# The distribution and molecular diversity of the Eastern Atlantic and Mediterranean chthamalids (Crustacea, Cirripedia)

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The three chthamalids *Chthamalus stellatus*, *C. montagui* and *Euraphia depressa* are common inhabitants of the intertidal zone in the Eastern Atlantic, Mediterranean Sea and Black Sea. In this study, we investigated the occurrence of these barnacles in a wide range of their distribution. Population divergences of these two species have been inferred using three molecular markers — internal transcribed spacer (ITS), elongation factor 1α (EF-1α) and cytochrome oxidase subunit I (COI). ITS sequences of *C. stellatus* were identical throughout the species range, whereas ITS sequences of *C. montagui* indicated that the Black Sea and Mediterranean populations are isolated from the Atlantic population. The COI and EF-1α sequences were the most variable and informative. They indicated a high genetic divergence between Atlantic, Mediterranean and Black Sea populations for *C. montagui*. In addition significant genetic structure was found among the populations of *C. stellatus* based on EF-1α but not COI. Interestingly, our molecular dating analysis correlated the pattern of diversification in *C. montagui* to major geological changes that occurred in the Mediterranean during the end of the Messinian and Pleiocene periods. We suggest that palaeohistory shaped the divergences between *Chthamalus* populations that have probably been maintained by current hydrographic conditions. Finally, COI phylogenetic analysis placed the genus *Euraphia* within the *Chthamalus* clade, suggesting the need for a taxonomic revision of *Euraphia*. This study represents the most detailed phylogeographical analysis of intertidal Mediterranean species to date, and shows that geological events have strongly shaped the current diversity pattern of this fauna.

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## Introduction

The marine realm seems to be continuous, with no barriers to gene flow. Hence, it was long expected that populations of marine species may reach panmixia over a wide geographical range. Briggs (1974) already noted the existence of subdivisions and barriers within seas and oceans. In the marine realm, the barriers to gene flow are far less obvious compared to terrestrial biota. However, past geological events and present hydrographic barriers in the marine environment are reflected in the distribution pattern of species (Longhurst 1998). When Darwin (1854) reported the distribution of *Chthamalus stellatus* he described this species as nearly cosmopolitan species, with five varieties, occurring on all shores with the exception of Australia. He noted differences in the shell, opercular valves and even in cirri and trophi, but nevertheless concluded (p. 459): 'I must believe that all the widely distributed forms here grouped together, do really belong to the same species'. Later, it was recognized that Darwin had lumped several distinct species. The distribution range of *C. stellatus* was described to be limited to the rocky shores in the subtropical and temperate Eastern Atlantic and the Mediterranean Sea, and to include two varieties, *C. stellatus* var. *communis* and *C. stellatus* var. *depressus*, which is a habitat form of *C. stellatus* (Utinomi 1959). Southward (1964) recognized Darwin's 'variety *e*' to be a distinct species, naming it *C. depressus*: this species is sympatric in the

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Mediterranean with *C. stellatus*. At a later stage *C. depressus* was assigned to another genus, *Euraphia* (Newman & Ross 1976). Kensler *et al.* (1965) noted differences between *C. stellatus* from the Mediterranean and the Eastern Atlantic but regarded them as a single species and described the distribution of these varieties at the interface between the Atlantic Ocean and the Mediterranean Sea (i.e. the Iberian Peninsula and the shores of Morocco). That study was published before *C. stellatus* and *C. montagui* were recognized as two distinct species. Southward (1976) clarified the picture and showed that *C. stellatus* comprises two distinct species, *C. stellatus* and *C. montagui*. In some cases, mixed populations of these two species of *Chthamalus* may be found on the same shore, while in other localities only one of the species is present. Crisp *et al.* (1981) summarized the known distribution range of *C. stellatus*, *C. montagui* and of *Euraphia depressa*, but their detailed study was restricted to the distribution of the population of the British Isles. This information was subsequently refined by adding more data on the distribution of these species (Pannacciulli *et al.* 1997); however in north east Africa and the northern Levant, the distribution remained unclear, mainly due to insufficient data.

It is difficult to separate species of the genus *Chthamalus* on the basis of morphology, and it became evident that what was considered in many cases to be a single *Chthamalus* population in fact represented distinct sympatric species. In some cases, the separation of these cryptic species requires genetic determination. The separation of *C. stellatus* and *C. montagui* was confirmed by allozyme studies (Dando & Southward 1980). Dando *et al.* (1979) compared the populations of *C. stellatus* and *C. montagui* from the Adriatic and south-west England and found that *C. stellatus* from all sites had similar allele frequencies, whereas *C. montagui* from the Adriatic differed from the English population. Dando & Southward (1981) showed that a sharp change in phosphoglucomutase (*pgm*) allozyme frequency occurred along the south-east coast of Spain. They suggested that during periods of glaciations and interglaciations associated with marine regression and transgression in the Pleistocene, the Atlantic and Mediterranean populations of *C. montagui* became physically separated and then diverged genetically. The maintenance of separation of the two populations was explained by the presence of opposing coastal currents leaving long sandy beaches which created a reproductive barrier between the two populations. Based on the differences in allozyme frequencies of *pgm*, Pannacciulli *et al.* (1997) suggested that the Atlantic and Mediterranean forms of *C. montagui* are sibling species. The present oceanographic conditions are the main cause for the genetic separation between the Atlantic and Mediterranean organisms, as the Almeria–Oran front restricts larval dispersion. In addition, genetic differences were also identified between the Atlantic and the Mediterranean populations of *C. stellatus*. Differentiation between the populations of *C. stellatus* was identified by variation at the glutamate oxaloacetate transaminase (*got*) locus. However, *C. stellatus* shows no discernible differentiation between the Atlantic and Mediterranean populations at two out of four loci examined (Pannacciulli *et al.* 1997), while *C. montagui* differs for all loci. One drawback of these studies is the lack of information from the south-eastern Mediterranean, and the use of allozymes as molecular markers for divergence. Allozymes have several limitations as markers: first, they have a low level of polymorphism; second, the alleles are scored on a gel only as fast, slow or intermediate, and their phylogenetic relationship cannot be determined. Indeed, Pannacciulli *et al.* (1997) suggested that the observed absence of genetic variability based on most allozyme loci between *C. stellatus* populations might be due to the limitations of this marker.

In addition to the separation between the Atlantic and Mediterranean populations, analysis of allozyme polymorphism of *C. montagui* from Malta revealed that the Maltese population too differs from the Mediterranean and Atlantic ones. Dando (1987) suggested that the salinity barrier between Cyrenaica and Tunisia might also contribute to the isolation of the Maltese population, which cannot exchange larvae with other parts of the Mediterranean. Similarly, Pannacciulli *et al.* (1997) found that the Black Sea population of *C. montagui* shows great genetic similarity to the Mediterranean population. This genetic similarity between the Black Sea and the Mediterranean populations is present despite the low salinity of the Black Sea and the hydrographic barrier of the Bosphorus, and was explained by the possible exchange of larvae between these basins during autumn, when saline Mediterranean waters flow into the Black Sea.

In this study, we revise the distribution of *E. depressa*, *C. montagui* and *C. stellatus*, and use both mitochondrial DNA (mtDNA) and nuclear DNA markers to confirm the genetic differences between populations of *C. montagui* and *C. stellatus*, initially based on differences in allozymes (Pannacciulli *et al.* 1997). Those authors pointed out that a full description of the population structure of the Mediterranean *Chthamalus* also requires the sampling of individuals from the Eastern Mediterranean and from the coasts of Africa. Our study partly fills this gap. Finally, we conducted a Bayesian molecular dating analysis in order to estimate the divergence timescale of the two species of *Chthamalus*.

## Materials and methods

### *Collection of samples*

Most of the samples used in this study were collected between the years 2000 and 2006 by the authors during various site visits. Some samples were obtained from local colleagues. When sea conditions permitted, all species present in the entire chthamalid zone were sampled. The barnacles were

removed from the substratum, fixed and kept in 95% ethanol. Upon arrival at the laboratory, the barnacles were identified using shell morphology or, in cases of doubt, examination of cirral setation, the samples were stored at –20 °C. After dissection, the soma of a single animal was used for each DNA study. Voucher specimens were deposited at the Hebrew University Zoological Museum.

***DNA amplification and sequencing***

DNA was extracted using High Pure PCR Template Preparation Kit (Roche; Germany). For PCR amplification, 50 ng of DNA was used (Saiki *et al.* 1988). Three gene fragments were amplified, the cytochrome oxidase subunit I (COI), the elongation factor 1α (EF-1α), and the region which encompasses the internal transcribed spacer 1 (ITS 1), 5.8S rDNA and ITS 2. For *E. depressa* only COI was amplified (see Results). Universal primers HCOI2198 and LCOI1490 (Folmer *et al.* 1994) were used for PCR and sequencing of the COI gene fragment (approximately 390–590 bp) (35 cycles, annealing 50 °C, 30″, extension 72 °C, 45″). For the EF1-α gene we used the *Balanus glandula* primers of Sotka *et al.* (2004) (35 cycles, annealing 58 °C, 30″, extension 72 °C, 30″) to amplify a fragment of approximately 400 bp. Primers for the ITS region were newly designed: ITS-EL1f 5′-GGTCGTCTAGAGGAAGTAAAAGTC-3′ and ITS-EL1r 5′-TTCCCGAACACCACATTGCACGACG-3′ (35 cycles, 54 °C 30″, extension 72 °C 45″). The ITS fragments comprised approximately 408 bp. All amplifications were carried out in a personal combi-thermocycler (Biometra, Germany). PCR products were purified using the High Pure PCR Product Purification kit (Roche; Germany). Forward and reverse sequencing of PCR products was performed by the Sequencing Unit of Tel-Aviv University (Israel), and by Macrogen Inc. (Seoul, South Korea), using automatic sequencing and the Taq DyeDeoxy Terminator Cycle sequencing system (Applied Biosystems). For EF-1α, when multiple polymorphic sites were detected in a sample (as evidenced on chromatograms by superimposed pairs of peaks, typically half-height and confirmed by sequencing in both directions), PCR products were ligated into the pGEM vector (Promega, Madison, WI). The plasmid was inserted into competent *Escherichia coli* cells, strain DH5α, and cultured on Fast Media Lab Agar IPTG/X Gel plates (Fermentas, Vilnius, Lithuania). Three to eight clones for each sample were sequenced. The appropriate alternative haplotypes were then determined. For sequences with only one heterozygous site, haplotypes were designated as the two alternative resolutions.

***DNA sequence analysis***

Arlequin 3.0 (Schneider *et al.* 2000; <http://lgb.unige.ch/arlequin>) was used to conduct an AMOVA on each three genes for each species. Using pairwise differences, the total molecular variance was partitioned into 'seas' (i.e. Black Sea, Mediterranean Sea and Atlantic Ocean) and 'within sea' components. A median-joining network (Bandelt *et al.* 1999) was inferred using COI, ITS and EF-1α haplotypes and the program NETWORK v 2.0 (available at http://www.fluxes-engineering com/sharenet.htm).

***COI dating analysis***

*Reconstruction of the best ML tree.* The COI data set used to reconstruct the phylogenetic relationships contains 64 sequences. To reduce computation time, only 23 COI haplotypes of *C. montagui* and 19 of *C. stellatus* representative of the genetic variability of these species were included in the tree reconstruction. The sequence of *Catomerus polymerus* (AY428048) was chosen as outgroup, based on the phylogenetic trees of Fisher *et al.* (2004) and Pérez-Losada *et al.* (2008). To obtain a more accurate estimation of the branch length between the outgroup and the ingroup various *Chthamalus* and *Euraphia*, sequences available in the databanks were also included.

Alignment was performed with CLUSTALX (Thompson *et al.* 1997) using default parameter settings and manually refined. The best probabilistic model of sequence evolution was determined with the program MODELTEST 3.07 (Posada & Crandall 1998), using the Akaike information criterion (AIC). ML searches for the best trees were performed with the program PAUP* (Swofford 2000). The parameters of the model and the best ML tree were determined in an iterative manner. The initial parameter values were those estimated by MODELTEST 3.07; those values were used for a first round of heuristic search starting with a neighbor-joining (NJ) tree and using tree bisection-reconnection (TBR) branch-swapping. Parameters were then estimated on the resulting tree and used for the subsequent round of heuristic search. The process was repeated until all parameters were stable. Bootstrap percentages (BP) were computed using the best parameters after 200 replicates starting with an NJ tree and with the TBR branch-swapping option.

*Dating analysis.* The program TREE-PUZZLE 5.2 (Schmidt *et al.* 2002), was used to test the molecular clock hypothesis. Computations were performed using the best ML topology, and the model parameters estimated with PAUP*. Two time constraints that had been previously used in molecular dating studies were selected to calibrate the molecular phylogeny. Based on Wares (2001), the divergence between the Panama trans-isthmian species pair *E. rhizophorae* and *E. eastropacensis* was constrained between 2.9 and 3.5 Mya. Based on Pérez-Losada *et al.* (2008) and Buckeridge (1983), the divergence of *Chamesipho brunnea* (i.e. the time separating the ingroup root from the present) was estimated to be 16–23 Mya (i.e. 19.5 + 3.5 Mya).

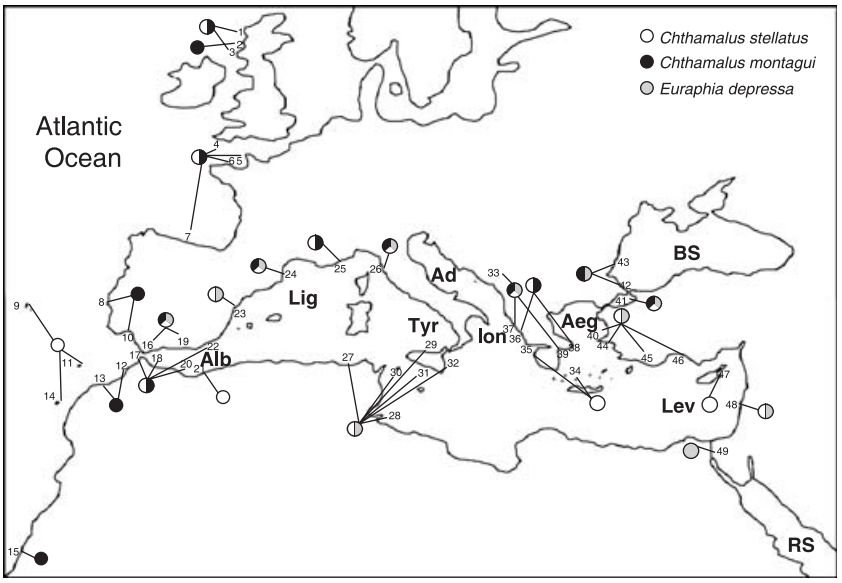


**Fig. 1** North Eastern Atlantic Ocean and Mediterranean indication map, Division of the Mediterranean to its main basins is indicated: Aeg, Aegean Sea; Alb, Alboran Sea; BS, Black Sea; Ion, Ionic Sea; Lig, Ligurian Sea; Tyr, Tyrrenian Sea; Lev, Levantine Sea; RS, Red Sea. Each sampling locations and occurrence of *Euraphia depressa*, *Chthamalus stellatus* and *C. montagui* in the Mediterranean, the Black Sea and the Eastern Atlantic is indicated. Number indicates one collecting site but when two collecting sites are very close they are indicated by a single number. 1. Seil Island, Scotland. 2. Loch Sween, Scotland. 3. Loch Caolisport, Scotland. 4. Plymouth, England. 5. St Malo and Cap Frehel, France. 6. Gijon, Spain 7. Cascais, Portugal. 8. Azores, Portugal. 9. Cadiz, Spain. 10. Madeira Island, Portugal. 11. Rabat, Morocco. 12. Agadir, Morocco. 13. Tenerife, Spain. 14. Dakar, Senegal. 15. Gibraltar, Gibraltar. 16. Cape Spartel, Tanger, Morocco. 17. Ceuta, Morocco. 18. Malaga, Spain. 19. Al Hoceima, Morocco. 20. Melilla and Kariet Arkeman, Morocco. 21. Cabo de Palos and Portman, Spain. 22. Valencia and Denia, Spain. 23. Banyuls sur Mer, France. 24. Villefranche sur Mer, France. 25. Ajaccio, Corsica, France. 26. Bastia, Corsica, France. 27. La Spezia, Italy. 28. Bizerte, Tunisia. 29. Jerba, Tunisia. 30. Pantelaria Island, Italy. 31. Agrigento, Sicily, Italy. 32. Cefalu, Sicily Italy. 33. Taormina, Sicily, Italy. 34. Dubrovnik, Croatia. 35. Igoumenitsa, Greece. 36. Parga, Greece. 37. Preveza, Greece. 38. Kamena Vourla, Greece. 39. Volos, Greece. 40. Koufonisi, Greece. 41. Buyuk Ada, Turkey. 42. Kilyos, Turkey. 43. Sozopol, Bulgaria. 44. Bodrum and Turgutreis, Turkey. 45. Marmaris, Turkey. 46. Patara, Turkey. 47. Anatalia, Turkey. 48. Cyprus. 49. Great Bitter Lake, Egypt. 50. Michmoret, Israel.

Molecular dating analyses were performed according to the Bayesian relaxed molecular clock approach (Thorne *et al.* 1998; Kishino *et al.* 2001) as implemented in the MULTIDIVTIME package (Thorne & Kishino 2002). First, the best fitting parameters for the COI gene were calculated via PAML (Yang 1997), using the F84 model (more complex models are not supported by the ESTBRANCHES program), five discrete gamma categories, and the topology identified with PAUP* as described above. These parameters were then entered in the ESTBRANCHES program to calculate the branch lengths of the rooted ML tree (*Tetraclita* was used as outgroup) and the variance–covariance matrix. Next, the Bayes Markov Chain Monte Carlo (MCMC) analysis was performed with the MULTIDIVTIME program, to approximate the posterior distributions of substitution rates and divergence times. Parameters of the analysis were set following the program guidelines. More specifically, we chose rttm = 1.95, rttmsd = 0.35, rtrate and rtratesd = 0.5987, brownmean and brownsd = 0.513 big time = 7.0. Other parameters were set to their defaults. The posterior distributions of node times were approximated by sampling values at every 100 steps over 100 000 MCMC steps, after discarding an initial burn-in of 100 000 steps. To verify that the program converged, computations were run twice, with the MCMC runs started using different initial values. In all cases, similar results were obtained for the two independent runs (the results of one of the two runs are presented).

## Results

### *Review of species distribution*

Our data on the distribution of the three species of chthamalids is based on a survey of 50 locations in the reported range of distribution of these species. Figure 1 summarizes our sampling stations and the occurrence of the three chthamalids, *E. depressa*, *C. stellatus* and *C. montagui*, at these locations. This data set represents a nearly complete collection within the described range of distribution of *E. depressa*, *C. stellatus* and *C. montagui*.

*Euraphia depressa*. *Euraphia depressa* was found only along the Mediterranean and Black Sea shores. This species is usually found in the splash zone, above the *C. stellatus* belt, but it is also found at lower tidal levels as a hypobiotic population in more sheltered zones. In this study, the most westward population of *E. depressa* was found to be at Kariet Arkmane, about 10 km east of Melilla, Morocco. Kensler *et al.* (1965) reported a hypobiotic population from Cape Spartel, at the western outskirts of Tanger, and from the splash zone near Ceuta; we could not confirm this finding. In Jerba, Tunisia, *E. depressa* occupies a lower belt and is found immersed in the water rather than at its typical habitat, the splash zone. At this locality *C. stellatus* is quite rare. The atypical relative distribution of these species in Jerba indicates the probable occurrence of a classical competitive interaction between intertidal barnacles. This interaction is comparable to the competition, described by Connell (1961), between *Semibalanus balanoides* and *C. stellatus* in Scotland, and *C. fragilis* and *S. balanoides* in New England (Wethey 1984).

In the Black Sea, *E. depressa* is quite common and is found together with *C. montagui* in Sozopol, Bulgaria and in Kilyos, Turkey. In Buyuk Ada, Sea of Marmara, it is found together with the other two chthamalids. Southward (1976) noted the presence of *E. depressa* in many samples from the northern coasts of the Black Sea, indicating that this species is widespread from the Danube delta to the Crimea. On the European shores, Kensler *et al.* (1965) reported the presence of this barnacle at Cape Trafalgar and Tarifa, which were not included in our sampling sites. Those authors even reported a small population in Cadiz on the Iberian Peninsula, but we could not confirm this finding; the only chthamalid found by us at this locality was *C. montagui*. Finally, Achituv & Safriel (1980) reported the presence of *E. depressa* in the great Bitter Lake (Suez Canal), but it is not known whether it has penetrated further south to the Gulf of Suez.

*Chthamalus stellatus*. Following the separation of *C. stellatus* into two distinct species, it has become evident that in some places both species are present, while in some localities only one can be found. Crisp *et al.* (1981) were the first to present maps of the distribution and relative abundance of the two species. Pannacciulli *et al.* (1997) refined this information by adding more data on the distribution of this species; however, in some areas the distribution has remained unclear, mainly due to lack of data from these areas.

Our own observations confirm the known distribution of *C. stellatus*. It is the most widely distributed species of the three chthamalids studied in this paper, and the only chthamalid found at the East Atlantic Islands: Madeira, the Azores and the Canary Island (Tenerife). It is not found along the Atlantic shores of Africa, but is present along all the Mediterranean shores examined during this study. It was absent from our sampling sites in the Black Sea and in Cadiz, St Malo, and at some of the western Scotland sites. A possible presence of *C. stellatus* in the Black Sea was indicated by Pannacciulli *et al.* (1997); however, we did not detect this species at our collection sites (Kilyos, Sozopol, Mamaia), and cannot confirm its presence in the Black Sea.

*Chthamalus montagui*. *Chthamalus montagui* is the only chthamalid species found along the western coast of Africa (i.e. Rabat, Agadir and Dakar). Samples from Cape Spartel, in the Tanger area, contained several specimens of *C. stellatus*, but the dominant chthamalid in these localities was *C. montagui*. At several sites along the Iberian Peninsula (i.e. Cadiz and Cascais), it is the sole chthamalid present. It is also the only chthamalid found by us at St Malo in Brittany and at Tayvalich in Western Scotland. In contrast, this species is not found east of Al Hoceima (Morocco) along the Mediterranean African shores. Based on our data, the eastern limit of *C. montagui* on the European coast of the Mediterranean is along the Aegean and Ionic coasts of Greece. However, we could not detect *C. montagui* in the vicinity of Patra on the Ionic Sea. It was not found by us in the island of Koufonisi in the Aegean basin, and from Cyprus, but it is found in the Sea of Marmara and in the Black Sea.

The present data set covers almost the entire distribution area of the three chthamalids. It includes material from the African coasts that were not surveyed previously. Moreover, in the present study we could refer to *C. stellatus* and *C. montagui* separately. Our observations agree with the chthamalid distribution reported by others (Dando & Southward 1980; Crisp *et al.* 1981; Pannacciulli *et al.* 1997), and provide information from newly sampled localities. The small differences noted, for example, the absence of *C. stellatus* in Cadiz, might be the result of temporal fluctuations in the distribution of the barnacle; sampling errors due to the rarity of a certain species at the limit of its distribution area; or its occurrence in a temporarily inaccessible microhabitat due to rough sea or other physical limitations.

### *Population genetics results*

For analyses 227 gene sequences were amplified. List of locations of collection of material used for gene amplification, GenBank accession numbers and voucher numbers are presented in Appendix 1 (Appendix 1 in Supporting Information).

Among the three genes studied, the COI sequences were found to be the most variable, while the ITS data sets were the least variable (Table 1). Our EF-1α data sets were less variable than the COI data sets (Table 1); however, surprisingly, they were the most informative concerning population structure, dividing the population into three regions, (i) the Eastern Atlantic, (ii) the Mediterranean, and (iii) the Black Sea and Aegean Basin.

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**Table 1** Molecular diversity indices for *Chthamalus* populations based on ITS, COI and EF-1α region sequences. Bp, sequence lengths; *n*, sample size; NH, number of haplotypes; *S*, number of polymorphic sites; *h*, values of haplotype diversity; π, nucleotide diversity. Standard deviations are indicated in parentheses.

| Species/Gene | Population | bp | *n* | NH | *S* | *h* | π |
|---|---|---|---|---|---|---|---|
| *Euraphia depressa* | | | | | | | |
| COI | Atlantic | 617 | 2 | 2 | 4 | 1.000 (0.500) | 0.006 (0.0032) |
| | Mediterranean | 617 | 11 | 6 | 8 | 0.727 (0.144) | 0.003 (0.0010) |
| | Aegean–Black Sea | 617 | 4 | 3 | 9 | 0.833 (0.222) | 0.008 (0.0022) |
| | All regions | 617 | 17 | 9 | 13 | 0.860 (0.068) | 0.005 (0.0009) |
| *Chthamalus stellatus* | | | | | | | |
| ITS | All regions | 408 | 30 | 1 | 0 | 0 | 0 |
| COI | Atlantic | 390 | 6 | 4 | 9 | 0.867 (0.129) | 0.010 (0.0022) |
| | Mediterranean | 390 | 14 | 12 | 18 | 0.978 (0.035) | 0.012 (0.0011) |
| | Aegean–Black Sea | 390 | 3 | 2 | 3 | 0.667 (0.314) | 0.005 (0.0024) |
| | All regions | 390 | 23 | 17 | 23 | 0.964 (0.026) | 0.011 (0.0010) |
| EF-1α | Atlantic | 418 | 10 | 5 | 10 | 0.867 (0.071) | 0.008 (0.0018) |
| | Mediterranean | 418 | 28 | 7 | 13 | 0.857 (0.034) | 0.012 (0.0006) |
| | Aegean–Black Sea | 418 | 2 | 2 | 2 | 1.000 (0.500) | 0.005 (0.0024) |
| | All regions | 418 | 40 | 13 | 18 | 0.918 (0.020) | 0.013 (0.0007) |
| *Chthamalus montagui* | | | | | | | |
| ITS | Atlantic | 625 | 18 | 8 | 12 | 0.850 (0.060) | 0.005 (0.0013) |
| | Mediterranean | 625 | 3 | 1 | 0 | 0 | 0 |
| | Black Sea | 625 | 4 | 3 | 10 | 0.833 (0.222) | 0.010 (0.0031) |
| | All regions | 625 | 25 | 10 | 13 | 0.873 (0.039) | 0.007 (0.0008) |
| COI | Atlantic | 593 | 23 | 17 | 24 | 0.972 (0.020) | 0.007 (0.0006) |
| | Mediterranean | 593 | 12 | 10 | 18 | 0.955 (0.057) | 0.007 (0.0018) |
| | Aegean–Black Sea | 593 | 14 | 10 | 15 | 0.890 (0.081) | 0.004 (0.0008) |
| | All regions | 593 | 49 | 37 | 65 | 0.983 (0.009) | 0.023 (0.0007) |
| EF-1α | Atlantic | 408 | 24 | 4 | 4 | 0.576 (0.097) | 0.002 (0.0005) |
| | Mediterranean | 408 | 10 | 3 | 2 | 0.600 (0.131) | 0.002 (0.0005) |
| | Aegean–Black Sea | 408 | 16 | 2 | 2 | 0.458 (0.095) | 0.002 (0.0005) |
| | All regions | 408 | 50 | 6 | 4 | 0.740 (0.030) | 0.003 (0.0003) |

*Euraphia depressa*. The COI sequences of *E. depressa* obtained in this study came from samples collected at 16 localities (Appendix 1, Fig. 1) ranging from Gibraltar and Malaga on the European coast of the western Mediterranean, and Kariet Arkmane on the African coast, Sozopol and Kilyos in the Black Sea and the Israeli population of Michmoret, at the Eastern range of its distribution. The alignment of the COI sequences was 617 bp long, and 13 positions were variable (Table 1). No significant genetic structure was detected in the AMOVA analysis or visible on the median joining network (Fig. 2). The variance among populations represents a negligible part of the total variance (–2.16%) and none of the pairwise comparisons of populations was significant (data not shown). As COI is the most variable marker studied, the ITS and EF-1α genes were not sequenced for this species.

*Chthamalus stellatus*. The COI data set includes 24 sequences from 15 localities (Appendix 1, Fig. 1). No significant genetic structure was detected in the AMOVA analysis. The variance among populations represents only 5.94% of the total variance ($F_{ST}$ = 0.059; *P*-value = 0.142). However, pairwise comparisons of populations indicate that Mediterranean and Aegean populations are marginally significantly different ($F_{ST}$ = 0.173; *P*-value = 0.041), while the Atlantic population is not significantly different from the Mediterranean or the Aegean populations (Table 2). Correspondingly, the *C. stellatus* median joining network (Fig. 3A) does not show any pattern of separation between populations.

The EF-1α data set, in contrast, shows significant genetic structure. The AMOVA analysis reveals that the variance among populations represents 22.18% of the total variance ($F_{ST}$ = 0.222; *P*-value < 0.001). The data set includes 40 haplotypes from 17 localities (Appendix 1, Fig. 1). Pairwise comparisons of populations indicate that the Atlantic population significantly differs from the Mediterranean and Aegean populations ($F_{ST}$ = 0.223–0.391; *P*-value < 0.016; Table 2). However, the Mediterranean and Aegean populations are not significantly different ($F_{ST}$ = 0.156; *P*-values = 0.133; Table 2). This might be the consequence of the small sample available for the Aegean Sea (only two sequences). In agreement with the AMOVA results, the median joining network (Fig. 4A)

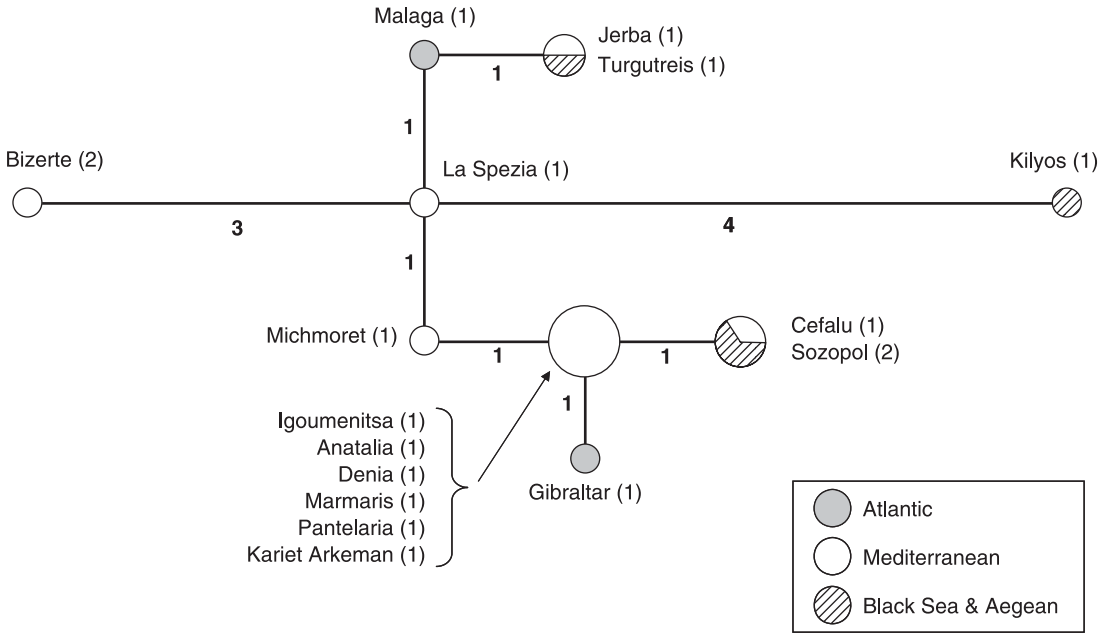


**Fig. 2** *Euraphia depressa* median joining network of COI haplotype. Numbers in parentheses indicate the number of haplotypes at each locality. Numbers along each branch designate the number of base differences among haplotypes. Each circle corresponds to a different haplotype, and its size is proportional to the corresponding haplotype frequency. Missing intermediates are indicated by small solid circles. For locations see Fig. 1.

**Table 2** Population pairwise $F_{ST}$ obtained with three molecular marker COI, EF-1α and ITS. *Chthamalus montagui* (below diagonal) and *C. stellatus* (above diagonal) (NS: nonsignificant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

| | Atlantic | Mediterranean | Aegean–Black Sea |
|---|---|---|---|
| COI | | | |
| Atlantic | — | –0.026$^{NS}$ | 0.146$^{NS}$ |
| Mediterranean | 0.779*** | — | 0.173* |
| Aegean–Black Sea | 0.854*** | 0.735*** | — |
| EF-1α | | | |
| Atlantic | — | 0.223*** | 0.391* |
| Mediterranean | 0.269** | — | 0.156$^{NS}$ |
| Aegean–Black Sea | 0.623*** | 0.505*** | — |
| ITS | | | |
| Atlantic | — | | |
| Mediterranean | 0.640*** | — | |
| Aegean–Black Sea | 0.218* | 0.300$^{NS}$ | — |

based on EF-1α sequences indicates that most haplotypes are not shared between populations.

The ITS data set of *C. stellatus* contains 30 samples from 26 localities. (Appendix 1, Fig. 1). All the sequences obtained were identical and it became apparent that this gene cannot be used to separate *C. stellatus* populations.

*Chthamalus montagui.* The COI data set of *C. montagui* contains 47 samples from 30 localities (Appendix 1, Fig. 1). The AMOVA analysis of the COI data indicates that the genetic variation among seas explain 81.21% of the total variance ($F_{ST} = 0.812$; $P$-value < $1.10^{-5}$). In addition, all pairwise comparisons between the three populations show significant $F_{ST}$ indices ($F_{ST} = 0.854$–$0.735$, $P$-values < $1.10^{-5}$; Table 2). This divergence is clearly visible in the median-joining network (Fig. 3B), which divides the COI haplotypes into three groups, 23 sequences belong to the East Atlantic–Alboran Sea, 12 to the Mediterranean and 14 to the Aegean–Black Sea.

The EF-1α data set of *C. montagui* contains 50 haplotypes from 14 localities (Appendix 1, Fig. 1). The AMOVA analysis indicates that the genetic variation among seas explains 53.69% ($F_{ST} = 0.537$; $P$-value < $1.10^{-5}$) of the total variance. All pairwise comparisons between the three populations show significant $F_{ST}$ indices ($F_{ST} = 0.269$–$0.623$, $P$-values < 0.002; Table 2). Similar to the COI sequences, these divergences too are clearly visible in the median-joining network (Fig. 4B).

The ITS data set of *C. montagui* contains 25 sequences from 20 localities (Appendix 1, Fig. 1). The AMOVA analysis indicates a significant population structure based on the ITS gene. The genetic variation among seas explains 44.3% ($F_{ST} = 0.443$; $P$-value = 0.003) of the total variance. Pairwise comparisons of populations indicate that the Aegean–Black Sea and Mediterranean populations are not significantly different ($F_{ST} = 0.300$; $P$-value = 0.26), while both populations

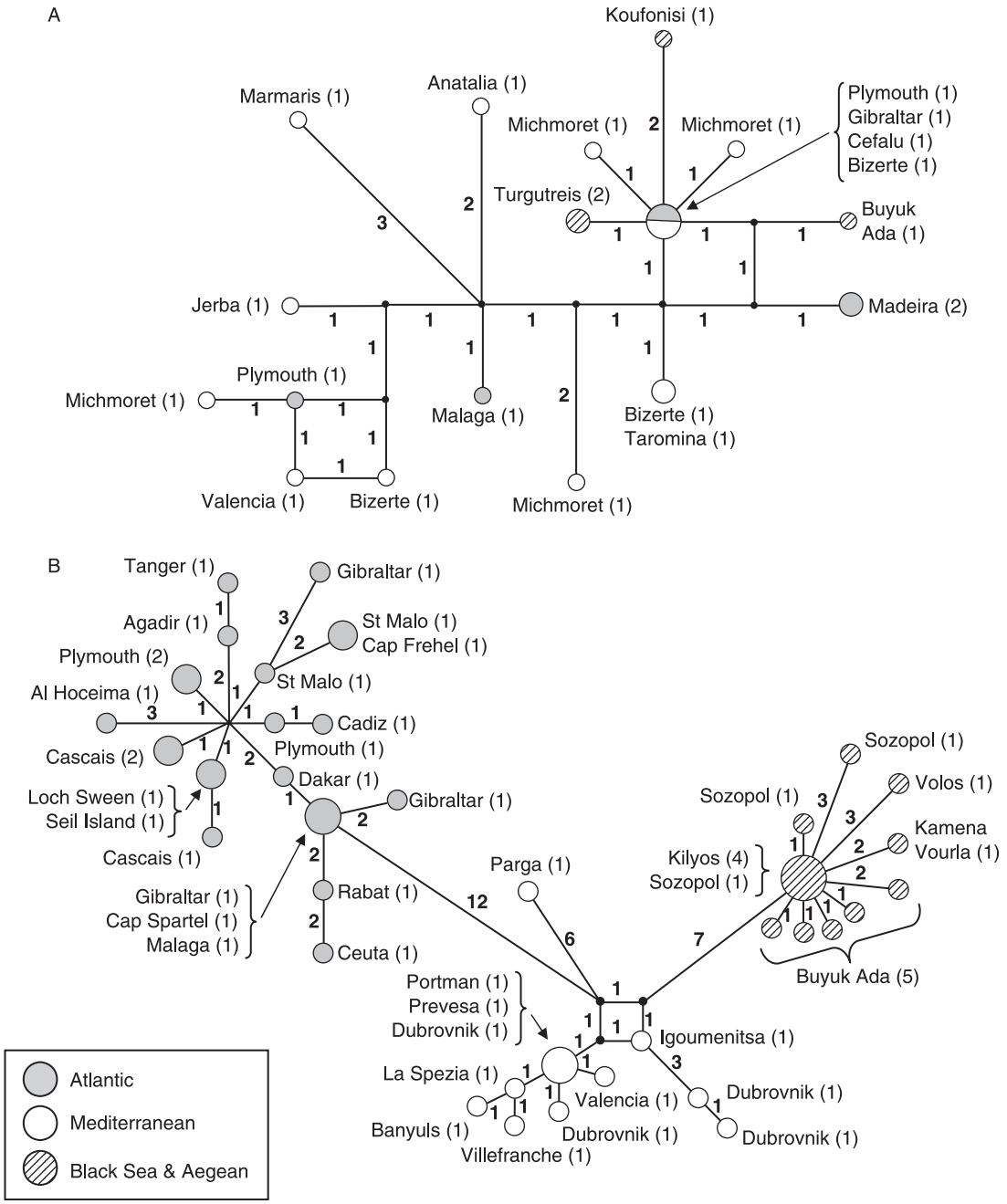


**Fig. 3** A, B. —A. *Chthamalus stellatus* median joining network of COI haplotype. —B. *Chthamalus montagui* median-joining network of the COI data. Numbers in parentheses indicate the number of haplotypes at each locality. Numbers along each branch designate the number of base differences among haplotypes. Each circle corresponds to a different haplotype, and its size is proportional to the corresponding haplotype frequency. Missing intermediates are indicated by small solid circles. For locations see Fig. 1.

are significantly isolated from the Atlantic population ($F_{ST}$ = 0.218–0.640; *P*-values < 0.03; Table 2). However, when indels are included in the analysis all pairwise comparisons between populations are significant (data not show). In agreement with the ANOVA result, the median-joining network (Fig. 5) based on this data set indicate that most Atlantic haplotypes are not shared by the Aegean–Black Sea and Mediterranean populations.

### *Phylogenetic and dating results*

The best model selected by AIC in MODELTEST 3.5 for COI was the TIM + I + Γ. The length of the aligned sequences

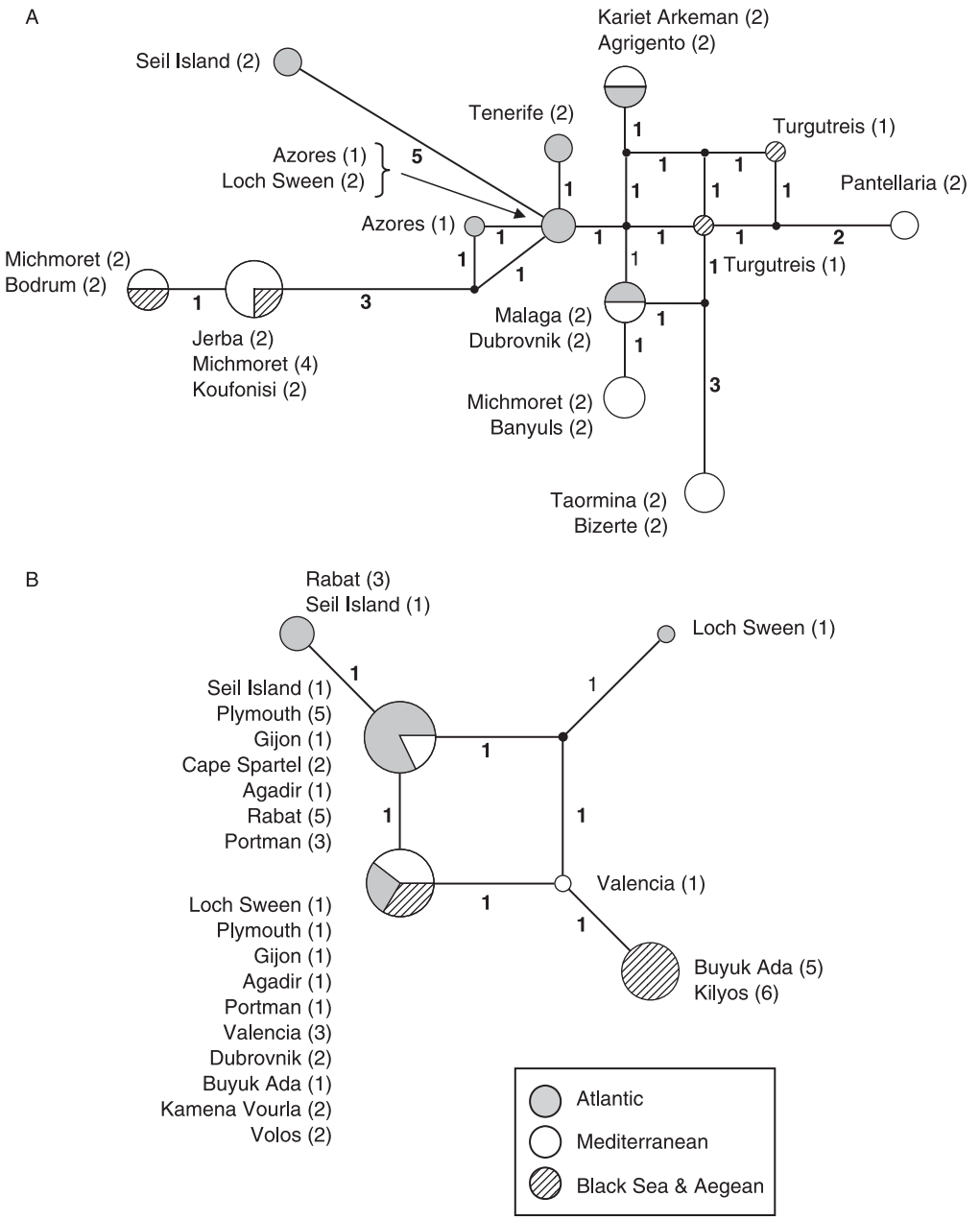


**Fig. 4** A, B. —A. *Chthamalus stellatus* median joining network of EF-1α haplotype. —B. *Chthamalus montagui* median-joining network of the EF-1α data. Numbers in parentheses indicate the number of haplotypes at each locality. Numbers along each branch designate the number of base differences among haplotypes. Each circle corresponds to a different haplotype, and its size is proportional to the corresponding haplotype frequency. Pies are indicative and do not represent frequencies of haplotype in sea basins. Missing intermediates are indicated by small solid circles. For locations see Fig. 1.

was 590 bp, of which 356 were constant. Among the remaining variable characters, 216 were parsimony informative and 18 uninformative. The topology of the trees generated by three methods of analysis MP, NJ and ML, was similar (data not shown). Our trees place the *Euraphia* samples on two inner clades within the *Chthamalus* clade (Fig. 6). This does not agree with Wares (2001), who rooted the *Chthamalus* trees with *Euraphia*. According to our results, *Chthamalus* is a paraphyletic taxon and *Euraphia* should be synonymized with *Chthamalus*. However, because of the low bootstrap support

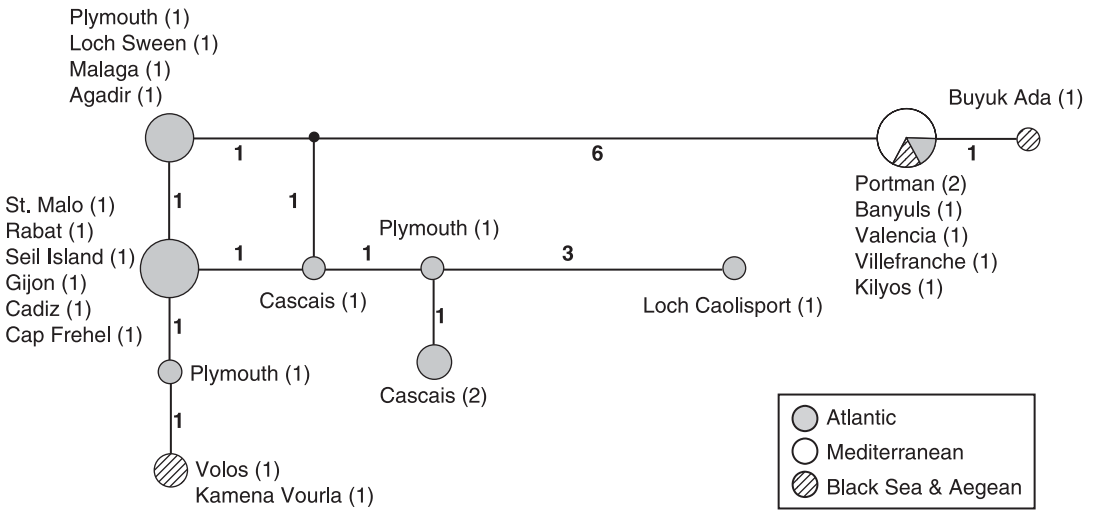


**Fig. 5** *Chthamalus montagui* median-joining network of the ITS data set. Numbers in parentheses indicate the number of haplotypes at each locality. Numbers along each branch designate the number of base differences among haplotypes. Each circle corresponds to a different haplotype, and its size is proportional to the corresponding haplotype frequency. Missing intermediates are indicated by small solid circles. For locations see Fig. 1.

additional gene sequences are necessary to confirm the phylogenetic position of *Euraphia*. Our findings call for a taxonomic revision of *Chthamalus* and *Euraphia*. In our previous analyses (Fisher *et al.* 2004), based on 18S rDNA sequences, *Euraphia*, *Chthamalus* and *Tetrachthamalus* were grouped on the same clade. However, this analysis included only two species of *Chthamalus* and two species of *Euraphia*. It is therefore hard to draw solid conclusions from this analysis. The present analysis indicates the need for a large-scale phylogenetic study of Chthamalidae.

The molecular clock hypothesis was significantly rejected ($P$-value < 0.01) based on COI sequences. This result supports the use of a relaxed-clock model for dating analysis. The divergence between the intertidal species pairs of chthamalids *C. proteus*/*C. mexicanus* and *E. eastropacensis*/*E. rhizophorae*, which are found on opposite sides of the Panama isthmus, has been estimated to correspond to the uplift of the Panama isthmus (Wares 2001). Our results do not support the sister clade relationships of *C. proteus* and *C. mexicanus*, suggesting that the divergence of these species is more ancient. Consequently, only the divergence between *E. eastropacensis* and *E. rhizophorae* was used as a calibration point. Our data indicate that *C. stellatus* and C. *montagui* diverged from a common ancestor 14 ± 4 Mya (Fig. 5). Within *C. montagui*, the Atlantic haplotypes and the Mediterranean haplotypes, including Aegean–Black Sea individuals, diverged 5.13 (± 2.13) Mya while the Aegean–Black Sea haplotypes split from the Mediterranean one 4.10 (± 1.91) Mya.

## Discussion

### *Current genetic structure and its maintenance*

*The divergence between the Atlantic and Mediterranean populations.* Our ITS, COI and EF-1α results regarding *C. montagui* agree with previous allozyme studies, indicating a significant genetic differentiation between the Mediterranean and East Atlantic populations (Dando & Southward 1981; Pannacciulli *et al.* 1997). The Atlantic population is a sister group of the Mediterranean–Black Sea population.

Dando & Southward (1981) suggested that, in the case of *C. montagui*, the history of the Mediterranean, combined with the present hydrographic pattern, might have promoted and maintained differentiation of the Mediterranean populations. Both they and Pannacciulli *et al.* (1997) pointed out that the separation between the Mediterranean and the East Atlantic populations of *C. montagui* is not found at the Straits of Gibraltar but rather more slightly eastward. In agreement, our own results confirm that the Gibraltar and Malaga samples are related to the Atlantic population and not to the Mediterranean one (Fig. 3B). Hydrographic barriers, in particular the Almeria–Oran front, may be involved in the observed separation. This front is a zone of turbulence in the Alboran Sea (Tintore *et al.* 1988), possibly restricting larval dispersal in both directions and forming a genetic barrier. Larvae of *C. montagui* from one side of the Almeria–Oran front may become trapped in hydrological structures and isolated from the other genetic pools. Genetic discontinuities at the Almeria–Oran front have also been reported, for example, for the mussel *Mytilus galloprovincialis* (Quesada

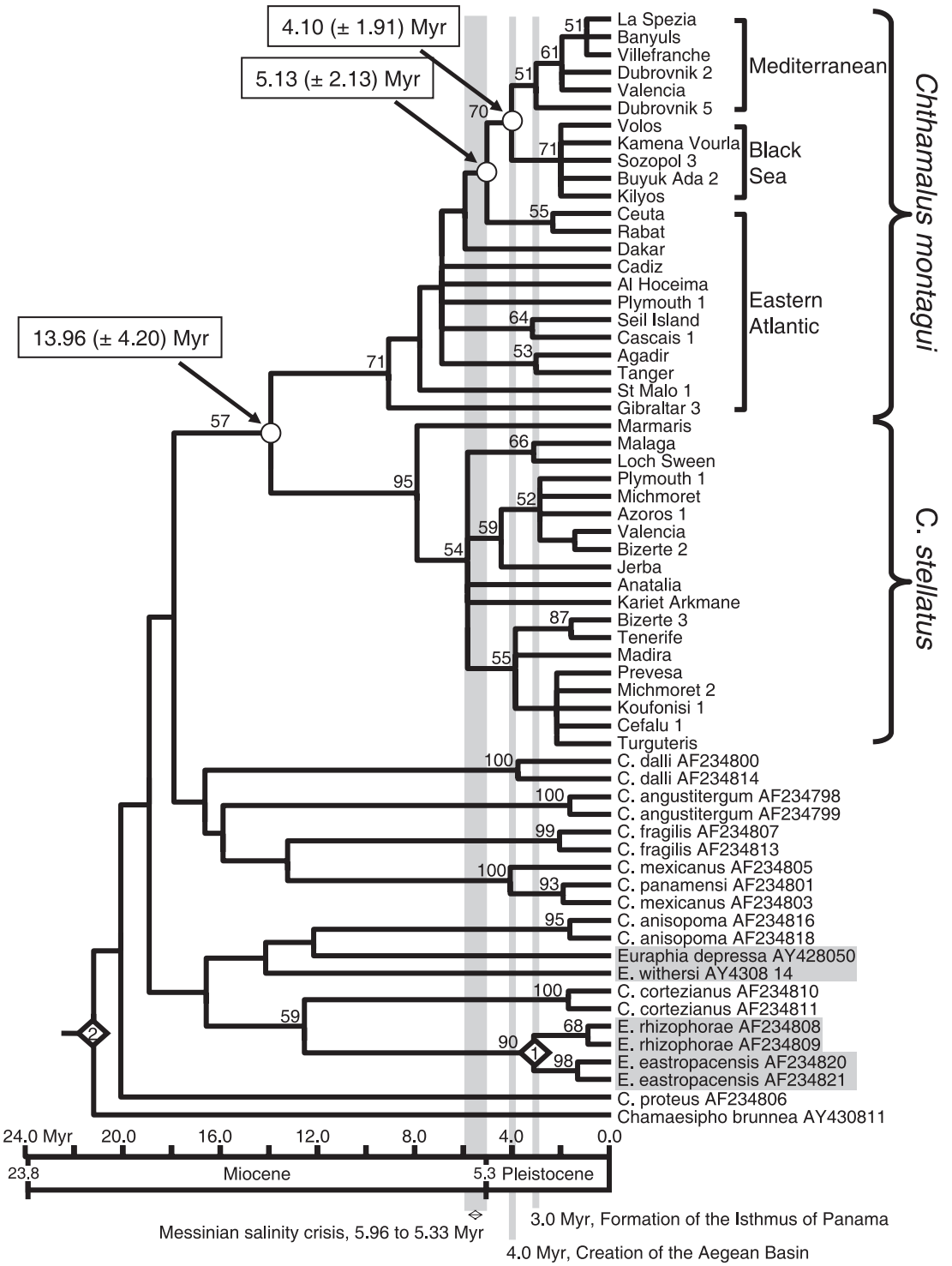


**Fig. 6** Molecular time scale of *Chthalamus* diversification. The chronogram was obtained by using the topology of the best ML tree, rooted with *Catomerus* sp. and using a Bayesian relaxed clock method. ML bootstrap supports are indicated above the corresponding branches. Fossil constraints are indicated by diamonds on the corresponding nodes: 1, 2.9–3.5 Mya; 2, 16–23 Mya (detailed references are provided in the Materials and methods section). The divergence date and the confidence interval of the main diversification events of *Chthalamus montagui* are indicated near the corresponding nodes. Major geological events are indicated below the time scale. Representative of the genus *Euraphia* are indicated in grey to stress the polyphyly of this genus.

*et al.* 1995a,b), and the cuttlefish *Sepia officinalis* (Perez-Losada *et al.* 2002). Additional data on the existence of a transition zone at the Almeria–Oran front are being acquired and have recently been summarized by Patarnello *et al.* (2007). However, not all biota follow this population structure.

While the EF-1α sequences of *C. stellatus* isolate the Atlantic population from the Mediterranean and Aegean ones; the COI and ITS data sets, surprisingly, do not show significant population structure for this species. This might be due to the differences in sample size (*e.g.* 23 COI sequences *vs*. 40 EF-1α sequences). It might also reflect different haplotype sorting for mitochondrial and nuclear genes. These results indicate that nuclear markers should indeed be sequenced; even if *a priori* they are less variable than mitochondrial ones.

Differences in the population genetics of the two species of *Chthamalus* might also be attributed to different larval dispersal. The larvae of *C. stellatus* are somewhat larger than those of *C. montagui* (Power *et al.* 1999). In culture, at 19 °C, *C. stellatus* develop more slowly than *C. montagui*, *C. stellatus* larvae reached stage VI in 16 days compared to 11 days for larvae of *C. montagui* at the same temperature (Burrows *et al.* 1999). It is possible that the size and slower development enable the larvae of *C. stellatus* to disperse more than those of *C. montagui* and cross hydrographic barriers.

*The divergence between Mediterranean and Aegean–Black Sea populations.* The maintenance of the genetic divergence observed between the Aegean–Black Sea samples of *C. montagui* and the Mediterranean ones can also be attributed to the existence of separate marine water masses. The limited water exchange between the Aegean–Black Sea and the eastern Mediterranean reduces the possibility of gene flow between these two basins. The circulation between the Ionian and Levantine basins of the Mediterranean and the Aegean basin is limited by the arc of the Cycladic archipelago. The surface circulation in the Aegean basin is influenced by cold northern winds and by upwelling of cold water along the Anatolian coast. These winds and currents move water from the Black Sea toward the Mediterranean. In addition there is an influx of low salinity water, 26‰, in the summer, from the Black Sea by the Straits of Bosphorus–Marmara Sea–Dardanelles corridor. The warmer and high-salinity (38‰–39‰) Mediterranean waters flow north along the eastern Aegean Sea, plunging beneath the low-salinity surface layer in the Aegean Sea (Pektas 1958). The Mediterranean waters occupy the entire Marmara basin only below 20–30 m depth. Consequently, most of the time, the planktonic larvae carried by the Mediterranean waters sink and do not reach the intertidal zone. Limited currents flow from the Mediterranean to the Aegean–Black Sea in autumn. However, these currents cannot create a gene flow between populations as they do not coincide with the breeding season of these species. Data on the breeding season suggests a summer reproduction, and indeed the population of *C. montagui* from Loch Sween, Scotland, breeds during the summer (Achituv & Barnes 1976). In the Eastern Mediterranean *C. stellatus* breeds in July–August (Mizrahi & Achituv 1990). Consequently, water circulation supports a limited one-way migration of larva from the Black Sea toward the Mediterranean. The smaller size of the Aegean–Black Sea population, as well as the different salinity conditions, suggests that a stronger genetic drift might be responsible for the presence of different haplotypes when compared with the Mediterranean population. However, adaptation and selection cannot be ruled-out as possible explanation to the genetic differences observed. Genetic divergences between Atlantic, Mediterranean and Aegean–Black Sea populations were also shown for the planktonic copepods *Calanus helgolandicus* and *C. euxinus* (Papadopoulos *et al.* 2005).

***Dating of the genetic differences***

Our estimates of divergence time indicate a separation of the Atlantic and Mediterranean populations of *C. montagui* approximately 5 Mya, while the Aegean–Black Sea population split from the Mediterranean population approximately 4 Mya. Interestingly, both dates correspond to major geological events in the Mediterranean basin (Fig. 6).

The separation between the Atlantic and East Mediterranean COI populations haplotypes of *C. montagui* coincide with the restoration of the marine fauna in the Mediterranean after the Messinian lacustrine 'Lago Mare' phase. We presume that the border between these two genetic populations was then maintained by the Almeria–Oran front. A similar example of allopatric speciation following the Messinian salinity crisis was described for the cyprinodont fish *Aphanius fasciatus* (Triantafyllidis *et al.* 2005). Additional examples have been given for terrestrial and fresh-water animals, which are generally unable to cross salt-water barriers (Beerli *et al.* 1996).

Similarly, the separation between the Aegean–Black Sea clade of *C. montagui* from the Mediterranean clade corresponds to the creation of the Aegean Sea but not to the creation of the Black Sea during the Pliocene. The epicontinental Aegean Sea with its maze of islands was created in the Pliocene by the movement of the Anatolian plate against the Eurasian plate. The establishment of the brackish-water Black Sea population occurred only at a later stage from the Aegean population. The Black Sea was a lacustrine shallow water basin not connected to the Mediterranean Sea (Newman & Ross 1976). The first invasion of Mediterranean waters into the Black Sea occurred during the Karadenizian transgression, between 0.6 and 0.5 Mya, and is correlated with the strong glaciations events in the northern hemisphere (Yanko *et al.* 1984; Yanko 1990). We suggest that the Aegean

waters brought larvae of benthic Mediterranean biota, including *C. montagui*, into the Black Sea. The separation of the populations is maintained by the present hydrographic regime, as described above. The fate of *C. montagui* during the periods of freshening episodes, in particular those associated with the spilling of Caspian waters and fauna into the Black Sea (Zubakov 1988), is unclear. We might speculate that during those periods a refuge population continued to exist within the Black Sea, or that the Black Sea was repopulated by larvae from the nearby Aegean Sea via the Marmara Sea.

This study represents the most detailed phylogeographical analysis of an ecologically important intertidal Mediterranean species to date, and shows that geological events have strongly shaped the current diversity pattern of this fauna. Further large-scale studies, including not only intertidal but also bentic and pelagic species, are needed in order to complete our understanding of the speciation events and gene flow occurring within the Mediterranean basin and between the Atlantic and Mediterranean populations.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix 1** List of sites of collection of material used in this study, for location see figure 1, number of site are indicated on map. Accession numbers of sequences are GenBank accession number Voucher numbers are collection number of the Invertebrate Section, Zoological Collection The Hebrew University of Jerusalem.

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