation within the longer Cox1 dataset (see results), the correlation between genetic and geographic distances was assessed by means of the Mantel test (Mantel, 1967), as implemented in ARLEQUIN version 3.1. A total of 10,000 random permutations were run in order to assess the statistical significance of the test.

Phylogeographic patterning within the longer Cox1 dataset (based on the analysis of 700 bp) was assessed and depicted through comparison of the two levels of population differentiation, G_{ST} (based solely on haplotype frequencies) and N_{ST} (considering the genetic relationship among haplotypes). These measurements were determined and compared in the software PERMUT & CPSRR version 2.0 (Pons & Petit, 1996), according to the methods described by Pons & Petit (1995; 1996). A significantly higher value of N_{ST} than that of G_{ST} usually refers to the occurrence of spatial population genetic subdivision and significant phylogeographic structure within the examined dataset (Pons & Petit, 1996; Petit *et al.*, 2005). The overall genetic diversity (H_T) and the average diversity within populations (H_S) were also estimated with the program PERMUT and compared.

Structured AMOVA (as implemented in ARLEQUIN) was also used to test hierarchical structuring of genetic variation (among populations, among populations within groups, and among groups) within the defined longer Cox1 dataset of *Clibanarius erythropus*, seeking for a potential occurrence of significant phylogeographic structure across postulated barriers to gene flow, such as the Gibraltar Strait, the Almería-Oran Oceanographic Front, the Siculo-Tunisian Strait, and the Peloponnese Hydrographic Break. For all tested biogeographic hypotheses across the aforementioned boundaries, population genetic structure was determined and statistically assessed based on both Tamura distance and haplotype frequency.

Reconstruction of evolutionary and biogeographic histories

In order to unravel the origin of the recorded pattern of genetic structure within *Clibanarius erythropus*, we intended to reconstruct its evolutionary and biogeographic histories. As such, the tempo and mode of genetic diversification within the studied decapod species could be comprehensively inferred.

In an attempt to reliably reconstruct the evolutionary history of the species, appropriate calibrations or substitution rates need to be provided and applied. For this purpose, and aiming at avoiding the overestimation of diversification times when implementing unspecific published mutation rates related to other decapod genera or families, we determined a mutation rate for the Cox1 mitochondrial gene in a species-level phylogenetic analysis, involving the examined dataset of Cox1 sequences (149) recovered in *Clibanarius erythropus* (with Atlanto-Mediterranean distribution) and three generated Cox1 sequences of *C. aquabilis* (being strictly confined to the East Atlantic: collected from the Canary Islands and Portugal). The comparison of these sequences resulted in a final cropped alignment of 406 bp defined by 55 sequences (with 52 sequences corresponding to *C. erythropus* and 3 sequences representing *C. aquabilis*). Based on the recorded deep mitochondrial split between these two species (as inferred from the high divergence rate of 14.04 % in Cox1 gene; 57 mutations were found to separate both species lineages according to the constructed TCS haplotype genealogy based on the analysis of 406 bp; data not shown) and their particular geographic distribution, we assumed that deep genetic divergence and speciation among the two hermit crab species could have taken root to the Messinian Salinity Crisis (MSC), following the biotic interruption between the Mediterranean Sea and the Atlantic Ocean. Therefore, the closure of the Gibraltar Strait at the onset of the MSC (5.59 million years ago; Krijgsman *et al.*, 1999) was used as a calibration point for mutation rate estimation within *Clibanarius* sp. It needs to be mentioned that even though the geographic distribution of *C. erythropus* is not entirely Mediterranean, encompassing the East Atlantic and Mediterranean littorals, the highlighted assumption is likely possible provided that marine species with similar distribution (as *C. erythropus*) were found to originate in the Mediterranean and then colonize the East Atlantic coast at historical times (Gysels *et al.*, 2004; Riesgo *et al.*, 2019). Besides, the contemporary geographic distribution of *C. aquabilis* (Madeira and the Canary and Cape Verde islands; Udekem d'Acoz, 1999; González-Gordillo *et al.*, 2001; Bartilotti *et al.*, 2008) covers what was regarded as refugial zones (Ibero-Moroccan and West African areas) for East Atlantic marine populations during the MSC (Klausewitz, 1973; Por, 2009). Accordingly, all of these insights likely ensure that implementing this biogeographic calibration may not lead to erroneous estimation of the mutation rate of Cox1 gene within *Clibanarius* sp. The software BEAST version 1.7.5 (Drummond *et al.*, 2012) was used to determine the Cox1 substitution rate. Prior to the analysis, we determined the optimal nucleotide substitution model for the examined dataset with the software MEGA X (Kumar *et al.*, 2018) using the Find best DNA models (ML) option in MEGA X (Nei & Kumar, 2000; Tamura *et al.*, 2011). The retrieved T92+G model was found to fit well the analyzed dataset (exhibiting the lowest Bayesian Information Criterion (BIC) score, according to the outcome of Maximum Likelihood fits of 24 different nucleotide substitution models tested by MEGA X). Because the BEAST program does not incorporate the T92 model, the second most optimal BIC model (specified as the HKY+G model, and also considered as equivalent to the T92+G model) was applied in the analysis. We used the two parameter Birth-Death model, regarded as an appropriate tree prior for species diversification (Nee *et al.*, 1994), and implemented an uncorrelated lognormal relaxed clock. A lognormal prior was specified for the root age of the monophyletic *Clibanarius* clade (5.59; alpha = 0.055; Marino *et al.*, 2011). The use of a normal distribution prior, for modeling uncertainty on the divergence time, is considered a useful calibration prior when applying a biogeographical date. A total run of 30 million generations were specified for the Markov Chain Monte Carlo (MCMC) simulations. The software TRACER version 1.5 (Rambaut & Drummond, 2009) was used to check the convergence of the runs (Effective Sample Sizes, ESS, of all parameters > 200) and exhibit the result corresponding to the mean mutation rate (and its 95% high posterior density interval (HPD)).

The estimation of genetic diversification time within *Clibanarius erythropus* haplotype dataset (based on the analysis of 700 bp of the Cox1 gene) was determined with the software BEAST version 1.7.5. Provided that the T92 model was identified as the most appropriate model of examined haplotype sequence data evolution by MEGA X, we implemented the second best fitting HKY model according to the outcome of MEGA analysis. The latter substitution model is considered as equivalent to the T92 model. The Bayesian Skyline coalescent model, known to fit a wide range of demographic scenarios while considering phylogenetic uncertainty (Drummond *et al.*, 2005), was applied as a coalescent tree prior, alongside a strict molecular clock model, regarded as the appropriate model for intraspecific datasets. The determined Cox1 gene mutation rate for *Clibanarius* was used to calibrate the haplotype phylogeny and to estimate the time to the most recent common ancestor (tMRCA) of the examined Cox1 dataset. MCMC simulations were run for 50 million generations (sampled every 1000 generations). The generated outputs of the Bayesian analyses were checked for convergence in TRACER version 1.5. After summarizing the resulting trees in TreeAnnotator version 1.7.5 (Drummond *et al.*, 2012), using maximum clade credibility, the obtained calibrated Cox1 haplotype genealogy was visualized and edited in FigTree version 1.4.0 (Rambaut, 2012).

The historical biogeography of *Clibanarius erythropus* in the East Atlantic and Mediterranean Sea was inferred from the reconstruction of ancestral geographic distributions of Cox1 haplotypes (based on the analysis of 700 bp of the examined mitochondrial marker), using the software RASP version 3.2 (Yu *et al.*, 2015). The mode of genetic diversification within *C. erythropus* (whether arising via dispersal or vicariance events) was determined by the S-DIVA (Statistical Dispersal-Vicariance Analysis) method (Ronquist, 1997), as implemented in RASP. For a comprehensive inference of the historical biogeography of *C. erythropus* across its distribution area, three biogeographic regions were defined according to the extant geographic distribution of Cox1 haplotypes in the East Atlantic and Mediterranean Sea. These delineated areas correspond to the East Atlantic, the western Mediterranean and the eastern Mediterranean. In order to conduct the S-DIVA analysis, the MCMC-based condensed tree exported from TreeAnnotator (included in BEAST) was loaded as ‘trees file’; while the distribution of the recorded Cox1 haplotypes through all defined biogeographical areas was loaded as ‘distribution file’. The function ‘Topology only’, included in RASP, was used to infer a Cox1 haplotype phylogeny. The likelihood of possible ancestral range at each generated node of the phylogeny was determined by means of the Most Likely States (MLS) function, as implemented in RASP.

Microevolutionary process of regional genetic diversification within Clibanarius erythropus across the East Atlantic-Mediterranean littoral

In order to unravel the microevolutionary process of genetic diversification within *Clibanarius erythropus* across its distribution range, we used the approximate Bayesian computation (ABC) method, implemented in the software DIYABC version 2.0.1 (Cornuet *et al.*, 2014). Based on the outcomes of RASP analysis and regional patterns of genetic diversity (see results), we assumed that gradual diversification, as mainly reflected by the analysis of regional genetic variability, could stem from historical dispersal and colonization from potential eastern Mediterranean penultimate glacial refugia, with the East Atlantic being recently colonized. In order to assess the likelihood of this assumption, four genetic diversification scenarios were tested. The first two scenarios (1 and 2) assumed dispersal origin from the eastern Mediterranean, with the East Atlantic being later colonized from either the eastern Mediterranean (scenario 1) or from the western Mediterranean (scenario 2). The remaining scenarios (3 and 4) propose an inverse scheme of diversification whereby the genetic makeup of *C. erythropus* was shaped by historical dispersal from the western Mediterranean. A set of summary statistics, describing genetic diversity and differentiation, was used to determine the most similar simulated dataset to the observed dataset, allowing an estimation of posterior distributions of the parameters of interest. The implemented summary statistics for each defined population included the following parameters: the number of haplotypes, the number of segregating sites, the mean of pairwise differences, and the number of private segregating sites. For summarizing statistics of population pairs, the number of haplotypes, the number of segregating sites, the mean of pairwise differences (W) and (B), and F_{ST} were used. A total of four million simulations (one million datasets for each scenario) were run for the ABC analysis. The posterior probabilities, corresponding to the four tested scenarios, were determined using the logistic regression method, as implemented in DIYABC. The most likely evolutionary scenario was identified as the one with the highest posterior probability (Beaumont, 2010).

Demographic history reconstruction

The demographic history of *Clibanarius erythropus* was comprehensively reconstructed for each examined population, each identified regional group (East Atlantic-western Mediterranean and eastern Mediterranean, as already considered for the regional genetic diversity estimates), as well as for the total mitochondrial dataset, using various approaches, based on the analysis of 700 bp of the mitochondrial Cox1 gene.

Within each kind of dataset, imprints of historical demographic changes were unveiled, through the assessment of departure from mutation-drift equilibrium, via the analysis of three neutrality tests (Tajima’s D (Tajima, 1989), Fu’s Fs (Fu, 1997), and Ramos-Onsins and Rozas’s R_2 (Ramos-Onsins & Rozas, 2002)). D and Fs statistics were determined with ARLEQUIN, while the R_2 index was calculated with DnaSP. A total of 1000 coalescent simulations were used to estimate these statistics as well as their level of significance. Significantly negative outputs of D and Fs, along with a significant value of the R_2 index, are indicators of a significant trend of demographic expansion.

Evidences of demographic and spatial expansion events were further assessed within the two identified regional groups (East Atlantic-western Mediterranean and eastern Mediterranean) by a mismatch distributions analysis, via the sum of squared deviations (SSD) (Rogers & Harpending, 1992; Rogers, 1995) between observed and expected distributions. The level of significance of this statistical procedure was computed in ARLEQUIN, using 1000 random permutations. Non-significant outcomes ($P > 0.05$) for SSD allowed the acceptance of the tested expansion (demographic or spatial) model. In order to determine the chronological order of each of these expansion events for the two studied regional groups (i.e., which region has initially undergone demographic and range expansion), times since demographic and spatial expansions, measured in mutational time units (τ), were highlighted for the two examined Cox1 datasets.

The detailed reconstruction of the historical demography, via the assessment of population size evolution through time, was carried out for the whole dataset (129 Cox1 sequences) of *Clibanarius erythropus* (found to be genetically homogenous, see results) using the coalescent-based Bayesian Skyline Plot approach (BSP; Drummond *et al.*, 2005) implemented in the software package BEAST version 1.7.5 (Drummond *et al.*, 2012). Given that the T92 model was identified as the best fitting model for the examined data and since the BEAST program does not include the T92 model, the analysis was conducted using the HKY substitution model (implemented as the second best fitting model according to the outcome of MEGA analysis and also regarded as equivalent to the T92 model) and a strict molecular clock as priors. The Cox1 gene mutation rate already determined for *Clibanarius* sp. was used to estimate time since the expansion for the investigated mitochondrial dataset. In order to ensure the convergence of the posterior distributions, we performed five independent Markov Chain Monte Carlo (MCMC) runs of 50 million generations each. These five MCMC simulations were combined by means of LogCombiner version 1.7.5 (Drummond *et al.*, 2012), after discarding the first 10 % iterations (5 million) as burn-in. Bayesian Skyline Plot for the studied Cox1

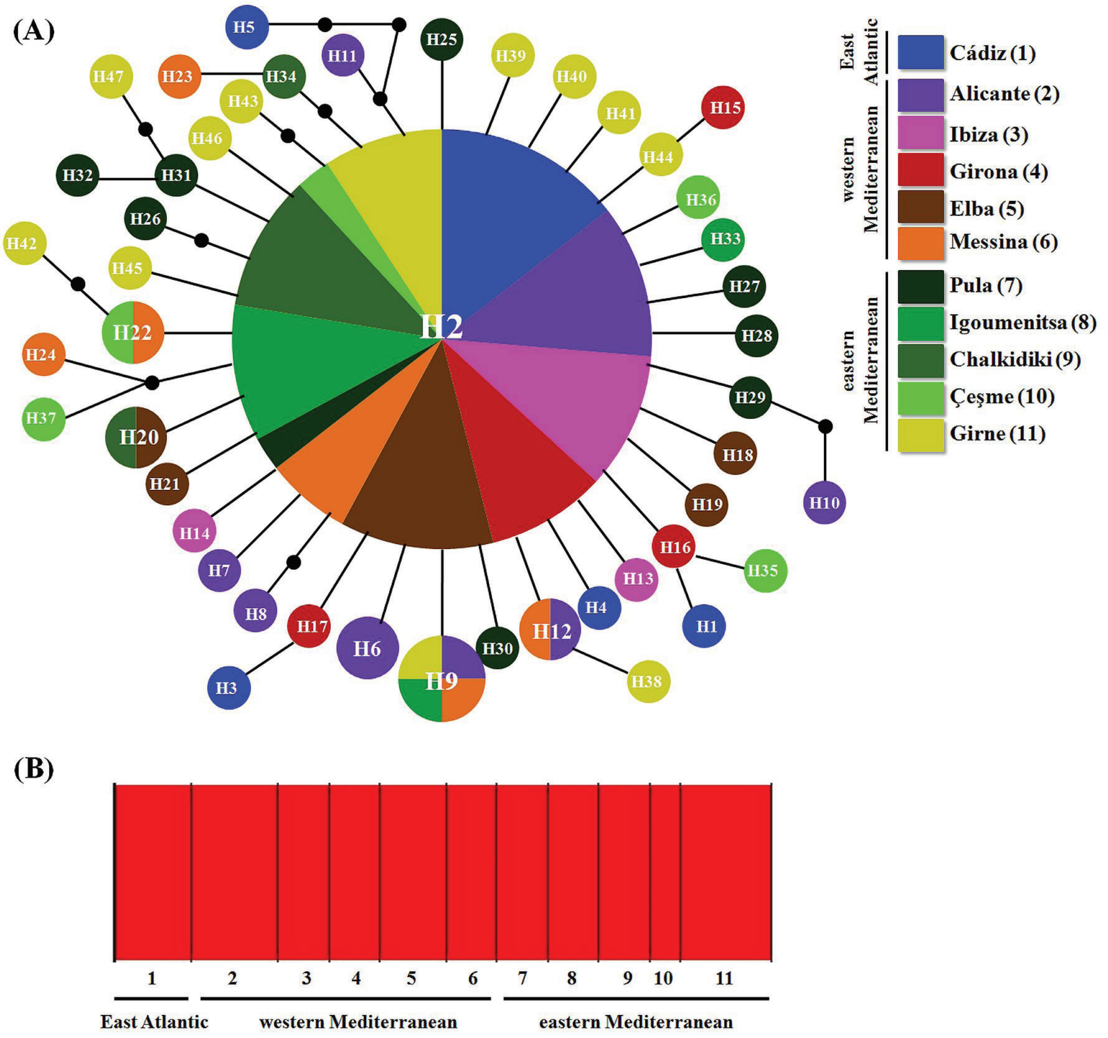

Fig. 2: (A) TCS parsimony network of *Clibanarius erythropus*, based on the alignment of 700 bp of the mitochondrial Cox1 gene, showing the evolutionary relationships among the recorded 47 haplotypes. Small black circles correspond to missing (or hypothetical) haplotypes. Each connecting line indicates one mutational step between haplotypes. Circle sizes depict proportions of haplotypes; the smallest corresponds to 1 and the largest to 76 individuals. (B) Identification of potential genetic clusters within *Clibanarius erythropus*, based on Cox1 gene (700 bp) sequences, using the Bayesian genetic assignment method implemented in BAPS version 6.0. The only retrieved single cluster is labeled in red. The numbers correspond to the populations (as already indicated in the network) used for the analysis.

dataset was generated in TRACER version 1.5, after confirming data convergence (effective sample size (ESS) > 200 for each parameter).

Results

Sequence diversity, population genetic variability, and regional pattern of genetic diversification

Analysis and comparison of 700 basepairs (bp) of the 129 Cox1 sequences of *Clibanarius erythropus* from the East Atlantic and Mediterranean Sea allowed the identification of 47 haplotypes (Table 2, Fig. 2). A total of 51 variable nucleotide sites allowed distinguishing these obtained haplotypes. Among the discerned variable sites, 18 were found to be parsimony-informative and 33 were shown to be autapomorphic.

The comparative analysis of population genetic diversity among the two defined Cox1 datasets revealed higher genetic variability measures in the longer Cox1 fragment dataset (stemming from a cropped and finally adjusted Cox1 fragment of 700 bp for a total of 129 sequences) (*N*h = 47, *h* = 0.654, *π* = 0.0017, K = 1.186; Table 2) than those retrieved in the shorter Cox1 fragment dataset (based on the final alignment of 316 bp within a total of 148 sequences) (*N*h = 28, *h* = 0.447, *π* = 0.0018, K = 0.575; Table S1). As such, a better resolution in capturing the maximum genetic variability within the analyzed mitochondrial marker in *Clibanarius erythropus* specimens can be assured by the longer Cox1 fragment dataset. Hence, the defined dataset, based on the analysis of 700 basepairs of the mitochondrial Cox1 gene, served to conduct the remaining statistical analyses.

The analysis of population genetic variability within *Clibanarius erythropus* from the East Atlantic and Mediterranean Sea, based on the analysis of 700 bp of the mitochondrial Cox1 gene, showed that the highest levels of genetic diversity measures were recorded in the eastern Mediterranean populations of Pula (*h* = 0.978) and Çeşme (*π* = 0.0029, K = 2.000) (Table 2). The lowest levels of haplotype diversity (*h*) were found in the three populations of Ibiza, Igoumenitsa, and Chalkidiki; while those corresponding to the nucleotide diversity (*π*) and the mean number of nucleotide differences (K) only characterized the two populations of Ibiza and Igoumenitsa (Table 2). Overall, eastern Mediterranean populations of *C. erythropus* exhibited higher levels of genetic diversity (*h* = 0.754, *π* = 0.0020, K = 1.396; Table 2) than their western Mediterranean and East Atlantic counterparts (*h* = 0.575, *π* = 0.0015, K = 1.036; Table 2). This spatial trend of genetic diversity was confirmed by the outcome of detailed assessment and comparison of genetic diversification levels among three regions corresponding to the East Atlantic, western Mediterranean and eastern Mediterranean after a rarefaction procedure (Table 3). Indeed, the eastern Mediterranean samples of *C. erythropus* were found to be significantly more diversified than their East Atlantic and western Mediterranean counterparts for all examined genetic diversity indices (haplotype diversity (*h*), nucleotide diversity (*π*), mean number of nucleotide differences (K), haplotypic richness (*Hap*r), number of private haplotypes (*N*p), and genetic endemism (*N*p/*Hap*r)) (Table 3). Besides, the western Mediterranean hermit crabs were shown to be genetically more variable than their East Atlantic counterparts for all analyzed parameters (*h*, π, K, *Hap*r, and *N*p) except genetic endemism, for which comparative analysis yielded non-significant output (Table 3), although the western Mediterranean region displayed slightly higher value (0.804) than that recorded in the East Atlantic region (0.800) (Table 3). The obtained findings suggest a longitudinal pattern of genetic diversi-

Table 3. Assessment and comparison of genetic diversification levels among the three examined regions (East Atlantic (EA), western Mediterranean (WM) and eastern Mediterranean (EM)) for *Clibanarius erythropus*. The computed values for each parameter were obtained after a rarefaction procedure to the smallest sample size (*N*) of 15 (from the East Atlantic).

Genetic diversity indices	**EA (15)**	**WM (15)**	**EM (15)**	**Assessment of inter-regional difference (unpaired *t*-test)**		
				EA *vs.* WM	**EA *vs.* EM**	**WM *vs.* EM**
Haplotype diversity (*h*)	0.4760	0.7130	0.8730	***	***	***
Nucleotide diversity (π)	0.0017	0.0019	0.0025	*	***	***
Mean number of nucleotide differences (K)	1.2000	1.3120	1.7320	*	***	***
Haplotypic richness (*Hap*r)	5.0000	7.4400	10.7000	***	***	***
Number of private haplotypes (*N*p)	4.0000	5.9800	9.2400	***	***	***
Genetic endemism (*N*p/*Hap*r)	0.8000	0.8040	0.8600	ns	***	***

*: Significant difference at $P < 0.05$; ***: Significant difference at $P < 0.001$; ns: Non-significant difference ($P > 0.05$). The values, highlighted for each diversity measure, correspond to the mean values obtained for the fifty conducted replicates. The number of private haplotypes, for each replicate, was determined according to the pattern of distribution of haplotypes in a dataset composed of 45 samples taken randomly from the East Atlantic (15), western Mediterranean (15) and the eastern Mediterranean (15).

fication within *C. erythropus* across its distribution range, with the eastern Mediterranean samples being the more genetically variable and the East Atlantic samples being the less genetically diversified.

Evolutionary relationships among the recorded Cox1 haplotypes and Bayesian inference of mitochondrial genealogy

Assessment of the evolutionary relationships among the discerned haplotypes of *Clibanarius erythropus*, via construction of the TCS statistical parsimony network, unveiled the existence of a single evolutionary lineage resolved by a star-like haplotype phylogeny (Fig. 2A). Within the retrieved TCS mitochondrial genealogy, based on the alignment of 700 bp of the mitochondrial Cox1 gene, rare haplotypes were found to radiate from the most common and proposed ancestral haplotype H2, which was found in all examined East Atlantic and Mediterranean populations (Fig. 2A, Table S2). Most derived haplotypes were shown to be directly separated from the common haplotype H2 by a single mutational step, except haplotypes H8, H11, H24, H26, H34, H37, and H43 which were distinguished from the ancestral haplotype by two mutational steps. A secondary set of derived haplotypes (H1, H3, H10, H15, H23, H32, H35, H38, H42, and H47) were gradually connected to the ancestral haplotype H2 by means of two to three mutational steps (Fig. 2A). The most divergent haplotype (H5) was recorded in the East Atlantic population of Cádiz. Notably, four mutational steps were found to connect this derived haplotype to the postulated ancestral haplotype H2 (Fig. 2A). All haplotypes, directly radiating the common haplotype H2, were singletons, being found in only one location, except haplotypes H6, H9, H12, H20 and H22 (Fig. 2A). Haplotype H6 was found in two individuals from the population of Alicante; whereas, haplotypes H9, H12, H20 and H22 were shared by hermit crab individuals from different locations (Fig. 2A, Table S2). Notably, the relatively more widespread haplotype H9 was found in four specimens from the populations of Alicante, Messina, Igoumenitsa and Girne (Fig. 2A). With the exception of haplotype H12 characterizing the western Mediterranean, the remaining three haplotypes H9, H20 and H22 were found in both western and eastern Mediterranean basins. Overall, the widespread geographic distribution of the common haplotype H2 and the sporadic occurrence of shared haplotypes by individuals from different regions (H9, H20, and H22) suggest a lack of geographic structure of genetic diversity and a high level of gene flow among examined populations of *C. erythropus*. The inference of haplotype genealogy, based on the final alignment of 316 bp of the Cox1 gene in all examined 149 sequences of *C. erythropus*, revealed the same trend as already observed in the genealogy of the longer Cox1 fragment dataset (Fig. S1). The output generated by the TCS statistical procedure for the shorter Cox1 fragment dataset was simpler than that obtained for the longer one, with all derived haplotypes (except H9, H10, H24, H25, and H26) being separated from the postulated ancestral haplotype H2 by only a single mutational step (Fig. S1), but with more yielded shared haplotypes among individuals (H3, H4, H7, H11, H12, H15, and H23; Fig. S1).

The Bayesian inference of mitochondrial genealogy, based on the analysis of 700 bp of the Cox1 gene, corroborated the pattern already discerned by the TCS Cox1 haplotype phylogeny. For instance, the outcome of BAPS analysis, as inferred from the Bayesian genetic assignment method, yielded a single evolutionary lineage which was found to characterize all examined East Atlantic and Mediterranean populations of *Clibanarius erythropus* (Fig. 2B).

Population genetic differentiation and phylogeographic structure

The analysis of molecular variance (one-level AMOVA), based on the analysis of the shorter Cox1 fragment dataset (comparison of 316 bp among the defined 12 populations from the East Atlantic and Mediterranean Sea, as indicated in Table S1), revealed a lack of inter-population genetic differentiation within the hermit crab *Clibanarius erythropus* (Φ_{ST} = -0.009, df = 147, P = 0.870, based on Tamura distances; F_{ST} = 0.018, df = 147, P = 0.131, based on haplotype frequencies). When surveying the longer Cox1 fragment dataset (700 bp), the overall genetic differentiation among the examined East Atlantic and Mediterranean populations of *C. erythropus* was found to be weak but significant based on haplotype frequencies (F_{ST} = 0.032, df = 128, P = 0.036). The general trend of partitioning of genetic variation among populations was not confirmed based on Tamura distances (Φ_{ST} = -0.004, df = 128, P = 0.762). The outcomes of pairwise comparison of genetic differentiation, for the longer Cox1 fragment dataset, showed that most pairwise genetic distances, estimated from nucleotide divergence and haplotype frequencies, were not significant except for a few comparisons (four based on nucleotide divergence, and five based on haplotype frequencies; Table 4). Notably, all the retrieved significant genetic distances were mainly attributed to the genetic distinctiveness of the eastern Mediterranean populations of Çeşme (based on Tamura distances) and Pula (remarkably according to haplotype frequencies, but also based on Tamura distances: Pula vs. Elba (Φ_{ST} = 0.030, $P < 0.01$)) (Table 4). Furthermore, most of the significant genetic distances, computed from nucleotide divergence and haplotype frequencies, were recorded between the western and eastern Mediterranean basins, but also between the East Atlantic and eastern Mediterranean (i.e., Cádiz vs. Pula: F_{ST} = 0.167, $P < 0.05$) (Table 4). The outcome of isolation by distance (IBD) analysis did not recover significant correlation between genetic and geographic distances (r = 0.011, P = 0.938), implying lack of contribution of geography to the unraveled significant pattern of genetic differentiation within *C. erythropus* across the East Atlantic-Mediterranean littoral.

The shallow pattern of population genetic differentiation along the East Atlantic and Mediterranean coasts,

Table 4. Pairwise comparisons of genetic differentiation, in East Atlantic and Mediterranean populations of *Clibanarius erythropus*, estimated from nucleotide divergence (Φ_{ST}, below the diagonal) and haplotype frequency ($F_{ST,}$ above the diagonal). Significant values in bold (*: $P < 0.05$; **: $P < 0.01$) were calculated from 10,000 permutations.

	East Atlantic	western Mediterranean					eastern Mediterranean				
	Cádiz	**Alicante**	**Ibiza**	**Girona**	**Elba**	**Messina**	**Pula**	**Igoumenitsa**	**Chalkidiki**	**Çeşme**	**Girne**
Cádiz	§	0.014	-0.036	-0.035	-0.029	0.019	**0.167***	-0.036	-0.036	0.111	0.058
Alicante	-0.000	§	0.026	-0.008	-0.002	-0.039	0.053	0.016	0.026	0.005	-0.005
Ibiza	-0.016	-0.013	§	-0.035	-0.030	0.037	**0.193***	-0.049	-0.049	0.144	0.076
Girona	-0.033	-0.004	0.000	§	-0.039	-0.008	0.121	-0.035	-0.035	0.065	0.028
Elba	-0.003	-0.000	-0.004	0.003	§	-0.002	**0.130***	-0.030	-0.048	0.073	0.035
Messina	0.005	-0.007	-0.000	-0.000	0.011	§	0.024	0.020	0.037	-0.041	-0.027
Pula	0.014	0.011	0.017	0.015	**0.030****	0.012	§	**0.193***	**0.193***	-0.025	0.005
Igoumenitsa	-0.016	-0.025	0.000	0.000	-0.004	-0.020	0.001	§	-0.049	0.144	0.069
Chalkidiki	-0.010	-0.008	0.000	0.000	-0.026	-0.038	0.016	0.000	§	0.144	0.076
Çeşme	0.013	0.026	**0.054***	0.007	**0.060***	-0.030	0.012	**0.054***	0.043	§	-0.027
Girne	-0.002	-0.010	-0.026	-0.024	-0.010	-0.022	-0.005	-0.034	-0.021	-0.016	§

as mainly revealed by the outcome of one-level AMOVA and pairwise comparison of genetic differentiation, showed a lack of population subdivision and phylogeographic structure within the hermit crab species across the surveyed region, as inferred from the outcome of PERMUT analysis. Indeed, the overall genetic diversity (H_T = 0.656) of *Clibanarius erythropus* was not considerably higher than the average diversity within populations (H_S = 0.633). Furthermore, the N_{ST} value (-0.003) was remarkably lower than the G_{ST} value (0.036), indicating the lack of phylogeographic structure within the examined Cox1 dataset of *C. erythropus.* This finding was confirmed by the outcomes of structured AMOVA. The examination of phylogeographic structure across potential barriers to gene flow, encompassing the East Atlantic and Mediterranean Sea, revealed a non significant genetic subdivision within *C. erythropus* across the Gibraltar Strait, the Almería-Oran Oceanographic Front, the Siculo-Tunisian Strait, and the Peloponnese hydrographic break (Table 5). The lack of genetic structure across these postulated biogeographic boundaries was shown according to both nucleotide divergence (Tamura distances) and haplotype frequencies (Table 5).

Reconstruction of evolutionary and biogeographic histories

When implementing the genetic divergence and speciation among *Clibanarius erythropus* and *C. aequabilis*, driven by the closure of the Gibraltar Strait at the onset of the Messinian Salinity Crisis (Krijgsman *et al.*, 1999), as a biogeographic calibration point, the outcome of the BEAST analysis unveiled a Cox1 mutation rate of 1.86 % per million years (Myr) (95% high posterior density interval (HPD): 0.65-3.01 %) within *Clibanarius*. The estimated rate was found to be within the range of mutation rates (1.11-6.58 % per Myr) recorded so far for the Cox1 gene in other decapod crustaceans (Knowlton & Weigt, 1998; Schubart *et al.*, 1998; Marino *et al.*, 2011; Crandall *et al.*, 2012; Deli *et al.*, 2018b; 2019a; González-Castellano *et al.*, 2020).

A calibrated Bayesian phylogeny of *Clibanarius erythropus* Cox1 haplotypes, based on the analysis of 700 bp of the mitochondrial gene Cox1, yielded a lack of resolution into clearly separated haplogroups. Instead, a pattern of gradual and rapid haplotype diversification was retrieved within the Cox1 genealogy (Fig. 3). Implementing the already determined Cox1 mutation rate within *Clibanarius*, the diversification of the recorded haplotypes within *C. erythropus* was found to start approximately at 0.131 million years ago (Mya) (95% HPD: 0.081-0.192 Mya), roughly coinciding with the beginning of the Last Interglacial (LIG) period (130,000-115,000 years ago; Wilson *et al.*, 1998). The main episodes of haplotype diversification are displayed in Figure 3. Notably, the initial and most representative episodes of these historical separation events were found to encompass a relatively short time period stretching from 0.131 Mya (95% HPD: 0.081-0.192 Mya) till approximately 0.112 Mya (95%

Table 5. Analysis of molecular variance (AMOVA) testing for partition of the genetic variance among the studied populations of *Clibanarius erythropus* under different biogeographic hypotheses.

Tested hypotheses of population genetic structure across potential barriers to gene flow	F-statistics based on Tamura distance	F-statistics based on haplotype frequency
1-Genetic structure across the Gibraltar Strait and Almería-Oran Oceanographic Front (GS-AOF):		
(Cádiz) vs. (Alicante, Ibiza, Girona, Elba, Messina, Pula, Igoumenitsa, Chalkidiki, Çeşme, Girne)	Φ_{SC} = -0.005 ns Φ_{ST} = -0.003 ns Φ_{CT} = 0.002 ns	F_{SC} = 0.036 * F_{ST} = 0.018 * F_{CT} = -0.019 ns
2-Genetic structure across the Siculo-Tunisian Strait (STS):		
(Cádiz, Alicante, Ibiza, Girona, Elba, Messina) vs. (Pula, Igoumenitsa, Chalkidiki, Çeşme, Girne)	Φ_{SC} = -0.004 ns Φ_{ST} = -0.004 ns Φ_{CT} = 0.000 ns	F_{SC} = 0.028 ns F_{ST} = 0.034 * F_{CT} = 0.007 ns
3-Genetic structure across the Peloponnese Hydrographic Break (PHB):		
(Cádiz, Alicante, Ibiza, Girona, Elba, Messina, Pula, Igoumenitsa) vs. (Chalkidiki, Çeşme, Girne)	Φ_{SC} = -0.005 ns Φ_{ST} = -0.004 ns Φ_{CT} = 0.001 ns	F_{SC} = 0.033 * F_{ST} = 0.029 * F_{CT} = -0.004 ns
4-Genetic structure across the GS-AOF+STS:		
(Cádiz) vs. (Alicante, Ibiza, Girona, Elba, Messina) vs. (Pula, Igoumenitsa, Chalkidiki, Çeşme, Girne)	Φ_{SC} = -0.004 ns Φ_{ST} = -0.005 ns Φ_{CT} = -0.001 ns	F_{SC} = 0.036 ns F_{ST} = 0.029 * F_{CT} = -0.007 ns
5-Genetic structure across the GS-AOF+STS+PHB:		
(Cádiz) vs. (Alicante, Ibiza, Girona, Elba, Messina) vs. (Pula, Igoumenitsa) vs. (Chalkidiki, Çeşme, Girne)	Φ_{SC} = -0.006 ns Φ_{ST} = -0.004 ns Φ_{CT} = 0.002 ns	F_{SC} = 0.044 * F_{ST} = 0.027 * F_{CT} = -0.018 ns

*: Significant difference at $P < 0.05$; ns: non-significant difference ($P > 0.05$).

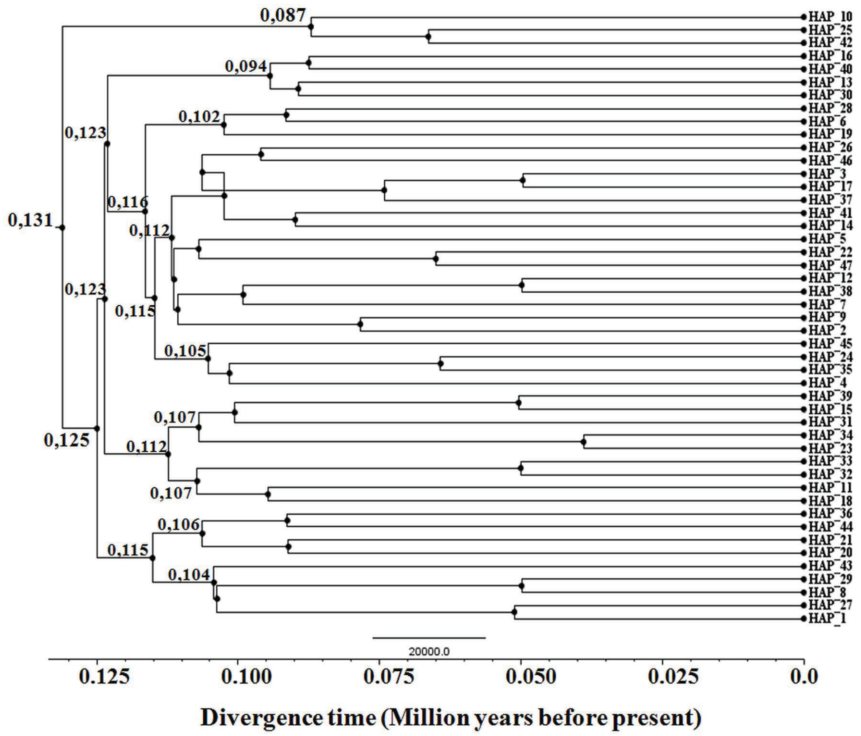

Fig. 3: Calibrated Bayesian phylogeny (as implemented in BEAST), based on the analysis of 700 bp of the mitochondrial Cox1 gene, showing diversification patterns of the recorded haplotypes within *Clibanarius erythropus* through time. The highlighted ages of basal nodes (mean values expressed in million years before present) correspond to the main diversification episodes.

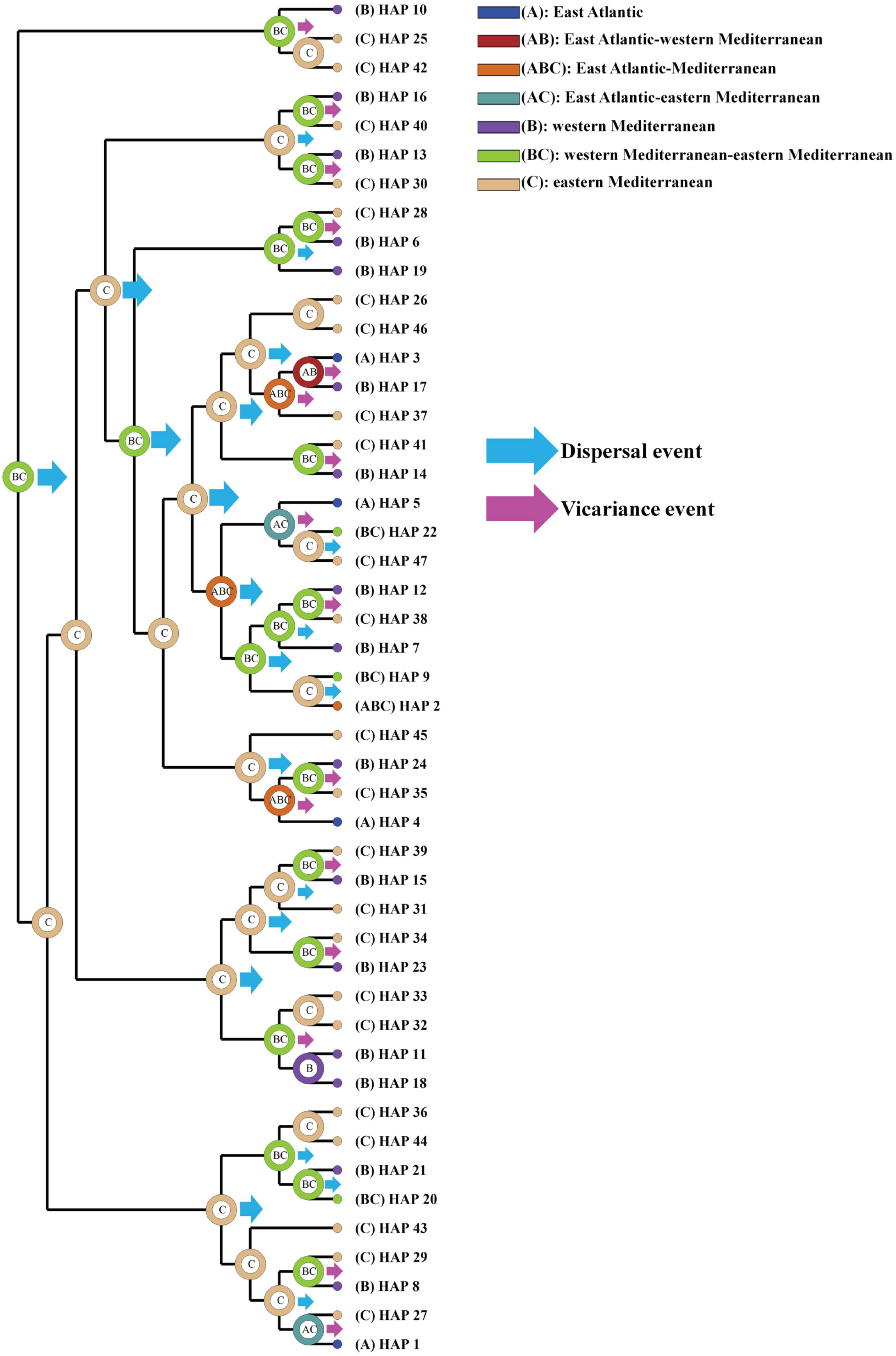

Fig. 4: Historical biogeography of *Clibanarius erythropus*, highlighting ancestral areas reconstruction of Cox1 haplotypes (based on 700 base pairs). Coloured circles on each node show the likelihood of occurrence of each ancestral haplotype at an inferred ancestral biogeographic region. Biogeographic regions are shown in different colors and denoted with alphabetic letters. Light blue arrows indicate dispersal events at the corresponding nodes, while pink arrows highlight possible vicariance events. The function 'Topology only', included in RASP, was used to infer the Cox1 haplotype phylogeny.

HPD: 0.073-0.143 Mya) (Fig. 3), matching once again the time span of the LIG. The marked increase of Cox1 haplotype diversity, mainly represented by the increase of the number of branch bifurcations and the explosive augmentation of Cox1 haplotypes, was shown to occur at the end of LIG period and continue to the glacial period of the Late Pleistocene (Würm glaciation: 110,000-12,000 years before present; Kukla, 2005) (Fig. 3).

The inference of historical biogeography of *Clibanarius erythropus*, through the reconstruction of ancestral geographic distributions of Cox1 haplotypes, showed that the diversification of the retrieved mitochondrial haplotypes was driven by a dispersal event from a geographic area encompassing the western and eastern Mediterranean basins, postulated to be the ancestral range for the studied hermit crab species according to the S-DIVA approach (Fig. 4). Despite this general finding, a closer look at the ancestral area reconstruction of Cox1 haplotypes, as depicted by the generated RASP haplotype phylogeny, showed that nearly all recorded haplotypes (44 out of the delineated 47 Cox1 haplotypes; Fig. 4) stemmed from the eastern Mediterranean, suggesting that this region was potential origin for the genetic polymorphism defined in *C. erythropus*. The outcome of S-DIVA analysis showed that frequent and successive episodes of dispersal events from the eastern Mediterranean to the western Mediterranean, but also occasionally to the East Atlantic (Fig. 4), were mainly at the origin of the onset of haplotype diversification. On the other hand, occasional backward dispersal events from the western Mediterranean to the eastern Mediterranean as well as those from the East Atlantic to both Mediterranean regions (western and eastern basins) (Fig. 4) were also shown to shape genetic polymorphism within *C. erythropus*. Within-regional dispersal events, depicted inside the western and eastern Mediterranean basins (Fig. 4), were also found to be potential drivers of haplotype diversity. Overall, dispersal events were the main evolutionary processes driving genetic diversification within the hermit crab species. The less frequent vicariance events were found to be successive to the major initial dispersal events and were mainly responsible for eventual separation of East Atlantic haplotypes and also involved in triggering genetic distinctness of some of the eastern and western Mediterranean haplotypes (Fig. 4).

Microevolutionary stems of the detected patterning of regional genetic diversity

Based on the collaborative findings inferred from the regional pattern of genetic diversification and the biogeographic history reconstruction through RASP analysis, four evolutionary scenarios of genetic diversification within *Clibanarius erythropus*, corresponding to gradual diversification through dispersal from the eastern Mediterranean (scenarios 1 and 2) or from the western

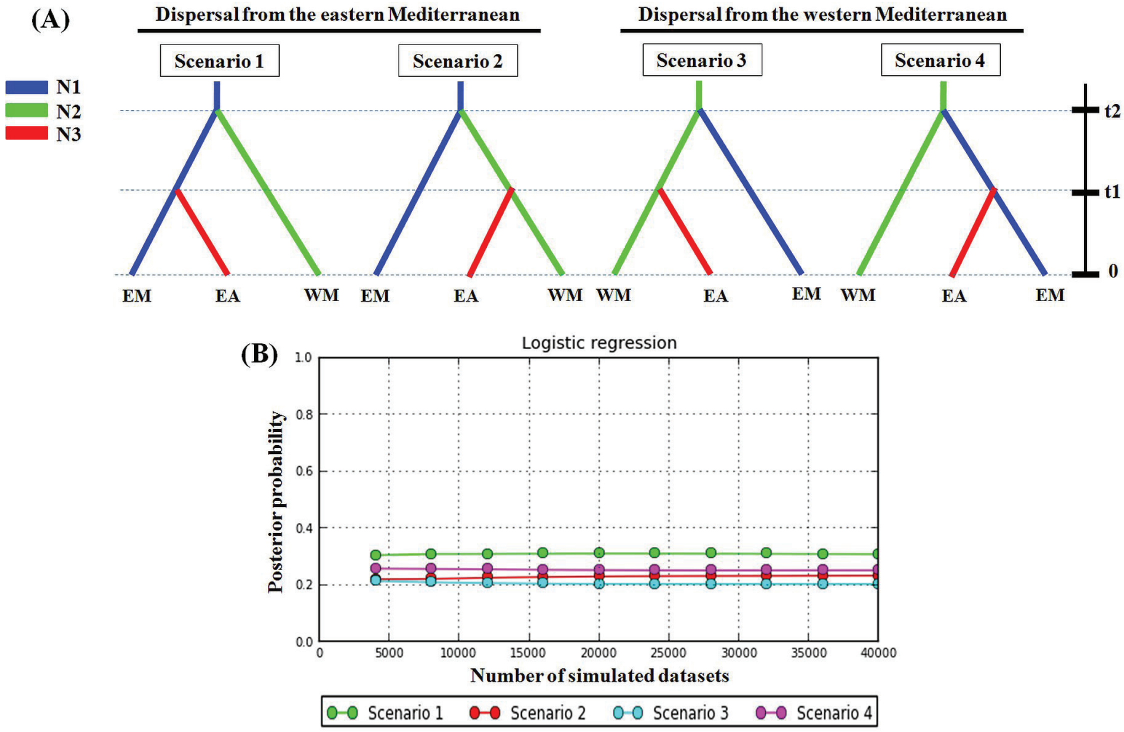

Fig. 5: Alternative tested evolutionary scenarios of genetic diversification within *Clibanarius erythropus* by means of the approximate Bayesian computation (ABC) method, implemented in the software DIYABC, based on mtDNA data. (A) Four tested scenarios corresponding to gradual diversification through dispersal from the eastern Mediterranean (scenarios 1 and 2) or from the western Mediterranean (scenarios 3 and 4). N1, N2, and N3 represent the effective population sizes of the eastern Mediterranean (EM), the western Mediterranean (WM) and the East Atlantic (EA) respectively. t2 and t1 correspond to the historical times at which gradual diversification occurred. (B) Logistic regression plot for the simulated scenarios. The x-axis represents the number of simulated datasets used to calculate the posterior probability of each scenario, as expressed in the y-axis.`

Mediterranean (scenarios 3 and 4), were tested (Fig. 5A). According to the outcome of the logistic regression plot, the estimated posterior probability for each tested evolutionary scenario indicated an unambiguous support for scenario 1 (Fig. 5B), in which hermit crabs from the western Mediterranean initially split from their eastern Mediterranean counterparts, followed by a more recent split of East Atlantic specimens of *C. erythropus* through a successive colonization from the eastern Mediterranean. The generated probability for the most supported scenario of genetic diversification (logistic approach: posterior probability (PP) = 0.3096, 95% confidence intervals (CI): 0.3029-0.3163) was found to lack overlap with those recorded for scenario 2 (PP = 0.2338, 95% CI: 0.2277-0.2399), scenario 3 (PP = 0.2040, 95% CI: 0.1982-0.2097), and scenario 4 (PP = 0.2526, 95% CI: 0.2463-0.2589). Based on these results, the significantly higher posterior probability, detected for scenario 1, allowed discarding with no doubt the possible western Mediterranean origin of hermit crabs genetic diversification. A founder effect following a historical colonization from the eastern Mediterranean could be rather a likely microevolutionary driver of the detected patterning of regional genetic variability across the East Atlantic-Mediterranean littoral.

Demographic history reconstruction

The outcome of the three neutrality tests (Tajima's *D*, Fu's F_S, and Ramos-Onsins and Rozas's R_2) analysis for each examined population, defined region as well as for the total dataset are shown in Table 2. At the population level, negative values of both Tajima's *D* and Fu's F_S were unveiled for all surveyed locations. Significant outputs of these indices were revealed for all populations (either in terms of Tajima's *D*, Fu's F_S or both) except for the population of Chalkidiki (Table 2). The Ramos-Onsins and Rozas's R_2 test was shown to be significant for the populations of Alicante, Elba, Messina, Pula, Çeşme and Girne (Table 2). At the regional scale, significant outputs of all analyzed neutrality tests (*D*, F_S, and R_2) were unveiled for the East Atlantic-western Mediterranean, the eastern Mediterranean, as well as for the total dataset (Table 2) highlighting a signature of past demographic expansion events within these defined genetic entities.

Statistical analyses of the mismatch distributions, via the sum of squared deviations (SSD), allowed accepting both models of demographic and spatial expansion for the two defined regions corresponding to the East Atlantic-western Mediterranean and the eastern Mediterranean (Table 6). According to the retrieved time since expansion, measured in mutational time units (τ), the eastern Mediterranean region was found to have undergone earlier demographic and spatial expansion events than the East Atlantic-western Mediterranean region (Table 6).

The detailed examination of the evolution of effective population size through time was found to corroborate previous analyses of demographic history and provided a comprehensive overview on the time since expansion. The generated Bayesian skyline plot for the metapopulation of *Clibanarius erythropus* provided clear evidence of demographic expansion within the examined total Cox1 dataset (129 Cox1 sequences derived from the alignment of 700 bp) (Fig. 6). The analysis of the noticeable trend of effective population size evolution through time unveiled a recent and sudden increase in effective population size, following a considerable phase of slight size decrease (Fig. 6). The recorded sudden expansion event in the analyzed Cox1 dataset started approximately at about 58,000 years ago (CI: 48,000-72,000 years ago). It should be noted that following a sharp and significant increase in effective population size, lasting until about 44,000 years ago, a subsequent phase of population dynamics of *C. erythropus* was characterized by progressive increase in effective population size (Fig. 6).

Discussion

The present mtDNA-based phylogeographic study is the first ever report on population genetic structure and evolutionary and biogeographic histories of the hermit crab *Clibanarius erythropus* across its distribution range spanning the north-East Atlantic and Mediterranean Sea. The obtained findings of the study revealed a marked macrogeographic genetic homogeneity across the East Atlantic-Mediterranean littoral, with noticeable lack of

Table 6. Assessment of both demographic and spatial expansion models for two examined regional datasets of *Clibanarius erythropus*.

Examined region/Tested expansion Model	Demographic expansion		Spatial expansion	
	SSD (*P*)	**τ (95 % CI)**	**SSD (*P*)**	**τ (95 % CI)**
East Atlantic-western Mediterranean	0.000 (0.991)	0.685 (0.000-2.746)	0.000 (0.954)	0.500 (0.267-2.977)
eastern Mediterranean	0.000 (0.968)	1.445 (0.769-2.146)	0.000 (0.938)	1.300 (0.703-2.046)

SSD: sum of squared deviations between observed and expected distributions under the tested expansion model. The probability of obtaining a simulated SSD greater than or equal to the expected was computed by 1000 random permutations. If this probability (*P*) was > 0.05, the expansion model is accepted. τ: time since expansion measured in mutational time units. CI: confidence intervals.

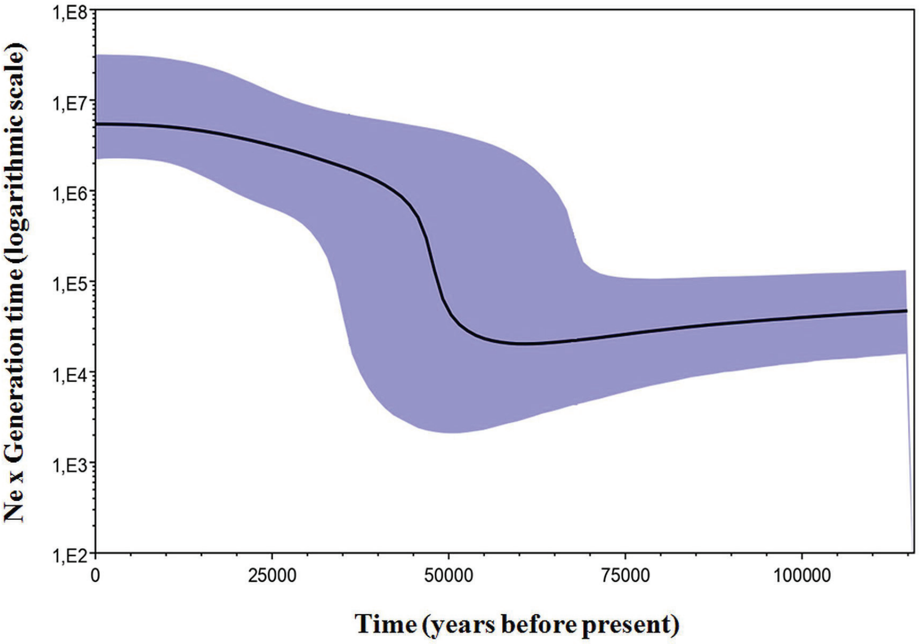

Fig. 6: Bayesian skyline plot (BSP) for the metapopulation of *Clibanarius erythropus* from the East Atlantic Ocean and Mediterranean Sea, showing changes in effective population size (Ne multiplied with generation time) over time (measured in years before present). The thick solid line depicts the median estimate, and the margins of the blue area represent the highest 95 % posterior density intervals.

population genetic structure across postulated biogeographic boundaries within the surveyed geographic spectrum. The retrieved results also showed that the discerned genetic signal stemmed from the residual effect of recent evolutionary history of the species, pointing out to the significant impact of Pleistocene climate oscillations on shaping the genetic variability and structure of the examined hermit crab species.

It has to be mentioned that we made sure during the sampling field trips to collect samples of the hermit crab species from geographically distant sites that cover the entire distribution range of the species. As such, the collected crab samples were representative enough to allow carrying out reliable statistics in population genetic investigations (more than five specimens per location). In fact, the sample size of all of the surveyed populations (except the population of Çeşme) was equal or higher than ten individuals, which is considered reliable for conducting statistical analyses (specifically those corresponding to population genetic diversity and differentiation analyses). On the other hand, evolutionary (temporal frame of genetic diversification), biogeographic (mode of genetic diversification) and demographic (effective population size evolution through time) history reconstructions of the species were inferred from the analysis of the whole dataset corresponding to 129 Cox1 sequences (derived from the alignment of 700 bp), which is also considered more than reliable for carrying out such analyses. For example, according to Grant (2015), a reliable BSP plot must be inferred from a dataset of at least 50 sequences. Hence, the examined data in this study are large enough to provide reliable and robust results.

Macrogeographic genetic homogeneity and lack of phylogeographic structure across postulated barriers to gene flow across the East Atlantic-Mediterranean littoral

The concordant outcomes of various population genetic analyses (TCS, BAPS, PERMUT, and AMOVA) unveiled a high genetic connectivity among populations of *Clibanarius erythropus* and a lack of phylogeographic structure across postulated barriers to gene flow spanning the East Atlantic and Mediterranean Sea. Macrogeographic genetic homogeneity has been previously shown for other hermit crab species across other parts of the globe such as *Coenobita brevimanus* across the north-western Pacific (Hamasaki *et al.*, 2015), *Clibanarius antillensis* across the western Atlantic (Nishikawa *et al.*, 2021), and *Clibanarius tricolor* across the Tropical West Atlantic (Stark *et al.*, 2021).

The striking pattern of high genetic homogeneity could stem from the interplay between the impacts of supposedly high larval dispersal potential within *Clibanarius erythropus* (Harms, 1992) and the oceanographic circulation patterns roaming across the surveyed geographic spectrum. In this context, we assume that the presumably long drifting larvae of the hermit crab species could reach geographically distant locations along the East Atlantic-Mediterranean littoral. Such extent of larvae transport and dynamics might be likely assured by the unidirectional surface current, called the Atlantic Current, originating from the East Atlantic, moving eastwards along the western Mediterranean coast and reaching into the eastern Mediterranean (Béranger *et al.*, 2004). As such, high genetic connectivity among East Atlantic and Mediterranean populations of *C. erythropus* is inevitable. This scenario could be likely supported by the concordant outcomes of TCS network genealogy, pairwise genetic differentiation, PERMUT and structured AMOVAs; whereby a considerable amount of gene flow (manifested by the wide geographic distribution of the common haplotype (H2), as well as a lack of genetic differentiation and phylogeographic structure) was detected between as far distant locations as those involving the East Atlantic population of Cádiz and the farmost eastern Mediterranean population of Girne. Future research investigation focusing on larval drift simulation of the hermit crab species, across the East Atlantic-Mediterranean littoral, with the Lagrangian analysis, is required in order to confirm this scenario.

Nevertheless, provided that other decapod species, with high dispersal potential as in *Clibanatius erythropus*, were shown to be genetically structured across biogeographic boundaries encompassing the East Atlantic-Mediterranean littoral (i.e., *Palaemon elegans* (Reuschel *et al.*, 2010) and *Carcinus aestuarii* (Deli *et al.*, 2016a; 2018a)), it is highly likely that larval dispersal is not solely responsible for the genetic makeup of the studied hermit crab species. Other factors, interacting with dispersal, could have driven the recorded macrogeographic genetic homogeneity. The high haplotype diversity but low nucleotide diversity, the low genetic differentiation and the lack of phylogeographic patterning, as well as the significant trend of historical demographic expansion, as recorded in *C. erythropus* (based on the alignment of 700 bp), are typical metapopulation characteristics, commonly observed in marine species (i.e., the warty crab *Eriphia verrucosa* (Deli *et al.*, 2019a), the black sea urchin *Arbacia lixula* (Deli *et al.*, 2019b), the hermit crab *Clibanarius antillensis* (Nishikawa *et al.*, 2021), and the mollusk gastropod *Ellobium chinense* (Shin *et al.*, 2021), reflecting the residual effect of recent evolutionary history. As such, it is reasonable to hypothesize that *C. erythropus* populations inhabiting the East Atlantic and Mediterranean Sea correspond to a single broad metapopulation that might have arisen due to repeating extinctions and colonization events driven by continuous and rapid changes in environmental and habitat conditions during the Pleistocene climate oscillations. The outcomes of evolutionary, biogeographic and demographic history reconstructions of *C. erythropus* are strongly in favor of this assumption and enable shedding further light on the cause of the striking genetic panmixia patterns retrieved within Atlanto-Mediterranean marine species.

In the following sections, we provide a comprehensive and detailed discussion of how historical processes might have exerted significant impact on geographic distribution of genetic variability of the species and contributed to the initial build-up to the contemporary pattern of population genetic structure discerned in *Clibanarius erythropus*. This would allow us eventually to come up with a consensus evolutionary history scenario of the hermit crab species that may explain the origin of the recorded high genetic connectivity among its populations across the distribution range.

Last Interglacial dispersal and colonization from potential eastern Mediterranean penultimate glacial refugia as potential initial driver of the recorded pattern of population genetic structure

Evolutionary and biogeographic histories reconstruction of *Clibanarius erythropus*, alongside the assessments of regional genetic diversity and ABC evolutionary scenarios of genetic diversification, revealed that the genetic structure of the population, as currently characterized, was potentially driven by the historical dispersal and colonization of the species from putative refugia in the eastern Mediterranean. This scenario is supported by several lines of evidences. First, calibrated Bayesian phylogeny showed that the genetic diversification within *C. erythropus* occurred approximately at 0.131 Mya. This recorded time frame for the genetic diversification roughly coincides with the Last Interglacial (LIG) period during the Eemian epoch (between 130,000-115,000 years ago; Wilson *et al.*, 1998). Second, the reconstruction of ancestral geographic distributions of Cox1 haplotypes suggests that the retrieved mitochondrial diversity within the Atlanto-Mediterranean *C. erythropus* was driven by a dispersal event from an ancestral geographic area located in the Mediterranean Sea. Third, the regional genetic diversity assessment and comparison unraveled a longitudinal pattern of genetic diversification within *C. erythropus* across its distribution range, with the eastern Mediterranean samples being the more genetically variable and the East Atlantic samples being the less genetically diversified. Fourth, the outcomes of DIYABC analyses approved the eastern Mediterranean as a center of genetic diversification; whereby a founder effect following a historical colonization from the eastern Mediterranean might have shaped the detected patterning of regional genetic variability across the East Atlantic-Mediterranean littoral.

On account of all these evidences, the pattern of Last Interglacial genetic diversification within *Clibanarius erythropus* not only invokes recent evolutionary history but also points out to the sensitivity of the hermit crab species to the Pleistocene palaeoenvironmental shifts. In this context, we hypothesize that the species might have retreated to potential eastern Mediterranean refugia when undergoing climate deterioration and sea-level significant dropping during the penultimate glacial period of the Pleistocene. Following this glacial epoch, the species might have tracked climate warming and sea-level restoration at the start of the LIG through the dispersal and recolonization of the whole Mediterranean Sea and north-East Atlantic Ocean. Evidences for the eastern Mediterranean origin of current genetic diversity (as unveiled in the studied hermit crab species) have started to emerge in other Atlanto-Mediterranean marine invertebrate species, such as in the emblematic sponge *Petrosia ficiformis* (Riesgo *et al.*, 2019). This commonly discerned historical biogeographic feature could be associated with the fact that the less dramatic seawater temperature decrease in the eastern Mediterranean during the Last Glacial Maximum (LGM, 3°C below current values) (Hayes *et al.*, 2005), and likely during previous glacial maxima, could have led to the onset of suitable refuge areas in this region for marine species (including the hermit crab *C. erythropus*) as previously wide suitable habitats disappeared (Patarnello *et al.*, 2007). On the other hand, the recorded time of genetic diversification and historical dispersal events, coinciding with the Last Interglacial period (130,000-115,000 years ago; Wilson *et al.*, 1998), could be ascribed to the fact that the LIG was a period characterized by a higher global sea level and a reduction in the ice sheet area (Zagwijn, 1996). It was also marked by warmer climate conditions (with the minimum winter surface temperature of the Mediterranean Sea remaining above 19°C for several thousands of years; Bardaji *et al.*, 2009), which might have been favorable for extended colonization and diversification of marine fauna (Muhs *et al.*, 2002). Temporal frames of genetic diversification, matching the Last Interglacial period, have been previously unraveled in various Atlanto-Mediterranean marine invertebrates with similar life history traits, as in *C. erythropus*, such as the black sea urchin *Arbacia lixula* (Wangensteen *et al.*, 2012) and the littoral prawn species *Palaemon elegans* (Deli *et al.*, 2018b).

Pre-LGM demographic expansion accentuating the effect of the recent LIG genetic diversification and promoting macrogeographic genetic homogeneity

The demographic history reconstruction of the metapopulation of the hermit crab *Clibanarius erythropus*, through the analyses of neutrality tests, mismatch distribution, and Bayesian Skyline Plot, unveiled a recent population demographic expansion. This finding was also consolidated by the star-like pattern of mitochondrial Cox1 genealogy coupled with a relatively high haplotype diversity and low nucleotide diversity (based on the alignment of 700 bp). The time since demographic expansion was estimated to occur around 58,000 years ago. The recorded temporal frame of population dynamics was found to predate the Last Glacial Maximum (LGM) period (between 26,500-20,000 years before present; Clark *et al.*, 2009). This pattern of pre-LGM expansion has also been reported in a wide variety of marine species (Hoarau *et al.*, 2007; Marko *et al.*, 2010; Muller *et al.*, 2012; von der Heyden *et al.*, 2013; Ni *et al.*, 2014; Reynolds *et al.*, 2014; Almada *et al.*, 2017; Wood *et al.*, 2017). Provided that this commonly detected pattern of population expansion has been shown to fall within the last glacial period of the Pleistocene (Würm glaciation: 110,000-12,000 years before present; Kukla, 2005), it could be attributed to the fact that the sea level dropping during the last glacial cycle did not occur uniformly towards the level discerned at the LGM, but rather oscillated rapidly over a period of 60,000 to 30,000 years ago (Lambeck *et al.*, 2002). Hence, the detected signature of population expansion could likely reflect one of the sudden increases in sea level during the last glacial period. The sudden and sharp increase in effective population size of the metapopulation of *C. erythropus* (from 58,000 till 44,000 years ago; Fig. 6) strongly supports this assumption. Notably, the difference in demographic signals exhibited by *C. erythropus* and other Atlanto-Mediterranean decapods (such as the warty crab *Eriphia verrucosa* displaying postglacial demographic expansion; Deli *et al.*, 2019a) could be the residual effect of species-specific life history strategies. In this context, we may hypothesize that the hermit crab *C. erythropus* was able to survive in greater portions of available habitat during periods of climatic change occurring in the last glacial cycle. This could be evidenced from the star-burst like Cox1 network of the species, suggesting that the discerned genealogy pattern might reflect the potential tolerance of the species to historical environmental conditions. Besides, the difference in temporal frame of regional demographic and spatial expansions, with the eastern Mediterranean region having undergone earlier expansion events than the East Atlantic-western Mediterranean region, could be attributed to the potential impact of climate fluctuations during the Würm glaciation on habitat availability and suitable ecological niche in the aforementioned two defined regions. Overall, the discerned pattern of the hermit crab metapopulation dynamics during the last glacial cycle might have significantly affected the genetic structuring of *C. erythropus*. Indeed, whether limited genetic differences could have or not been accumulated during the short laps of time period since the LIG diversification, following a dispersal from putative eastern Mediterranean refugia and consequent founder effect, a successive historical demographic expansion might have erased any resulting slight patterns of genetic divergence, therefore, homogenizing the gene pools between source and founded populations and restoring gene flow across the biogeographic boundaries encompassing the East Atlantic-Mediterranean littoral. As such, the detected pattern of regional demographic and spatial expansions might have intensified the effect of the recent LIG genetic diversification and promoted the macrogeographic genetic homogeneity as currently observed in *C. erythropus*.

The interplay between the impacts of recent evolutionary history and contemporary abiotic and biotic homogenizing processes on the retrieved pattern of genetic structure

Several lines of evidence support the impact of recent evolutionary history of the hermit crab *Clibanarius erythropus* on its population genetic structure across the distribution range. First, the time and mode of diversification of the species showed that the recent dispersal event at the beginning of the Last Interglacial period might have not allowed sufficient time for the onset of significant genetic divergence and complete lineage sorting. Residual effects of such an event are still figured out in the detected trend of population genetic differentiation; whereby the lack of phylogeographic structure was discerned across the main biogeographic boundaries encompassing the East Atlantic-Mediterranean littoral and only few pairwise significant genetic distances were unveiled. Notably, most of these significant outputs were detected among the eastern Mediterranean basin and the rest of the distribution range, consolidating the potential occurrence of the penultimate glacial refugium in the eastern Mediterranean as previously discussed. Second, the pre-LGM demographic expansion event might have significantly contributed to the homogenization of gene pool following range expansion. The prevalence of the common haplotype (H2) in the distribution range of the species (being present in the East Atlantic, western Mediterranean and eastern Mediterranean), alongside the single identified mitochondrial BAPS cluster characterizing the East Atlantic and Mediterranean populations, strongly corroborate this scenario. Third, the lack of significant IBD (Isolation by Distance) pattern, recorded within the examined mitochondrial dataset of *C. erythropus*, likely reflects the residual effect of recent population history and suggests that the hermit crab populations might have not yet reached migration-drift equilibrium.

These aforementioned lines of evidence add to the growing body of phylogeographic investigations unveiling the crucial role played by historical processes in driving patterns of genetic panmixia within marine biota, specifically those assigned to the Atlanto-Mediterranean region (Wangensteen *et al.*, 2012; Evangelisti *et al.*, 2017; Deli *et al.*, 2019a). These new insights do not only help with providing in-depth knowledge of the potential factors responsible for gene pool homogenization, but also revolutionize thoughts and views about the true origin of genetic homogeneity across larger geographic scales (i.e., those encompassing the north-eastern Atlantic and Mediterranean Sea littoral, known to harbor various kinds of barriers to gene flow such as oceanographic fronts as well as temperature and salinity gradients). Recently, observable intensive genetic panmixia within Atlanto-Mediterranean marine species has been found to stem from residual effects of a recent evolutionary history, involving a recent colonization from putative Atlantic refugia, as previously shown in the black sea urchin *Arbacia lixula* (Wangensteen *et al.*, 2012; Pérez-Portela *et al.*, 2019) and the littorinid gastropod *Echinolittorina punctata* (Evangelisti *et al.*, 2017). Consequently, populations of these marine panmictic systems might have not had enough time to accumulate significant genetic differences, that would allow for complete lineage sorting (Wangensteen *et al.*, 2012), and therefore exhibited the lack of genetic divergence, despite the documented potential isolating impact of abiotic processes in the East Atlantic and Mediterranean Sea (i.e., oceanographic circulation patterns (Duran *et al.*, 2004; Reuschel *et al.*, 2010; Sá-Pinto *et al.*, 2012; Deli *et al.*, 2016a) and sea water temperature and salinity gradients (Carreras *et al.*, 2020)). Another relatively new process, playing a crucial role in driving patterns of genetic homogeneity within marine species, was unveiled in the recent study of Deli *et al.* (2019a). Based on phylogeographic examination and evolutionary, biogeographic and demographic histories reconstructions of the Atlanto-Mediterranean crab *Eriphia verrucosa*, Deli *et al.* (2019a) unraveled the prevalence of high genetic connectivity among north-eastern Atlantic and Mediterranean populations and lack of phylogeographic structure across postulated biogeographic boundaries. Integrative evidences from the outcomes of comparisons of regional genetic diversification, as well as evolutionary and biogeographic histories reconstructions within *E. verrucosa*, allowed the authors to reveal genetic imprints of a Late Pleistocene vicariant event across the Gibraltar Strait, erased by subsequent postglacial expansion events for both the East Atlantic and Mediterranean regions (found to harbor refugial populations during the Würm glaciation), and admixture among refugial lineages. Based on these insights, Deli *et al.* (2019a) postulated that the Late Pleistocene climate oscillations might have had a significant impact on shaping the genetic variability and structure of this examined crab species. The authors also suggested that the resulting pattern of genetic panmixia (primarily induced by the potential effect of historical processes) is likely still maintained by the impact of contemporary factors, including oceanographic features, larval dispersal and behavior, and large effective population size.

These insights suggest a rather enhancing role (than direct one) of contemporary ecobiological features of marine populations in the chain of events kick-started by historical processes (that might have affected populations during their evolutionary history). Thus, we may hypothesize that the detected macrogeographic genetic homogeneity within the studied hermit crab species *Clibanarius erythropus* could be initially driven by historical events and then maintained by the impact of contemporary homogenizing processes (i.e., life history traits, including high larval dispersal potential, and oceanographic circulation patterns across the study area as previously discussed). The interplay between impacts of recent evolutionary history and contemporary abiotic and biotic homogenizing features was also discerned and discussed by Shin *et al.* (2021) for the mollusk gastropod *Ellobium chinense*; whereby, the recorded genetic homogeneity and metapopulation characteristics of the species (with pelagic larval phase) were found to be initially triggered by Late-Middle and Late Pleistocene expansion events (echoing cyclic extinction and dispersal events driven by continuous and rapid changes in historical environmental and habitat conditions) and later retained under the influence of the Kuroshio warm currents (facilitating the spread of larvae) across the north-western Pacific.

On account of these findings, trends of recent evolutionary histories of marine species, and their impact on population genetic structure, may invoke the sensitivity of these species to historical climate fluctuations (species-specific response). In this context, we hypothesize that the recorded trend of macrogeographic genetic homogeneity within *Clibanarius erythropus* could stem from population turnover leading to the erosion of potential genetically divergent lineages, accumulated over previous cyclic glacial maxima (Grant & Bringloe, 2020). In particular, this process might have led to the decrease of genetic variability within the studied hermit crab species following severe historical genetic bottlenecks driven by the loss of much of the suitable ecological niche of the species across its distribution range during palaeoclimate and palaeogeographic shifts of the Pleistocene glaciations. While the population-turnover model (local extinction and recolonization) might have played a crucial role in limiting the onset of new evolutionary lineages in *C. erythropus* across the East Atlantic-Mediterranean littoral, it may provide an explanation into why other Atlanto-Mediterranean decapod species with similar dispersal potential, as in *C. erythropus*, but with different responses to palaeoenvironmental conditions, display remarkable spatial patterning of population genetic structure (Reuschel *et al.*, 2010; Weiss *et al.*, 2018).

Evolutionary history scenario of Clibanarius erythropus across its distribution range

Based on the obtained findings, we may propose the following evolutionary history scenario that could explain the recorded pattern of population genetic structure within the hermit crab species *Clibanarius erythropus* across its distribution range encompassing the East Atlantic and Mediterranean Sea. During the Penultimate Glacial Period (PGP: between 192,000 and 135,000 years before present, Jakobsson *et al.*, 2001; Colleoni *et al.*, 2016), severe climate shifts might have led to the loss of much of the suitable ecological niche of the hermit crab species. Notably, the East Atlantic and western Mediterranean could have been the most affected regions by these brutal PGP environmental changes, opposed to the eastern Mediterranean where few suitable habitats were still standing. Hence, the hermit crab *C. erythropus* withstood the PGP abrupt climate changes by retreating to potential refugia within the eastern Mediterranean, while undergoing severe bottlenecks that might have led to the depletion of its genetic diversity and possibly the loss of established divergent genetic lineages over previous Pleistocene glacial and interglacial cycles. At the start of the Last Interglacial (LIG) period, an amelioration of environmental conditions associated with climate warming and sea-level restoration allowed hermit crabs to recolonize the Mediterranean Sea and East Atlantic from potential eastern Mediterranean refugia through successive dispersal events, interspersed by occasional vicariant events. Although such historical events are expected to promote the effects of founder effects and genetic bottlenecks, ultimately resulting in spatial patterning of genetic differentiation, the interplay between impacts of the short time period since the LIG diversification (insufficient for accumulated significant genetic differences) and the successive pre-LGM demographic expansion of the species might have played a crucial role in impeding the onset of genetic distinctness and/or erasing any pre-existing accumulated genetic divergence, leading to the macrogeographic genetic homogeneity as currently detected in the studied hermit crab species. The resulting lack of phylogeographic structure within *C. erythropus* across its distribution range, which reflects the residual effect of recent evolutionary history, is still likely maintained by the impact of contemporary abiotic (i.e., particular oceanographic circulation patterns across the surveyed geographic spectrum) and biotic (i.e., specific ecological requirement and life history traits of the species including high larval dispersal potential) homogenizing processes.

Conclusions

The findings of the present study are relevant and provide novel insights into the evolutionary history and microevolutionary processes underlying the recorded trend of population genetic structure as well as the tempo and mode of genetic diversification of the hermit crab *Clibanarius erythropus* across its distribution range. Notably, they contribute to deepening the knowledge about the impact of historical climate fluctuations on forging contemporary spatial patterns of population genetic diversity and structure within Atlanto-Mediterranean marine biota. Besides, they add evidence to the various and alternative evolutionary strategies causing genetic panmixia within marine species by unraveling a new genetic diversification process in the studied hermit crab species (interplay between impacts of LIG dispersal from potential eastern Mediterranean refugia and pre-LGM demographic expansion) in comparison to earlier recorded mechanisms in other marine species (i.e., recent colonization from putative Atlantic refugia (Wangensteen *et al.*, 2012; Evangelisti *et al.*, 2017), and Late Pleistocene vicariant event across the Gibraltar Strait erased by subsequent postglacial expansion and admixture among refugial lineages (Deli *et al.*, 2019a)). However, these novel insights need to be confirmed by the analysis of other variable nuclear markers provided that independent genes (such as mitochondrial and nuclear ones) may yield different evolutionary rates and coalescent histories. Notably, the analysis of a single gene could only provide insights into the coalescent history of that locus, which may not always be representative of population history. Besides, population genetic analyses of several independent genetic markers in an extended representative sampling dataset (covering the whole distribution range across the East Atlantic as well as across the Mediterranean Sea and Black Sea) shall provide a complete and comprehensive view on the pattern and the historical origin of population genetic diversity and structure of *C. erythropus*. The population genomic investigation of this Atlanto-Mediterranean hermit crab species, by means of Next Generation Sequencing analysis of SNPs, is currently planned to be carried out and would shed more light on the retrieved interesting evolutionary process causing genetic panmixia.

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Supplementary Data

The following supplementary information is available online for the article:

Table S1. Genetic diversity estimates for the examined hermit crab *Clibanarius erythropus* populations, based on the analysis of 316 base pairs of the mitochondrial Cox1 gene. Values reported for each population as well as for the total dataset are: sample size (*N*), number of polymorphic sites (*N*ps), number of haplotypes (*N*h), haplotype diversity (*h*), nucleotide diversity (π), and mean number of nucleotide differences (K).

Table S2. Geographic distribution of the 47 haplotypes of *Clibanarius erythropus* recorded at the 11 populations from the East Atlantic Ocean and Mediterranean Sea, based on the analysis of 700 base pairs of the mitochondrial Cox1 gene.

Fig. S1: TCS parsimony network of *Clibanarius erythropus*, based on the alignment of 316 bp of the mitochondrial Cox1 gene, showing the evolutionary relationships among the recorded 29 haplotypes (obtained from the comparison of a total dataset of 149 Cox1 sequences). Each connecting line indicates one mutational step between haplotypes. Circle sizes depict proportions of haplotypes; the smallest corresponds to 1 and the largest to 110 individuals.