

A Star Is Torn – Molecular Analysis Divides the Mediterranean Population of *Chthamalus stellatus*

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Abstract

The star barnacle, *Chthamalus stellatus* Poli, populates the Mediterranean Sea, the North-Eastern Atlantic coasts, and the offshore Eastern Atlantic islands. Previous studies have found apparent genetic differences between the Atlantic and the Mediterranean populations of *C. stellatus*, suggesting possible geological and oceanographic explanations for these differences. We have studied the genetic diversity of 14 populations spanning from the Eastern Atlantic to the Eastern Mediterranean, using 63 genomic polymorphic sites. We have found that these populations form four distinct clusters: Eastern Atlantic, Western Mediterranean, Mid-Mediterranean and Eastern Mediterranean, with evident connectivity between them. We examined here environmental conditions like surface currents, water salinity and temperature as probable factors that have formed the population structure. We suggest that *C. stellatus* is a suitable marine animal for studying how geological events and hydrographic conditions shape the fauna in the Mediterranean Sea.

INTRODUCTION

Barnacles of the genus *Chthamalus* are a major worldwide component of the rocky intertidal zone of tropical and sub-tropical shores, with few species penetrating into temperate latitudes. The star barnacle, *Chthamalus stellatus* Poli, is a species with wide geographical range, covering the Mediterranean Sea, the North-Eastern Atlantic coasts, and the offshore Eastern Atlantic islands: Madeira, Azores, and Cape Verde (Southward, 1976; Stubbings, 1967). Recently it was demonstrated that the Cape Verde Islands population is an independent Evolutionary Significant Unit (ESU), a sister clade of *C. stellatus* (Tikochinski, Motro, Simon-Blecher, & Achituv, 2020). *C. stellatus* is absent from the North-Western Africa Atlantic coast, where it is replaced by another species of *Chthamalus*, *C. montagui* Southward, which is widely distributed in the Mediterranean. Burrows, Hawkins, & Southward (1992; 1999) suggested that differences in the distribution of these two species and especially the absence of *C. montagui* from the Atlantic islands are related to the lifespan of their pelagic stage. The larvae of *C. stellatus* are larger than those of *C. montagui*, they live longer and can disperse further offshore. This strategy appears to allow *C. stellatus* to maintain populations on offshore islands. However, *C. stellatus* could not spread further south to the North-West African shores and the neighboring islands, presumably because of incompatible salinity and temperature conditions (Bhatnagar & Crisp, 1965). Several molecular studies have aimed to resolve the population structure of Chthamalids in the Mediterranean Sea and the oceanographic forces leading to this structure. Pannacciulli, Bishop, & Hawkins (1997) showed that in both species there is a separation between the Atlantic and Mediterranean populations, with *C. montagui* exhibiting a greater separation than that of *C. stellatus*. Since the Mediterranean Chthamalids that are located close to the Strait of Gibraltar resembled the Atlantic ones, they hypothesized that the Almeria–Oran Front in the Alboran Sea is a major barrier to larval dispersion and therefore restricts gene flow between the Atlantic and the Mediterranean of the two *Chthamalus* species. The Almeria–Oran

Front is regarded as a major transition zone between the Atlantic and the Western Mediterranean for 58 marine species, including *C. montagui* (Patarnello, Volckaert, & Castilho, 2007). Using three molecular markers, Shemesh, Hochon, Simon-Blecher, & Achituv (2009) examined the distribution of three Chthamalid species, *C. stellatus*, *C. montagui* and *Euraphia depressa*, in the Eastern Atlantic, the Mediterranean Sea and the Black Sea. Their wide selection of sampling sites revealed a significant genetic structure among the populations of these Chthamalids. However, for *C. stellatus*, the structure was based only on one marker, EF1.

In our presented study we used two molecular markers to examine the distribution of *C. stellatus* in the Mediterranean. While the number of collecting sites (14) is slightly smaller than in the abovementioned studies, they adequately represent both the Eastern and the Western basins of the Mediterranean, as well as a few locations in the Eastern Atlantic. Sample sizes from each location, nevertheless, are quite large, enabling us to perform population genetic analyses and to construct dendrograms which are based on population samples, and not on just a single or a very few individuals from each location. Thus, we get a more accurate and more reliable picture of the population structure than that depicted by previous studies, which used only a single or a very few individuals to represent an entire local population.

Due to its life cycle and possible mode of settlement and distribution, we suggest that *Chthamalus stellatus* is a suitable marine animal for studying how geological events and hydrographic conditions shape the fauna in the Mediterranean Sea.

MATERIALS AND METHODS

Samples of *Chthamalus* were collected in the intertidal rocks by us or were donated by colleagues; the barnacles were fixed and stored in 96% ethanol. The samples used for this study are stored at the Israeli National Natural History Collections at the Hebrew University of Jerusalem (for details see Supplementary Material 1).

We examined *Chthamalus stellatus* populations from 14 different locations (Figure 1): Five locations are on the Eastern Mediterranean basin – Bodrum (Aegean Sea, Turkey), Dubrovnik (middle Adriatic Sea, Croatia), Fažana (north Adriatic Sea, Croatia), Larnaca (Cyprus) and Rethymno (Crete). Three locations are on the Western Mediterranean basin – Bastia (Corsica), Málaga (north Alboran Sea, Spain) and Melilla (south Alboran Sea, Spain). Three locations are in-between the Eastern and the Western basins, in the Mid-Mediterranean zone – Birżebbuġa (Malta), Bizerte (Tunisia) and Pantelleria (Strait of Sicily, Italy). The three remaining locations are in the Northeastern Atlantic Ocean – Biarritz (Bay of Biscay, France), Canary Islands (Macaronesia) and Madeira (Macaronesia).

Individuals from these 14 populations were characterized by two nuclear genetic markers – EF1 and NaKA. Each population included at least 15 individuals for each marker (See Table 1). GenBank accession numbers used in our study are MT296012-247 and MT633576-654 for EF1, MT296286-518 and MT633655-737 for NaKA.

DNA Extraction

DNA was extracted from soft tissue of 349 specimens of *Chthamalus stellatus* that were collected from 14 different locations in the Mediterranean and the Eastern Atlantic Sea using the AccuPrep® genomic DNA extraction kit (Bioneer, Daejeon, Korea).

PCR and Sequencing

Three gene segments were amplified using previously described primers for the nuclear genes Na-K-ATPase (NaKA) and elongation factor 1 α (EF1) (Wares, Pansky, Pitombo, Daglio, & Achituv, 2009). PCR reactions were carried out in 25- μ l reaction volumes containing 1X PCR buffer (including 1.5 mM MgCl₂), 0.2 mM of each dNTP, 1 μ M of each primer, 1 unit of Super-Term Taq polymerase (Hoffmann-La Roche), and about 100 g template DNA. PCR reactions were processed in an MJ Research thermal cycler with the following thermal regime: an initial step of 2 min at 95°C followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 57°C,

and 1 min at 72°C, followed by 3 min at 72°C and then held at 15°C. PCR products were visualized on 1.5% agarose gels and sequenced bidirectionally using the PCR primers on an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

Statistical Analyses

After alignment, we discarded the monomorphic nucleotide positions, and considered only those polymorphic. Thus, we were left with 30 positions for EF1 and 33 positions for NaKA, and the following analysis was done for each marker separately: For each of the 14 populations, we calculated the distribution of the four different nucleotides (A, C, G and T) in each of the nucleotide positions. Note that if x_1, x_2, x_3 and x_4 are the proportions of A, C, G and T in a position, then is a point in the four-dimensional space, whose sum of coordinates is 1. The corresponding point $x^* =$ lies on the surface of the four-dimensional unit sphere. Next we compared, for each position, the distribution of the four different nucleotides between the 14 different populations, by using a distance or a similarity metric (see below). Thus, we get for each position, 91 pairwise distances (or similarities). These distances (or similarities) were averaged over all relevant positions of the marker, to obtain the final pairwise distances (or similarities) for the marker. The results were arranged in a 14×14 symmetric distance (or similarity) matrix. We then we added the two distance (or similarity) matrices (one for each marker) to obtain the comprehensive distance (or similarity) between the populations. This final matrix served for constructing population dendrograms or for performing a Principal Coordinates (PCoA) analysis.

We used three different distance measures, the squared Euclidean distance, a modified squared chord distance and the Manhattan (or city block) distance, and one similarity measure, a modified Morisita's similarity coefficient. If x_1, x_2, x_3 and x_4 are the proportions of A, C, G and T in population 1, and y_1, y_2, y_3 and y_4 are these proportions in population 2, then: the squared Euclidean distance = ; the modified squared chord distance = (which is actually the squared length of the chord connecting x^* and y^* on the unit sphere); the Manhattan distance = ; and the modified Morisita's similarity coefficient = .

We considered three different amalgamation procedures – UPGMA (unweighted pair group method with arithmetic mean), minimum variance (Ward's method) and furthest neighbor (complete-linkage clustering), as well as PCoA analysis, using the MVSP software, Kovach Computation Services 2013. We thus can construct ten different unconstrained trees (i.e., all different combinations, except that minimum variance is only applicable for the squared Euclidean or the squared chord distances).

For each population we calculated, separately for each marker, the mean number of different alleles per position. We then averaged over the two markers to obtain the overall mean number of different alleles per position in this population. In addition, we calculated for each population the mean expected heterozygosity of a marker, defined as, where p_k and q_k are the proportions of A, C, G and T in position k ($k = 1, 2, \dots, N$, where N is the number of positions in the marker). We then averaged the measures of the two markers, to obtain the expected heterozygosity of the relevant population. Similarly, for each population, we calculated the percentage of polymorphic positions in each marker, and then averaged the percentages of the two markers, to obtain the polymorphism measure of the relevant population.

We call an allele which is present in a population X (with a frequency of at least 1%), an *exclusive* to that population, if it is present in that population but not in other population or populations to which we compare population X.

Statistical tests were carried out using IBM SPSS Statistics 26. All p -values are given for a two-tailed alternative.

RESULTS

The four genetic distance/similarity matrices are presented in Supplementary Material 2. They are the basis for constructing the population dendrograms. Six of the ten different dendrograms exhibit the same topology of two main branches, where each is divided into two clusters. Cluster 1, henceforth the Eastern Atlantic cluster (EA), consists of Biarritz, the Canaries and Madeira; cluster 2, the Western Mediterranean cluster

(WM), consists of Bizerte, Málaga and Melilla. Clusters 1 and 2 both belong to the same main group. Cluster 3, the Mid-Mediterranean cluster (MM), consists of Bastia, Birżebbuġa and Pantelleria; Cluster 4, the Eastern Mediterranean cluster (EM), consists of Bodrum, Dubrovnik, Fažana, Larnaca and Rethymno. Clusters 3 and 4 both belong to the same main group. Two representatives of this configuration are given in Figs. 2 and 3. We checked the robustness of the topology presented in this group of dendrograms by repeating the Ward's analysis of Fig. 2, 14 times – each time omitting a different location (thus remaining with 13 locations). Except for the omitted location, the remaining structure of the four above-mentioned clusters did not change. Further demonstration of this division into four clusters is displayed by the PCoA analysis in Fig. 4. The two axes account for 86.5% of the variance.

Two of the ten different dendrograms exhibit a slightly different topology of two main branches. One consists of the Eastern Atlantic cluster only, whereas the other is divided into two sub-branches. One of these sub-branches consists of the Eastern Mediterranean cluster and the other is further divided into two clusters – the Western Mediterranean and the Mid-Mediterranean clusters. Thus, the setup of the four above-mentioned clusters also exists in these two dendrograms. A representative of this configuration is given in Fig. 5.

The remaining two dendrograms place Pantelleria in the Western Mediterranean cluster, next to the geographically nearby Bizerte. A representative of this configuration is given in Fig. 6.

The mean number of different alleles per position, the mean expected heterozygosity measures and the mean percentage of polymorphic positions in each population are presented in Table 2.

The distribution of the number of alleles per position is not statistically different from a normal distribution (Kolmogorov-Smirnov test, $p = 0.364$). In accordance with the results of the multi-variate analysis, as presented by the genetic dendrograms, we divide the 14 populations into four groups: EA (the Eastern Atlantic cluster), WM (the Western Mediterranean cluster), MM (the Mid-Mediterranean cluster) and EM (the Eastern Mediterranean cluster). The means ($\pm$ se) of each group are: 1.361 ± 0.030 , 1.782 ± 0.042 , 1.857 ± 0.039 and 1.942 ± 0.020 , for EA, WM, MM and EM, respectively (see Fig. 7a). Using a one-way ANOVA for testing the differences in the mean number of alleles per position between these four groups, we get $F_{3,10} = 68.849$, $p < 0.001$. Post-hoc: EA vs. WM $p < 0.001$; EA vs. MM $p < 0.001$; EA vs. EM $p < 0.001$; WM vs. MM $p = 0.841$; WM vs. EM $p = 0.020$; MM vs. EM $p = 0.408$.

The distribution of the expected heterozygosity is not statistically different from a normal distribution (Kolmogorov-Smirnov test, $p = 0.464$). The means ($\pm$ se) of each group are: 0.069 ± 0.008 , 0.154 ± 0.007 , 0.205 ± 0.005 and 0.230 ± 0.003 , for EA, WM, MM and EM, respectively (see Fig. 7b). Using a one-way ANOVA for testing the differences in expected heterozygosity between these four sub-clusters, we get $F_{3,10} = 169.899$, $p < 0.001$. Post-hoc: EA vs. WM $p < 0.001$; EA vs. MM $p < 0.001$; EA vs. EM $p < 0.001$; WM vs. MM $p = 0.001$; WM vs. EM $p < 0.001$; MM vs. EM $p = 0.041$.

The distribution of the percentage of polymorphic positions is not statistically different from a normal distribution (Kolmogorov-Smirnov test, $p = 0.295$). The means ($\pm$ se) of each group are: $33.9\% \pm 2.7\%$, $67.2\% \pm 3.0\%$, $72.6\% \pm 3.4\%$ and $77.5\% \pm 1.62\%$, for EA, WM, MM and EM, respectively (see Fig. 7c). Using a one-way ANOVA for testing the differences in the percentage of polymorphic positions between these four groups, we get $F_{3,10} = 57.722$, $p < 0.001$. Post-hoc: EA vs. WM $p < 0.001$; EA vs. MM $p < 0.001$; EA vs. EM $p < 0.001$; WM vs. MM $p > 0.999$; WM vs. EM $p = 0.084$; MM vs. EM $p > 0.999$.

Focusing more on the differentiation between the populations of the Eastern and the Western Mediterranean basins, we compared the number of exclusive alleles in each. Since the number of exclusive alleles is influenced by the number of locations in a basin, for a balanced comparison we considered only three of the five locations in the East (i.e., Bodrum, Larnaca and Rethymno), and compared them to the three locations in the West (i.e., Bizerte, Málaga and Melilla). In the East, we counted a total of 15 exclusive alleles (that is, 10 alleles of EF1 and 5 alleles of NaKA that are present in the East, but not in the West), compared to only 3 alleles (3 of EF1 and none of NaKA) that are exclusive to the West. Using the exact binomial test, with equal number of exclusive alleles in each basin as the null hypothesis, we obtained a p -value of 0.008.

DISCUSSION

Our study presents for the first time a detailed and an accurate picture of *Chthamalus stellatus* population distribution in the Mediterranean Sea. Previous studies (Crisp, Southward, & Southward, 1982; Pannacciulli et al., 1997; Shemesh et al., 2009) used only a single or a very few individuals to represent each local population. Using 63 SNPs in 14 populations, each consisting of 15–28 specimens, enabled us to get a more reliable picture of this barnacle’s populations in the Mediterranean as well as the Eastern Atlantic.

The various analyses performed in our study clearly demonstrate four distinct clusters (see fig. 2-5). The Eastern Atlantic (EA) cluster, consists of the western peripheral populations of *C. stellatus*, namely, Biarritz, the Canaries and Madeira. This cluster was long-established in previous studies that show the separation between the Atlantic and the Mediterranean populations of *C. stellatus* (Crisp et al., 1982; Pannacciulli et al., 1997; Shemesh et al., 2009). The Western Mediterranean (WM) cluster, which consists of Bizerte, Málaga and Melilla, is a sister cluster to the EA cluster. Two of the cluster’s locations, Málaga and Melilla, are geographically adjacent to the Strait of Gibraltar, and therefore naturally influenced by the surface current entering from the Atlantic and flowing along the Northern Africa coast (Millot & Taupier-Letage, 2005). The third location (Bizerte) is geographically quite distant, closer to the Mid-Mediterranean locations studied here, but probably still influenced by the flow along the coast. The resemblance between the Atlantic populations and a distant Mediterranean population of barnacles is reported here for the first time. The Eastern Mediterranean (EM) cluster, that consists of Bodrum, Dubrovnik, Fažana, Larnaca and Rethymno, is well-defined in all our different analysis approaches. The Mid-Mediterranean (MM) cluster consists of Bastia, Birżebbuġa and Pantelleria. Bastia, at the northwestern coast of Corsica, belongs geographically to the Western Mediterranean basin. The other two MM locations are also close to the Western Mediterranean. Nevertheless, the MM cluster emerged as a sister group to the EM cluster in all our analysis approaches. While this is not surprising for Birżebbuġa and Pantelleria, the Bastia population is an integral part of this cluster. This finding is supported by a previous *C. stellatus* study, clustering Bastia, Genoa and nearby locations with MM populations and not with the Atlantic cluster (Pannacciulli et al., 1997). The only exception to this cluster analysis is presented in two of the 10 trees (Fig. 6) in which the population of Pantelleria is clustered with the WM.

Establishment of new barnacle populations, as well as other sessile animals, mainly occurs by current-assisted larval distribution (Johannesson, 1988). The pelagic stage of *C. stellatus* is about 22 days, allowing for extensive connectivity between populations (Pannacciulli, Manetti, & Maltagliati, 2009). The well-documented surface current, coming from the Atlantic Ocean and entering the Mediterranean through the Strait of Gibraltar can easily bring barnacle larvae to the shores of Málaga and Melilla. Previous studies have included these locations as part of the Atlantic region population, separated from the rest of the Mediterranean by the Almeria-Oran Front (Pannacciulli et al., 1997). But, according to our results, it appears that the Almeria-Oran Front is not impermeable to the propagules of *C. stellatus* which is reflected in the resemblance of the Western Mediterranean populations of both sides of the front. Patarentello et al. (2007) reviewed over 20 population studies of 58 different marine species across the Atlantic-Mediterranean range, aiming to comprehend phylogeographical patterns, including potential barriers in the Mediterranean Sea. The patterns obtained from their data were very diversified, even between closely related species. The three major patterns were (i) full congruence between Atlantic and Mediterranean clades; (ii) distinct Atlantic and Mediterranean clades, where the Almeria-Oran front serves as the Atlantic-Mediterranean phylogeographical break; (iii) an Eastern Mediterranean clade that is distinct from the Western Mediterranean and Atlantic Ocean clade where the Sicily Strait and the Messinian Strait serve as a phylogeographical boundary (see also Villamor, Costantini, & Abbiti, 2014). Our results further expand the second and third models of Patarentello et al. (2007) and divide the populations to four clusters. In contrast to the previous above-mentioned analyses, that are based on a limited number of markers and small samples of specimens representing each population, our results that are based on a larger set of informative markers and a large population sample, look more reliable.

The Atlantic current, entering through the Strait of Gibraltar, extends east along the shores of Northern

Africa (Hamad, Millot, & Taupier-Letage, 2006; Millot & Taupier-Letage, 2005; Poulain et al., 2013) (Fig. 8) elucidates the resemblance between Bizerte and the other WM cluster populations and the influence by the sister cluster of the EA populations. This Atlantic current system is likely to assist larval distribution entering the Mediterranean. It appears that the influence of this current does not spread further east and we can only find traces of the connection between the Bizerte and the Pantelleria populations (Fig. 6), which can also be explained by local gyres. The EM cluster is well defined and spans over a large part of the Mediterranean, from the eastern shores through the Aegean Sea and north into the Adriatic Sea. This part of the Mediterranean is influenced by the Asia Minor current as well as some cyclonic and anti-cyclonic gyres (Pinardi, Arneri, Crise, Ravaioli, & Zavatarelli, 2006) and we therefore witness increased connectivity between the different populations. The northern part of the Western Mediterranean is influenced by currents from the area of Sicily (Pinardi et al., 2006), and therefore it is not clear why the population resembles the MM populations and not a WM one like Bizerte. The mixing of water by the mesoscale gyres in the Tyrrhenian sea (Fig. 8) may contribute to the resemblance between the Bastia population and that of the other MM populations. Pantelleria is very close to the border between the well-defined branches of the EA/WM and the MM/EM, and indeed found to be genetically close to both. Although belonging to the WM cluster in most analysis, it is part of the WM cluster in two of the population trees, right next to its geographically neighboring population of Bizerte.

We may also speculate that the separation between the Western and Eastern Mediterranean populations of *C. stellatus* is a result of the geological history of the Mediterranean. One of the most conspicuous events that shaped the composition of flora and fauna of the Mediterranean is the Messinian Salinity Crisis (MSC) that started at the end of the Miocene, about 6 million years ago (Krijgsman, Hilgen, Raffi, Sierro, & Wilson, 1999) when the connection between the Mediterranean and the Atlantic was cut. The water balance of the Mediterranean was, and still is, deficient and is compensated by the Atlantic inflow through the Strait of Gibraltar. The Messinian Salinity Crisis ended in the Zanclean flood, occurred 5.33 million years ago when the Strait of Gibraltar opened and have refilled the Mediterranean Sea. During the MSC, the water level and salinity of the Mediterranean fluctuated and it was dried up and refilled repeatedly during the few million years of the Messinian stage, leaving behind lakes of different salinities (Hsu, Ryan, & Cita, 1973). Some of these lakes might be a refuge, inhabited by resistant organism like intertidal barnacles that naturally withstand fluctuating temperatures and salinities. We may propose that eastern and middle populations of *C. stellatus* are a relic of the MSC lakes while the WM populations resemble the Atlantic “invaders” arriving like many other organisms in the Zanclean flood.

In order to better understand how these populations were shaped, the selective pressures of the habitats, like salinity and temperature, must be considered. When compared to other species of barnacles (Bhatnagar & Crisp, 1965), *C. stellatus* was found to be better adapted to higher temperatures while less inhabiting lower salinity niches. Low salinity has been correlated with reduced number of eggs per brood in *C. stellatus* (Barnes and Barnes, 1965). These adaptive advantages can contribute to a bigger, stable and more polymorphic population in the higher salinity and temperature conditions of the EM. Indeed, the EM populations have more alleles per position and their expected heterozygosity is significantly higher than all other populations (Figs. 7a–b). The EA populations, on the other hand, have significantly smaller number of alleles per position, lower expected heterozygosity and lower percent of polymorphic positions (Figs. 7a–c), presumably reflecting the adaptive difficulties of *C. stellatus* in the lower salinity and temperature of the East Atlantic Ocean. The Atlantic current influence on the Mediterranean is also evident in the low salinity region stretching from the Strait of Gibraltar along the Northern Africa shores to Bizerte (Fig. 8). As expected from this, the WM populations do have less alleles per position and their expected heterozygosity is significantly lower than the EM populations. The environmental conditions may also explain the establishment of EM populations of *C. stellatus* in the western part of the Mediterranean. Mean surface salinity and temperature of the Northern Tyrrhenian and the Ligurian Seas are the highest in the Western Mediterranean Basin (UNEP/MAP 2012, p 23). These favorable conditions could allow for the establishment of a polymorphic population like the one in Bastia.

Patarentello et al. (2007) have concluded that genetic diversity does not necessarily decrease in a direction

either from the Atlantic Ocean to the Mediterranean or even to the Adriatic Sea. The higher genetic variability of the EM population can be partially explained by the central-marginal hypothesis, also termed the Carson hypothesis (Carson, 1959; Eckert, Samis, & Loughheed, 2008; Sagarin & Gaines, 2002). The hypothesis claims that range margins exhibit less genetic diversity and greater inter-population genetic differentiation compared to range cores. Since the Eastern Mediterranean shores are not a naturally occurring edge of the population this effect can only be seen in the Atlantic where *C. stellatus* does not spread further south to the Western Africa shore and even the Cape Verde *Chthamalus* was shown to be a different species (Tikochinski et al., 2020).

Yet another explanation may be the advantage of variability within the eastern basin populations in overcoming the higher temperature changes as well as other rapid condition changes and processes typical to this part of the Mediterranean, especially since the opening of the Suez Canal.

Our study presents a unique opportunity to study processes of population settlements in sessile animals, the influence of oceanographic conditions and processes including selection and genetic variation. In order to better understand and solidify some of our speculations, more studies of this range of geographic distribution, population size and genetic polymorphism are needed.

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AUTHOR CONTRIBUTIONS

Y.T., U.M. and Y. A. designed the study. N.S.B. and Y.A. collected the specimens. S. T. and Y.T. obtained the molecular data. U.M. analysed the data with help from Y. T. Y.T., U.M. and Y. A. wrote the paper.

DATA AVAILABILITY STATEMENT

All DNA sequences can be downloaded from the NCBI. GenBank accession numbers are MT296012-247 and MT633576-654 for EF1, MT296286-518 and MT633655-737 for NaKA.

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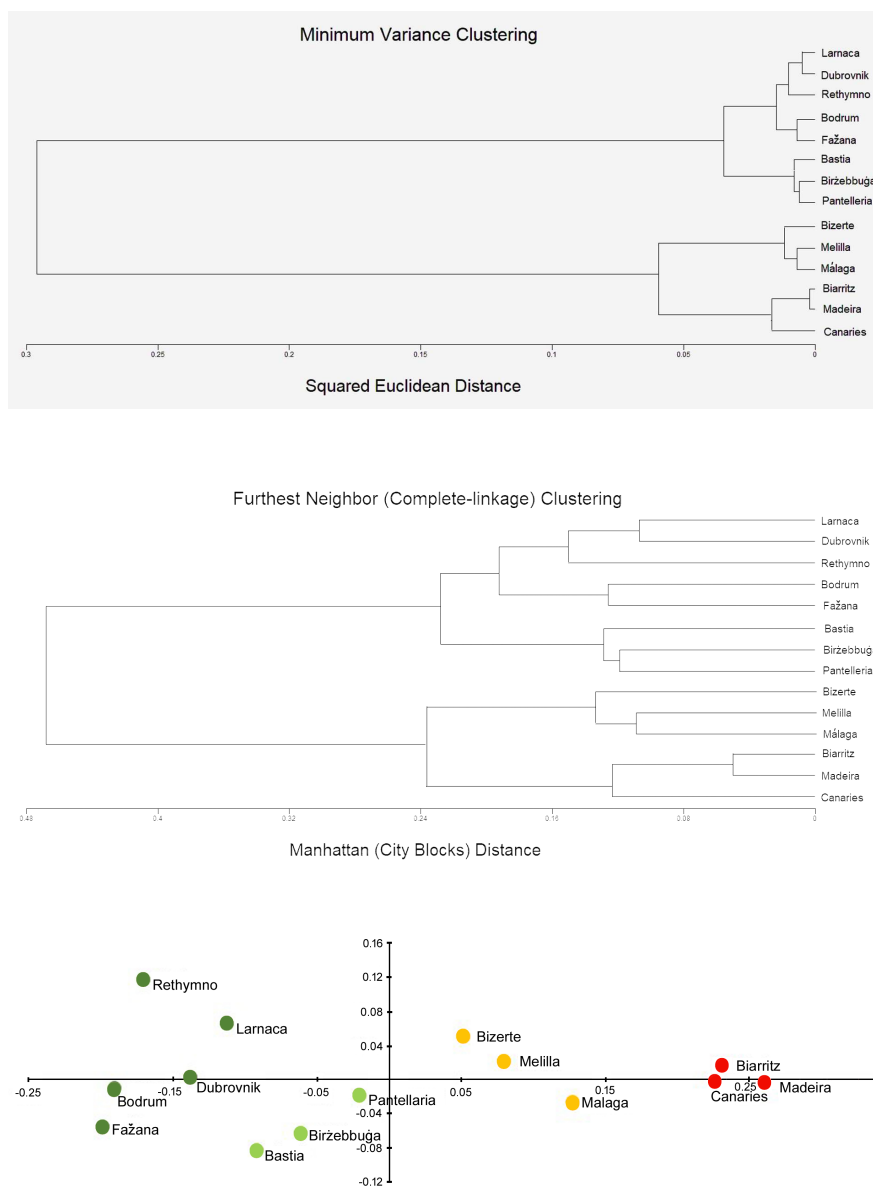
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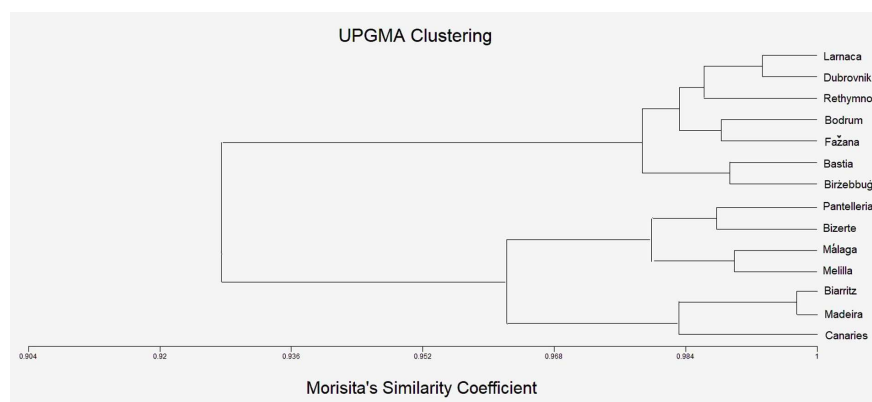
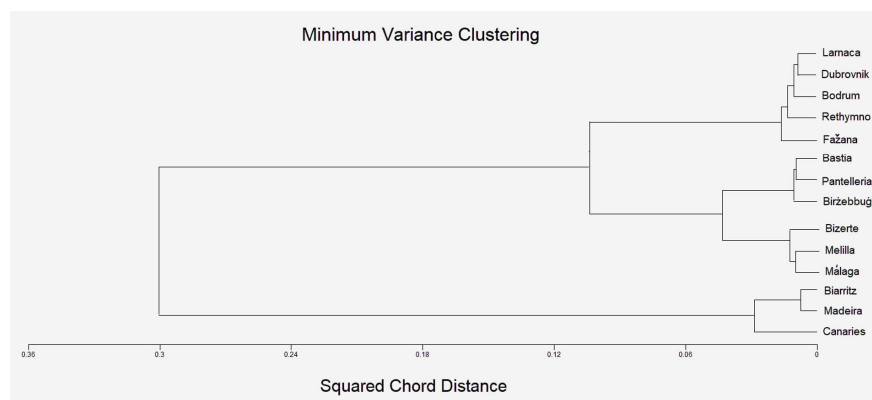
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