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Drivers of population structure of the bottlenose dolphin (*Tursiops truncatus*) in the Eastern Mediterranean Sea

Stefania Gaspari[a], *Aviad Scheinin*[b,c], *Draško Holcer*[d,e], *Caterina Fortuna*[f], *Chiara Natali*[a] *Tilen Genov*[g,h], *Alexandros Frantzis*[i], *Guido Chelazzi*[a], *André E. Moura*[j]

[a]*Department of Biology, University of Florence, Florence, Italy*
[b]*The Leon Recanati Institute for Maritime Studies and the Department of Maritime Civilizations, the Leon H. Charney School for Marine Sciences, University of Haifa, Haifa, Israel*
[c]*IMMRAC (Israel Marine Mammal Research and Assistance center)*
[d]*Department of Zoology, Croatian Natural History Museum, Demetrova 1, Zagreb, Croatia*
[e]*Blue World Institute of Marine Research and Conservation, Kaštel 24, Veli Lošinj, Croatia*
[f]*Italian National Institute for Environmental Protection and Research, Rome, Italy*
[g]*Morigenos, Slovenian Marine Mammal Society, Piran, Slovenia*
[h]*Institute for Biodiversity Studies, Science and Research Centre, University of Primorska, Koper, Slovenia*
[i]*Pelagos Cetacean Research Institute, Vouliagmeni, Greece*
[j]*School of Life Sciences, University of Lincoln, Lincoln, UK*

Corresponding author: Andre E. Moura, amoura@lincoln.ac.uk, Tel: +44 (0)1522886805

Abstract

The drivers of population differentiation in oceanic dispersal organisms have been crucial for research in evolutionary biology. Adaptation to different environments is commonly invoked as an alternative to geographic isolation, as a driver of differentiation in the oceans. In this study, we investigate the population structure and phylogeography of the bottlenose dolphin (*Tursiops truncatus*) in the Mediterranean Sea, using microsatellite loci and the entire mtDNA control region. By further comparing the Mediterranean populations with the well described Atlantic populations, we addressed the following hypotheses: 1) bottlenose dolphins show population structure within the environmentally complex Eastern Mediterranean Sea; 2) population structure was gained locally or otherwise results from chance distribution of pre-existing genetic structure; 3) strong demographic variations within the Mediterranean basin have affected genetic variation sufficiently to bias detected patterns of population structure. Our results suggest that bottlenose dolphin exhibits population structures that correspond well to the main Mediterranean basins. Furthermore, we found evidence for fine-scale population division within the Adriatic and the Levantine seas. We further describe for the first time, a distinction between populations inhabiting *pelagic* and *coastal* regions within the Mediterranean. Phylogeographic analysis, suggests that current genetic structure results mostly from stochastic distribution of Atlantic genetic variation, resulting from a recent post-glacial expansion. Comparison with Atlantic mtDNA haplotypes, further suggest the existence of a metapopulation across North Atlantic/Mediterranean, with pelagic regions acting as source for coastal environments.

Key words: *population structure, phylogeography, Tursiops truncatus, Mediterranean Sea, gene flow*

Introduction

Despite an apparent lack of physical barriers to dispersal, many marine organisms exhibit population structure over scales smaller than their dispersal potential (Norris 2000; Bierne *et al.* 2003). The Mediterranean basin in particular is a global biodiversity hotspot (Almada *et al.* 2001; Bograd *et al.* 2010), and several marine species exhibit complex population structure patterns over relatively short geographic distances (e.g. Ascids: Perez-Portela and Turon 2008; Echinoderms: Zulliger *et al.* 2009; Molluscs: Perez-Losada *et al.* 2007, Calvo *et al.* 2009; Marine turtles: Carreras *et al.* 2006; Cetaceans: Natoli *et al.* 2005; Gaspari *et al.* 2007a,b; Fish: Carreras-Carbonell *et al.* 2006; Charrier *et al.* 2006; Domingues *et al.* 2007). The Mediterranean is thus a particularly interesting region to investigate the drivers of population structure in marine organisms.

Population structure in the Mediterranean Sea is often suggested to result from differential adaptation to the environmental complexity of the basin (Borsa *et al.* 1997; Naciri *et al.* 1999; Bahri-Sfar *et al.* 2000, Domingues *et al.* 2005; Galarza *et al.* 2009; Zulliger *et al.* 2009). However, alternative mechanisms such as isolation-by-distance (Zulliger *et al.* 2009; Casado-Amezúa *et al.* 2012), or strong regional demographic

variations (Rolland *et al.* 2006) have also been proposed. This complexity is further emphasized by the lack of consistent patterns of differentiation across the Strait of Gibraltar for various marine taxa (Patarnello *et al.* 2007).

The bottlenose dolphin (*Tursiops truncatus*) represents a good model to test different hypotheses regarding the drivers of genetic differentiation. In European waters (Mediterranean, Eastern North Atlantic, and North Sea), its population structure appears to correlate strongly with environmental differences (Natoli *et al.* 2005), consistent with suggestions that differences in habitat requirements drive population structure in cetaceans (Mendez *et al.* 2011; Amaral *et al.* 2012). In the Atlantic, populations typically segregate between lineages inhabiting pelagic and coastal environments (Hoelzel *et al.* 1998, Natoli *et al.* 2004), and mitogenomic analysis showed that in European waters, these two ecotypes show incomplete lineage sorting (Moura *et al.* 2013b), suggesting recent establishment of the observed population structure patterns. Several communities inhabiting estuarine/bay environments, are genetically differentiated from individuals sampled in open waters, both coastal and pelagic (Parsons *et al.* 2002; Nichols *et al.* 2007; Fernandez *et al.* 2011, Mirimin *et al.* 2011; Louis *et al.* 2014a), which is thought to result from occupation of newly formed habitats after the Last Glacial Maxima (LGM) (Louis *et al.* 2014b).

It has also been suggested that the Mediterranean populations of bottlenose dolphin have recently occupied the area from Atlantic populations (Natoli *et al.* 2005). However, preliminary data shows evidence of fine-scale population structure within the Mediterranean basin (Gaspari *et al.* 2013), consistent with morphological variation described between basins (Sharir *et al.* 2011), and the existence of groups with different levels of site fidelity (Bearzi *et al.* 2005; Bearzi *et al.* 2009). Previous analyses have suffered from low power, both in terms of the markers used, and the number and geographic representation of samples. To date, no study has compared fine scale genetic structure within the Mediterranean, with the well described genetic structure in the North Atlantic (Natoli *et al.* 2004; Tezanos-Pinto *et al.* 2008; Louis *et al.* 2014a). This information can thus allow to distinguish between genetic structure that results from random distribution of ancestral variation, from that established within the Mediterranean due to local adaptation.

Furthermore, several studies suggest that strong demographic history has confounded population structure patterns in other North Atlantic cetaceans, including common dolphin (*Delphinus delphis*) (Natoli *et al.* 2008; Moura *et al.* 2013a), white-beaked dolphin (*Lagenorhynchus albirostris*) (Banguera-Hinestroza *et al.* 2010), white-sided dolphin (*Lagenorhynchus acutus*) (Banguera-Hinestroza *et al.* 2014), bowhead whale (*Balaena mysticetus*) (Foote *et al.* 2013), and the killer whale (*Orcinus orca*) (Hoelzel *et al.* 2002; Moura *et al.* 2014). Given recent suggestions of population size changes in some Mediterranean regions (Bearzi and Fortuna, 2006, Pleslić *et al.* 2013), further detailed analysis of the patterns and processes of population structure is required to advise appropriate conservation measures, and enable an accurate appreciation of the potential for recolonization of local populations by vagrant individuals.

In this study we investigate population structure and phylogeography of the bottlenose dolphin within the Mediterranean Sea, using both nuclear and mitochondrial markers. We employed a comprehensive sample set that covered most of the main Mediterranean basins, namely the Tyrrhenian, Adriatic, Ionian, Aegean and Levantine seas (largely unexplored). These basins all represent unique oceanographic features, characterized by differences in bathymetry, temperature, salinity, and productivity, among others. We evaluated the following key hypotheses: 1) Bottlenose dolphins show fine-scale population structure within the environmentally complex Eastern Mediterranean Sea; 2) Population structure was gained locally or otherwise results from chance distribution of pre-existing genetic structure; 3) Strong demographic variations within the Mediterranean Sea have affected genetic variation sufficiently to bias detected patterns of population structure. Because the bottlenose dolphin is a top predator in the Mediterranean Sea (Bearzi *et al.* 2009), phylogeographic patterns of this species will reflect wider changes in the environment, and thus be crucial in understanding the biogeographic history of the basin.

Materials and Methods

Sample collection and DNA extraction and amplification

Tissue samples from 194 adult common bottlenose dolphins were collected between 1992 and 2011 from the five main eastern Mediterranean basins (Figure 1) through biopsies of free-ranging animals (fr) and stranded specimens (str). Samples numbers are as follows: Adriatic Sea (Adriatic north: 7 fr and 50 str; Adriatic central-south: 21 fr and 9 str), Ionian Sea (14 str), Aegean Sea (10 str), Tyrrhenian Sea (16 str), and Levantine Basin (68 str). One important consideration, is that the Ionian Sea is considerably deeper than all other basins and is, in this respect, similar to pelagic regions. DNA was extracted with phenol/chloroform and ethanol precipitation from tissue samples preserved in salt saturated 20% DMSO or 95% ethanol.

Samples were genotyped at 12 microsatellite loci, namely EV37Mn, EV14Pm (Valsecchi and Amos, 1996), TtruGT6 (Caldwell *et al.* 2002), D08 (Shinohara *et al.* 1997) and Ttr04, Ttr11, Ttr19, Ttr34, Ttr58, Ttr63,

TtrRH1 and TtrRC12 (Rosel *et al.* 2005). Genotypes were determined using an ABI 3100 genetic analyser with Genotyper (Applied Biosystems). A binning procedure was performed to ensure that all alleles were identified correctly across populations. Genotyping accuracy was assessed by randomly re-amplifying 30% of the samples as controls. Gender was determined through differential amplification of the zinc finger gene regions present in the X and Y chromosomes (ZFX and ZFY, respectively), as described by Bérubé and Palsbøll (1996).

The mitochondrial DNA (mtDNA) control region (920 bp) was amplified and sequenced using the primers TURCRL5483 (5' - GGTCTTGTAAACCGGAAAAGG - 3') and TURCRH6379 (5' - GCAGACTTACACATGCAAGCA - 3') designed in this study, as described in Gaspari *et al.* (2013).

Raw sequence chromatographs from both strands were edited and aligned using CODONCODE ALIGNER (CodonCode Corporation).

Summary statistics

Duplicate samples were identified using the EXCEL MICROSATELLITE TOOLKIT (Park, 2008), and by calculating probabilities of identity P(ID) and P(ID)sib for each basin (Figure 1) using GENALEX (Peakall and Smouse, 2006). In the presence of population substructure or in small populations where related individuals may remain in proximity and be sampled, P(ID)sib provides a more conservative estimator of the probability of finding the same multi locus genotype at random within the population.

Genetic diversity in each basin (Figure 1) was assessed by calculating number of alleles, mean number of private alleles, observed and expected heterozygosity, autocorrelation coefficient (r) and inbreeding coefficient (F_{IS}) using GENALEX (Peakall and Smouse, 2006). Allelic Richness was calculated in F_{STAT} (Goudet 2001) based on the minimum sample size. Departure from Hardy-Weinberg Equilibrium (HWE) was tested for each microsatellite locus in each population using the Fisher exact test with 1000 permutations, as implemented by ARLEQUIN (Excoffier *et al.* 2005).

For mtDNA, unique haplotypes were identified with ARLEQUIN. Genetic diversity in each basin was assessed by calculating number of polymorphic sites, number of haplotypes, pairwise identity (π) and haplotype diversity (H).

Analysis of genetic differentiation

The presence of fine scale population structure among the main Mediterranean basins (Figure 1) was investigated through the analysis of molecular variance (AMOVA), carried out using ARLEQUIN for both mtDNA and microsatellites using F_{ST} as an estimator.

For mtDNA, genetic structure was further analysed by constructing a minimum-spanning network using NETWORK (Bandelt *et al.* 1999). A second network was constructed including sequences from previous studies, in order to assess the relationships between Mediterranean and North Atlantic mtDNA haplotypes. GenBank was queried using the expressions Tursiops + "control region" and Tursiops + D-Loop, while retaining only those entries that were over 500 bp and corresponded to animals sampled in the North Atlantic (accession numbers in Table S1; Western North Atlantic Coastal ecotype was excluded).

For microsatellites, we used two methods to determine the most likely number of distinct genetic clusters, without a priori assignment of individuals to populations. We used the Bayesian clustering methods implemented in STRUCTURE (Pritchard *et al.* 2000), using the admixture model with correlated allele frequencies, without specifying sampling locations or geographic origin of samples. The model was run for cluster number (K) from 1 to 15, using a burn-in period of 150,000 Markov Chain Monte Carlo (MCMC) iterations followed by 1,000,000 iterations. Five independent runs were conducted for each value of K to check for convergence of results, analysed using STRUCTURE-HARVESTER (Earl and vonHoldt, 2012).

Correlation between genetic structures and basin of origin, was assessed using the software OBSTRUCT (Gayevskiy *et al.* 2014). This method analyses how much a given pattern of inferred population structure is explained by a predetermined population assignment, by calculating the statistic R^2, based on the sum of squares. The levels of K with the highest support from the STRUCTURE runs were used as input, and the 5 different oceanographic basins were used as the predetermined population assignment.

In addition, we used the spatially explicit method implemented in TESS to investigate fine-scale population structure within the Adriatic and the Levantine basins (Tyrrhenian samples were not used to avoid biases resulting from the rectangular shape of the Italian Peninsula, which means the linear distance between Tyrrhenian and Adriatic is much smaller than the distance of the oceanic route separating these two basins, which involves covering the entire coastline), by running the conditional autoregressive (CAR) admixture model, using burn-in of 20,000 steps followed by 120,000 MCMC steps. The number of cluster (K) to test was set from 1 to 10, with 10 replicates run for each K. The spatial interaction parameter was set to 0.6 and the degree of trend to linear (which are the default parameters). The most likely number of clusters was selected by plotting Deviance Information Criterion (DIC) values against K and by examining plots of individual

assignment probabilities. When K was defined, the run with the lowest DIC was used and individuals were assigned to clusters based on maximum assignment probabilities.

Analysis of gene flow within the Mediterranean

Recent and asymmetric migration rates among the five main basins were estimated using the Bayesian method implemented in BAYESASS (Wilson and Rannala 2003). Preliminary runs were performed to adjust the MCMC mixing parameters of migrations rates, allele frequencies and inbreeding coefficients, to ensure proposed acceptance rates around 30 %. We then performed 10 runs with a burn in of 1×10^6 iterations followed by 2×10^7 MCMC iterations and a sampling frequency of 1,000. Consistency of the results between the runs was also checked. In addition, sex-biased dispersal was analysed in GENALEX, by calculating gender-specific Assignment Index correction (AIc) and testing difference for statistical significance using a Mann–Whitney U-test (Mossman and Waser 1999).

Historical demography

For microsatellite data, Fu's Fs and Tajima's D neutrality tests were carried out in GENALEX, as well as tests for recent reduction in population size using the software BOTTLENECK (Cornuet and Luikart 1996). Tests were carried out using both the Stepwise Mutation Model (SMM) and the Infinite Allele Model (IAM), as well as a combined model assuming 70% SMM and variance set to 30. This was complemented by testing for a shift in the mode of allele frequency distribution, which is more adequate for identifying recent bottlenecks.

For mtDNA, a mismatch distribution was constructed (Rogers and Harpending 1992) using the software ARLEQUIN. Time of expansion was calculated using the formula $T = \tau /2U$, where U represents the mutation rate over the total length of the sequence used in the mismatch distribution (calculated by multiplying the calibrated mutation rate μ by 918 bp). The mutation rate μ was calculated based on the biogeographical method used in Moura *et al.* (2013b), using the software IMA (Hey and Nielsen 2007). We compared the estimated time of expansion obtained from two sources of mutation rate variation: different values for the closing of the Bosporus Strait (the biogeographical calibration used in Moura *et al.* (2013b); and inference derived from calculating mutation rate using the whole mtDNA as in Moura *et al.* (2013b), and using the control region only. This was done to provide an idea of the error introduced in our interpretations, resulting from using an inappropriate mutation rate, a well described source of phylogeographic bias (Ho *et al.* 2008).

Historical variation in effective population size was reconstructed using the Bayesian skyline method implemented in the software BEAST (Drummond *et al.* 2012), using the mutation rate estimated from cetacean whole mtDNA by independent studies (Ho and Lanfear, 2010; Moura *et al.* 2013b), and an alternative mutation rate devised for control region only using the same method as in Moura *et al.* (2013b).

Results

Measures of genetic diversity

The test for duplicates yielded two pairs of samples with matching genotypes, but the haplotype sequences were different for both pairs and were therefore kept. Probabilities of identities across microsatellites loci were low in all populations, which suggests enough power to differentiate between individuals. For microsatellites, diversity levels were similar between the different basins analysed. Observed heterozygosity was usually lower than expected, but differences were not significant and samples from all basins did not significantly deviate from Hardy-Weinberg equilibrium (Table 1). For mitochondrial DNA, pairwise identity (π) was similar between all basins (0.010 ± 0.003), except for Levantine where it was lowest (0.003). Conversely, haplotype diversity (H) was lowest in the Tyrrhenian Sea (0.714), but high overall (Table 1).

Microsatellite genetic structure and gene flow estimates

A significant level of genetic differentiation was detected among all basins in the Eastern Mediterranean Sea. Analyses of Molecular Variance (AMOVA) revealed significant divergence among populations ($F_{ST} = 0.071$ $P > 0.001$), although most genetic variation occurred within rather than among populations ($V_{WI} = 75\%$, $V_{AI} = 18\%$,$W_{AP} = 6\%$). No evidence of spatial autocorrelation (r) was found among samples from all basins (Table 1). Pairwise F_{ST} comparisons were also applied to the Adriatic basin, both as a uniform basin, and as a subdivided basin to check for potential sub-structure within the Adriatic, given its environmental heterogeneity. Between basins all were significant, except for comparisons involving the Aegean Sea (Table S2). Within the Adriatic Sea, the significant F_{ST} differences were found between samples from the Gulf of Trieste (GT) and the rest of the Adriatic, and between the East and West coasts, both keeping or excluding the GT area (respectively: $F_{ST} = 0.016$, $P = 0.000$; $F_{ST} = 0.024$, $P = 0.000$). Furthermore, the comparison between the two coasts was also significant when only the North Adriatic was considered, both keeping and excluding the GT area (respectively: $F_{ST} = 0.026$, $P = 0.002$; $F_{ST} = 0.034$, $P = 0.000$).

Bayesian clustering analyses collectively suggest genetic differentiation between all main basins, although most clusters include individuals from multiple regions (Figure S1 and S2). From the STRUCTURE analysis, the highest posterior probability was obtained for K=15 (Figure S3), although at K=15 no further resolution was detectable relative to K=9 (Figure S1). ObStruct analyses for K=9 resulted in an average R^2=0.31, with pairwise values ranging from 0.03 (between Adriatic and Aegean) to 0.31 (between Adriatic and Levantine). PCA plot revealed some degree of overlap between the different basins cluster assignment, but different basins generally correspond well to genetic clusters. Exceptions are the Aegean and Ionian Seas, which exhibit a high degree of overlap with samples from most basins. Individuals ancestry plots for K=9 clearly separate the main geographic areas analysed (Figure S1), with further subdivision found within the Adriatic and the Levantine regions (Figure S1). Samples from the GT separate clearly from other Adriatic Sea samples, but cluster together with samples from the Aegean Sea (Figure S1). Within the Adriatic Sea, different patterns can be seen between North/ Central/ South regions of the basin (Figure S1), but the pattern is not clear. In the Levantine basin, three well separated clusters can be identified, which roughly segregate between North/Central/South of the basin (Figure S1). However, spatial resolution is low due to all samples resulting from strandings, which limits inference (Bilgmann *et al.* 2011).

TESS results were consistent with STRUCTURE (Figure S2) in separating the Gulf of Trieste from the rest of the Adriatic, however this cluster is shared with individuals from North Adriatic, South Adriatic and Aegean Seas. No further structure was identified within the Levantine. The Ionian Sea shared clusters with all other basins. Both STRUCTURE and TESS individual ancestry plots organized by sample origin are included in the supplementary material (Figure S1 and S2).

BAYESASS showed generally low recent migration rates between basins. Exceptions are migration rates from the Ionian into Tyrrhenian/Adriatic/Aegean, which are all above 0.10, and from Adriatic into Ionian/Aegean, also all above 0.10 (Table 2). Sex biased dispersal suggested females are the dispersing gender, with negative AIc for females and positive AIc for males (Figure S4). However, Mann-Whitney U test was not significant (P = 0.7).

mtDNA Genetic Structure

Analyses of molecular variance (AMOVA) revealed significant divergence among populations (F_{ST} = 0.285; P > 0.001), although most genetic variation occurred within rather than among populations (V_{WP} = 71,47% W_{AP} = 28,53%).

The median-joining network showed a main torso composed of well differentiated and equally represented haplotypes (mean 5.4 mutations) with no clear geographic correspondence, and two terminal star shaped sections. In both these sections, the central haplotype is found in multiple basins (Tyrrhenian/Adriatic for one, and Levantine/Aegean/Adriatic for the other), but haplotypes branching from those central ones were generally private to either Tyrrhenian/Adriatic/Aegean in one case, or Levantine in the other (Figure 3).

The network for North Atlantic *Tursiops* is characterized by several equally differentiated haplotypes at the centre of star shaped phylogenies, but there is no clear correspondence between network lineages and geographic origin. Haplotypes found in the Ionian Sea (which is considerably deeper than other basins) were generally closely related to haplotypes from the Western North Atlantic Pelagic (WNAP) ecotype (as defined in Hoelzel et al 1998), though none was shared (Figure S5). In contrast, haplotypes from Mediterranean basins with depth profiles similar to coastal regions (Tyrrhenian, Adriatic, Aegean and Levantine), were often shared with samples obtained from oceanic locations (Azores and Madeira), as well as open water coastal locations (mainland Portugal, Bay of Biscay, Gulf of Cadiz, Iroise Sea) and the English Channel. Interestingly, only two haplotypes are shared between WNAP and other oceanic locations (Azores and Madeira), although they tend to be separated by a small number of mutational steps. Haplotypes that are shared between multiple locations are generally found at the centre of star shaped phylogenies, while terminal haplotypes are usually private to specific locations, including both Mediterranean coastal basins and Atlantic open water coastal regions.

Historical demography

Summary statistics suggested a recent expansion accompanied by low inbreeding. Tajima's D and Fu's F were both generally negative, consistent with demographic expansion for most basins, except for the Aegean where a positive value suggests contraction. Consistently, F_{IS} values were positive for all basins except the Aegean Sea where it was negative, with only the Tyrrhenian and Levantine being significantly different from zero (Table 1).

Mismatch distribution showed a bimodal profile that did not significantly differ from the expected under a spatial expansion model (P = 0.162; Figure 4), but it did significantly deviate from expectations under a demographic expansion model (P = 0.02). Estimates of expansion time suggest this has occurred recently, likely after the Last Glacial Maximum (LGM; Table 3). Using a mutation rate calibrated for the whole mitogenome, the estimated time of spatial expansion centres around the Eemian interglacial roughly 155 kya (Table 3; note

that the most likely time for the opening of the Bosporus strait is around 5-7 kya). However, using the mutation rate calibrated for control region only (the fragment used in this study), this time moves forward to after the LGM.

Bayesian skyline plots also retrieve an increase in effective population size close to the LGM. Using the mutation rate calibrated for the entire mitogenome, the increase in population size starts roughly around 35 kya (Figure S6a), and it shifts to around 5 kya using the mutation rate calibrated for control region only (Figure S6b). Note that the error associated with this calculation is likely to be large, due to the fact that only modern samples were used. Consistently, calculating the time of expansion using τ =1 for the first modal peak (corresponding to difference between the star-shaped regions of the network), the time of demographic expansion is also after the LGM for both mutation rates (Table 3).

Discussion

Population structure within the Mediterranean

The results of our study collectively suggest that the bottlenose dolphin in the Mediterranean Sea exhibits fine-scale population structure. Geographical distribution of the main population groups appears to correspond well to the main Mediterranean basins, namely the Tyrrhenian, Ionian, Adriatic, Aegean and Levantine seas (Figure 2 and S1). The R^2 statistic was not as high as observed in strongly structure populations according to geographic region, but it was still distinctively higher than expected if structure is not organized geographically (Gayevskiy *et al*. 2014). The OBSTRUCT visual plot (Figure 2) is also consistent with this by showing strong levels of correlation between geographic origin of samples and inferred genetic cluster, although it appears weaker for some geographic regions (i.e. Ionian; see below for more details). Furthermore, we found evidence of fine-scale population division within the Adriatic and the Levantine. However, this pattern is likely confounded by patterns of migration and phylogeographic history (see below for details). In the Adriatic, samples from the Gulf of Trieste (GT) clearly differentiate from other Adriatic samples, and within the Adriatic Sea our results indicate division both between North/Central/South basins, consistent with previous preliminary results (Gaspari *et al*. 2013).

Patterns of population structure across such small distances are commonly described for bottlenose dolphins around the world (e.g. Rosel *et al.* 2009; Ansmann *et al.* 2012; Kiszka *et al.* 2012), and our results of fine-scale population structure within the Adriatic Sea are consistent with local reports of strong site fidelity (Bearzi *et al*. 1997; Genov *et al.* 2008; Pleslić *et al*. 2013). However, this fine-scale structure is likely to have been established recently, and it is not clear how stable it will be in the long term.

The separation between the GT and the remaining Adriatic is similar to that observed elsewhere in European waters, such as the Moray Firth (Scotland), the Shannon estuary (Ireland), and the Sado estuary (Portugal). In all locations, small populations show strong site-fidelity to semi-enclosed bays, with limited interaction with populations outside the bays (Ingram and Rogan 2002; Augusto *et al.* 2011; Cheney *et al.* 2013). Genetically they are differentiated from the closest populations, but are often similar to those found further apart (Parsons *et al.* 2002; Fernández *et al*. 2011; Mirimin *et al.* 2011), just as observed in our study. In our case, the similarity between GT and the Aegean Sea is likely due to the stochastic distribution of genetic variation during a recent colonization of the Mediterranean (see below for details).

Phylogeographic history and Mediterranean invasion

Our study suggests that population structure within the Mediterranean largely results from stochastic distribution of genetic variation, through a series of founder events (either sequential or concurrent) during a recent invasion of the Mediterranean Sea. An Atlantic origin of Mediterranean populations has been proposed earlier (Natoli *et al.* 2005; Moura *et al.* 2013b), and our study confirms this by showing that haplotypes private to individual basins occur in low frequencies and branch off from star shaped phylogenies, whose central haplotypes are shared across the Mediterranean/Atlantic. Our study further estimates a timing for this colonization, which likely occurred after the Last Glacial Maxima (LGM), particularly if a faster mutation rate is used.

Several other marine organisms in the Mediterranean exhibit star shaped networks (Natoli *et al.* 2005, Carreras *et al*. 2006; Charrier *et al*. 2006; Perez-Losada *et al*. 2007; Sušnik *et al*. 2007; Perez-Portela and Turon 2008; Zulliger *et al*. 2009) similar to those found in our study. In most cases, this expansion has been dated to around the Eemian interglacial (consistent with our older age estimate of ~155 Kya), and never before the Pleistocene (Patarnello *et al*. 2007; Perez-Losada *et al*. 2007; Sušnik *et al*. 2007; Pujolar *et al*. 2010). However, mutation rates used in these earlier studies were not calibrated using biogeographical events, and are thus likely underestimated (Ho *et al.* 2005; Patarnello *et al*. 2007; Ho *et al*. 2008; Calvo *et al*. 2009). Indeed, recent studies on sand smelt (Pujolar *et al.* 2012) and green crab (Marino *et al.* 2011) using mutation rates derived from

biogeographical calibrations events, also time the Mediterranean colonization to after the LGM. In our case, due to the control-region being a known mutational hotspot (Stoneking 2000), the rate calculated for the whole mitogenome (Moura *et al.* 2013b) is likely inappropriately slow for our study.

Geological data indicates that during the LGM, water exchange through the Strait of Gibraltar was much reduced (Mikolajewicz 2011). This likely led to lower temperatures and oxygen levels, with corresponding increases in salinity compared to present day, particularly in the Eastern Mediterranean basin (Mikolajewicz 2011). Because the Mediterranean has a net deficit of water, and limited connectivity with the Atlantic, this further led to changes in the sea level, with a likely drying of the Adriatic Sea, and physical separation between the Eastern and Western Mediterranean basins (Thiede 1978; Hayes *et al.* 2005).

High salinity during the LGM could have been a limiting factor for the survival of fish species in the Eastern Mediterranean, especially in combination with high water temperatures (Morris 1960; Gonzalez 2011). Dolphins are large predators with high resting metabolic rates (Williams *et al.* 2001), which require a high energy diet to survive (Spitz *et al.* 2012). Therefore, even if some fish species managed to survive the harsh environmental conditions in the LGM Mediterranean, they might not have been present in sufficient numbers to support a viable bottlenose dolphin population.

The differentiation of the population occupying the Levantine basin, together with known morphological differences (Sharir *et al.* 2011), could reflect longer term survival of local refugial population that diversified in isolation. However, the number of unique haplotypes and private alleles was comparable to those found in the Adriatic, where a refugial population was very unlikely. Nevertheless, if present genetic structure patterns reflect two independent colonisations of the Mediterranean, still most of its diversity results from a recent expansion from the Atlantic, as haplotypes from the Adriatic and Aegean seas are more closely related to Ionian/Atlantic haplotypes. Our data thus support the interpretation that bottlenose dolphin colonization of the Mediterranean occurred after the LGM, and that current patterns of populations structure are the result of chance distribution of haplotypes due to founder events during the spatial expansion.

Research from other high dispersal animals is also consistent with a post-LGM expansion. Common dolphin (*Delphinus delphis*) mtDNA also shows a pattern consistent with a recent expansion into the Mediterranean from a larger Atlantic population (Natoli *et al.* 2008), but exhibits weak population structure possibly due to a more fluid social structure and lower natal philopatry (Moura *et al.* 2013a). Recent studies on white-sided dolphins (*Lagenorhynchus acutus*, Banguera-Hinestroza *et al.* 2014) and harbour porpoises (*Phocoena phocoena*, Fontaine *et al.* 2014) also found evidence for a post-LGM expansions in the North Atlantic, as well as in killer whales (*Orcinus orca*; Moura *et al.* 2014b) in association with a strong decline during the LGM (Moura *et al.* 2014a). Similarly, the low diversity observed in loggerhead sea turtles in Eastern Mediterranean (Carreras *et al.* 2006), has been linked with excessively low temperature for successful hatching during the LGM (Bowen *et al.* 1993), which would imply that present loggerhead turtles are descendent from post-glacial colonizers. Recently, comparison with simulated datasets also suggested that differentiation of certain estuarine/bay populations of bottlenose in the North Atlantic, has been achieved post LGM (Louis *et al.* 2014b). This suggests that the LGM might have had profound effects not only in the Mediterranean marine fauna, but also more broadly in the North Atlantic.

Integration with North Atlantic structure

Integration of our samples with mtDNA data from the North Atlantic available in the literature, showed significant haplotype sharing not only between Mediterranean and Atlantic, but also more broadly across the Atlantic. In spite of this, shallow water basins within the Mediterranean had high number of private alleles as compared to the deeper Ionian Sea, with mtDNA also showing close resemblance with the Western North Atlantic Pelagic (WNAP) ecotype (Hoelzel *et al.* 1998; Tezanos-Pinto *et al.* 2008). This suggests a pelagic *vs* coastal differentiation as described elsewhere in the world (Hoelzel *et al.* 1998, Segura *et al.* 2006; Tezanos-Pinto *et al.* 2008; Caballero *et al.* 2012) including the Eastern North Atlantic (Natoli *et al.* 2004; Moura *et al.* 2013b). This distinction is described here within the Mediterranean for the first time, and is consistent with reports of vagrant individuals being sighted only occasionally in regions where other groups show strong site fidelity (Bearzi *et al.* 2005; Genov *et al.* 2008; Pleslić *et al.* 2013). It is also ecologically consistent, as the Ionian Sea is the deepest of all basins analysed (Becker *et al.* 2009).

However, the sharing of haplotypes indicates a different dynamics to that found in the Western North Atlantic, where genetic differentiation is not only much deeper (Hoelzel *et al.* 1998, Moura *et al.* 2013b), but also accompanied by nearly complete spatial segregation (Torres *et al.* 2003). Instead, vagrant individuals with a fluid social structure are commonly reported in the same sites as coastal populations with tighter social structure (Bearzi *et al.* 2005; Genov *et al.* 2008; Pleslić *et al.* 2013; Martinho *et al.* 2014), and gene flow is detected between the two habitats (Quérouil *et al.* 2007, this study). Instead, population dynamics of bottlenose dolphin in the North Atlantic and Mediterranean Sea are more typical of a metapopulation (Nichols *et al.* 2007; Oremus *et al.* 2007). In such cases, populations that exhibit strong site fidelity and are demographically isolated (e.g., the

Tyrrhenian and the Adriatic populations), can appear to be part of a single large population in a mtDNA phylogenetic network (Oremus *et al.* 2007).

Recent expansion into unoccupied habitats contributes to the sharing of haplotypes across distant regions, but metapopulations are usually characterized by specific source-sink dynamics, for which there is evidence in our study. Microsatellite data suggest that gene flow is stronger from the Ionian *pelagic* basin to the other *coastal* basins than in the opposite direction, and also low between different *coastal* basins. This implies that gene flow between shallow water basins (e.g. Tyrrhenian, Adriatic and Aegean) might be mediated by the pelagic ecotype, and explains how coastal populations exhibiting strong site fidelity (Bearzi *et al.* 2005; Genov *et al.* 2008; Pleslić *et al.* 2013) can still be genetically similar (e.g. Tyrrhenian and Adriatic seas). Gene flow appears to be female mediated, which would explain why lineage sorting between pelagic and Mediterranean haplotypes is incomplete (Natoli *et al.* 2005; Moura *et al.* 2013b) in spite of the good differentiation seen here in nuclear DNA.

A metapopulation dynamics would account for the weak genetic differentiation found between oceanic and coastal open water samples in the Atlantic (Quérouil *et al.* 2007; Louis *et al.* 2014a). Regardless, our North Atlantic network shows that open water coastal areas still exhibit private alleles at the tips of star shaped phylogenies, suggesting that these regions too are characterized by local specializations following expansion. Populations inhabiting estuarine/bay habitats will differentiate faster due to a low carrying capacity of those environments, meaning large populations of a top predator cannot be supported and new individuals (either migrants or born locally) not readily accepted. Consistently, *coastal* populations found to have strong genetic differentiation, typically inhabit enclosed or semi-enclosed habitats and have low census and effective population sizes (Parsons *et al.* 2002; Fernández *et al.* 2011, Mirimin *et al.* 2011; Louis *et al.* 2014a). Therefore, population differentiation in North Atlantic/Mediterranean bottlenose dolphins is likely the result of the low carrying capacity of coastal environments, which makes them unable to act as sink populations for the larger pelagic source population.

Conservation implications

From our genetic data, no evidence was found for recent local population contractions in the Mediterranean, except for the Aegean Sea. This inference controverts previous concerns of up to 50% decline of bottlenose dolphins in the Adriatic Sea since the 1950s (Bearzi and Fortuna 2006). However, bottleneck tests often lack power if pre-bottleneck effective population size was low, and if the test is carried out a small number of generations after the bottleneck (Peery *et al.* 2012). A recent study in the Adriatic found evidence for an increase in abundance for one local population (Pleslić *et al.* 2013), suggesting that natural local fluctuations could be interpreted as reductions if quantitative data is limited to a short time period or sparse over a long period. For the Aegean Sea, genetic data support a recent bottleneck (although sample size is low), but no abundance estimates are currently available to corroborate the inference from genetic data. Our results highlight the need for accurate abundance estimates in the region, as it is possible that local declines are occurring undetected.

A metapopulation dynamics has important conservation implications. Although coastal populations could be replaced through migration from the source population, local declines likely reflect an inability of the environment to support a viable population, and thus reflect the need for conservation measures. Similarly, pelagic source populations should be seen as a valuable reservoir of individuals and genetic variation, and be the target of conservation measures well before its numbers appear to be depleted.

Concluding remarks

Our study suggests that present bottlenose dolphin genetic structure patterns in the Mediterranean Sea largely result from the stochastic distribution of Atlantic genetic diversity during a recent post-glacial expansion. Furthermore, North Atlantic and Mediterranean populations likely constitute a single metapopulation, with pelagic populations acting as genetic source for coastal ones. Current population differentiation appears to be the result of the combined effects of past climatic variations, local carrying capacity associated with differences in social structure and site fidelity, and potentially ecological differences, particularly between the *pelagic* and *coastal* populations. Adaptation might further contribute to differentiation, but this will likely not be possible to address using only neutral genetic markers.

Our results have important implication for the understanding of Mediterranean biodiversity. Previous studies have suggested that Mediterranean biodiversity was the result of endemism from glacial *refugia*. Our study further suggests that the Mediterranean might have also been a sink for many Atlantic species post-LGM. This could explain why some of the Mediterranean oceanographic boundaries do not appear to constitute genetic boundaries for all marine species (e.g. Patarnello *et al.* 2007). Patterns of population structure will likely results

from a combination of pre-expansion diversity, dispersal and colonization mechanisms, as well as specific limiting factors and behavioural characteristics in post-colonization environments.

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Table 1. Diversity index for both microsatellite (top) and mtDNA (bottom) in *Tursiops truncatus* samples from the 5 main Mediterranean basins. **N** - number of samples; **AR** - Allelic Richness; **NA** - number of alleles; **Mean PA** - mean number of private alleles; **He** - expected heterozygosity; **Ho** - observed heterozygosity; **r** - autocorrelation coefficient; F_{IS} - inbreeding coefficient; **NP** - number of polymorphic sites; **NH** - number of haplotypes; π - mean number of pairwise differences; **H** - haplotypic diversity; **D** - Tajima's D; **Fs** - Fu's Fs.

Microsatellites

Basin	N	AR	NA	Mean PA	He	Ho	r	F_{IS}	F_{IS} p-value
Tyrrhenian	16	5.8	7.17	0.08	0.74	0.68	0.13	0.17	0.01
Adriatic	86	6.19	11.60	1.33	0.75	0.83	0.06	0.05	0.05
Aegean	10	6	6.25	0.08	0.76	0.68	0.01	-0.02	0.63
Levantine	68	6.04	10.91	2.00	0.77	0.64	0.09	0.10	0
Ionian	14	5.92	7.00	0.25	0.79	0.69	0.08	0.10	0.47

mtDNA

Basin	N	NP	NH	π	H	D	Fs
Tyrrhenian	15	21	6	0.007	0.71	-0.02	2.63
Adriatic	70	51	32	0.010	0.92	-0.10	-6.51
Aegean	10	28	10	0.012	1	0.84	-3.04
Levantine	26	15	14	0.003	0.82	-0.91	-6.53 **
Ionian	12	29	8	0.010	0.91	-0.03	0.72

Table 2. Recent migration rate extimates between *Tursiops truncatus* sampled in the 5 main Mediterranean basins, using the software BayesAss. The first column represent the basin of origin, while the first row represent the destination basins. Marked in bold are all migration values above 0.10.

	Tyrrhenian	**Adriatic**	**Aegean**	**Levantine**	**Ionian**
Tyrrhenian	*0.69*	0.00	0.02	0.00	0.01
Adriatic	0.03	*0.73*	**0.14**	0.00	**0.12**
Aegean	0.01	0.00	*0.70*	0.00	0.01
Levantine	0.03	0.01	0.02	*0.99*	0.06
Ionian	**0.25**	**0.26**	**0.11**	0.00	*0.79*

Table 3. Calculation of spatial and demographic expansion times from values of τ obtained in the mismatch distribution analysis. Mutations rates were obtained using a credible range for a biogeographical calibration point as described in (Moura *et al.* 2013b). **μ** - mutation rate in substitutions/site/million years; **U** - mutation rate in substitution/locus/million years.

Calibration time (years)	**τ = 13.423 (spatial expansion model)**					
	whole mtDNA rate			***D-Loop only rate***		
	μ	*U*	*t (Kyears)*	*μ*	*U*	*t (Kyears)*
10,000	0.030	27.54	243.70	0.21	192.78	34.81
9,000	0.031	28.46	235.84	0.23	211.14	31.79
7,000	0.035	32.13	208.89	0.29	266.22	25.21
5,000	0.063	57.83	116.05	0.41	376.38	17.83
Calibration time (years)	**τ = 1 (first modal peak)**					
	whole mtDNA rate			***D-Loop only rate***		
	μ	*U*	*t (Kyears)*	*μ*	*U*	*t (Kyears)*
10,000	0.030	27.54	18.16	0.21	192.78	2.59
9,000	0.031	28.46	17.57	0.23	211.14	2.37
7,000	0.035	32.13	15.56	0.29	266.22	1.88
5,000	0.063	57.83	8.65	0.41	376.38	1.33

Figure 1. Map of the study area. Samples were obtained from stranded and free-ranging *Tursiops truncatus* from the main Mediterranean basins (dashed circles), namely: Tyrrhenian, Adriatic, Ionian , Aegean, and Levantine. Shaded areas represents the two main topographical discontinuities of the Adriatic Sea floor.

Figure 2. PCA plot reflecting the relation between geographic origin of sample and cluster assignment from Structure K=9. Plot made using OBSTRUCT (Gayevskiy *et al*. 2014) .

Figure 3. Phylogenetic network of mtDNA from Mediterranean *Tursiops truncatus*, obtained usin the software Network. Each circle represent a unique haplotype, with size being proportional to the number of samples carrying it. Links represent 1 point mutation between haplotypes, with longer links represented with 1 dark vertical bar for each mutation. Black circles represent haplotypes that wer inferred by the software, but not found in the population. Numbered haplotypes (15, 17, 20, 22, 25, 26, 48) are all similar to North Atlantic Pelagic ecotype haplotypes (Figure S4).

Figure 4. Mismatch distribution of pairwise differences between all Mediterranean *Tursiops truncatus* mtDNA haplotypes, calculated using the software Arlequin. Solid line represents the expected distribution under a spatial expansion model, which does not significantly differ from the observed data (see Results).

Figure 1

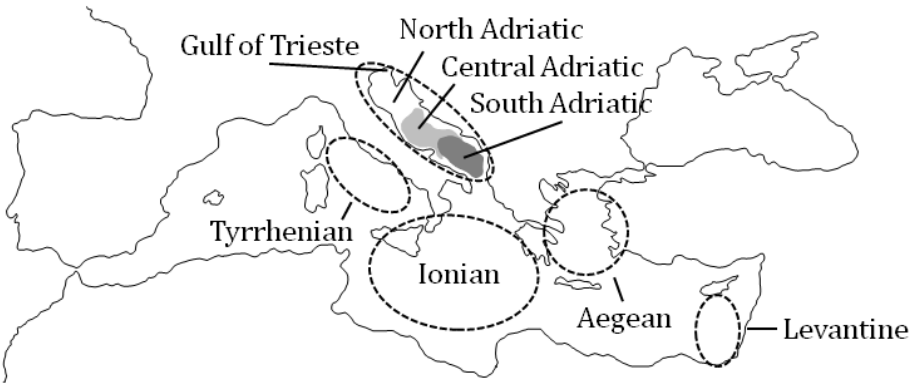

Gulf of Trieste
North Adriatic
Central Adriatic
South Adriatic
Tyrrhenian
Ionian
Aegean
Levantine

Figure 2

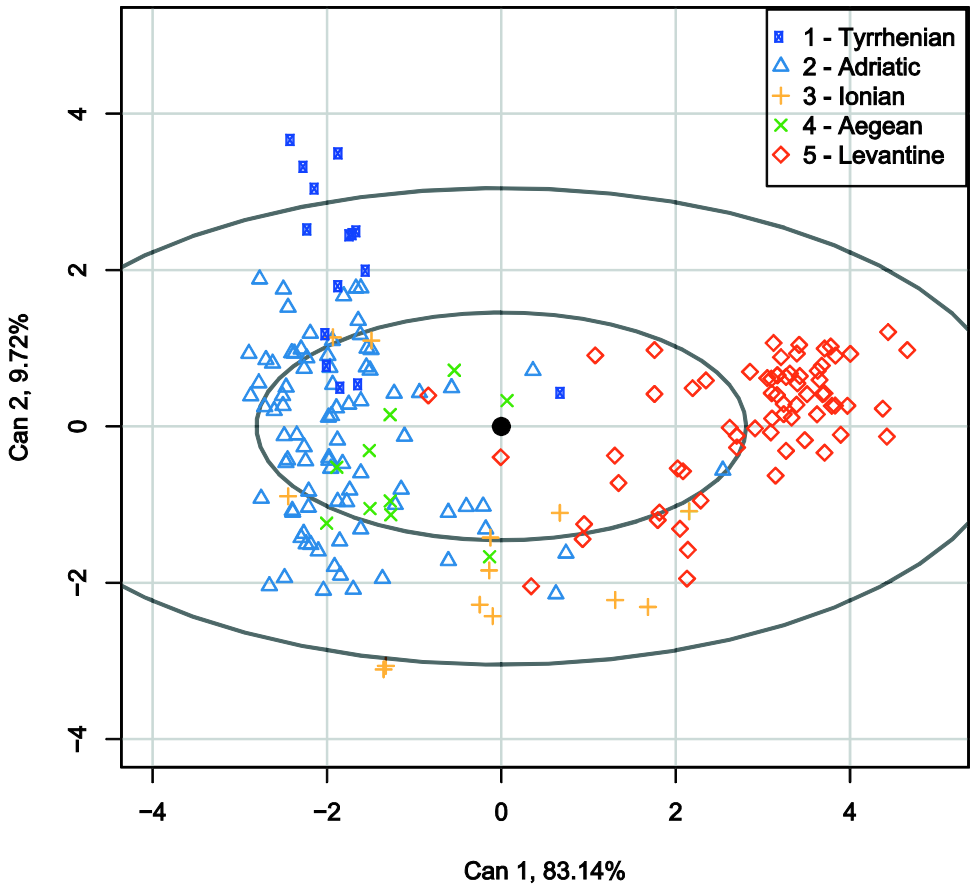
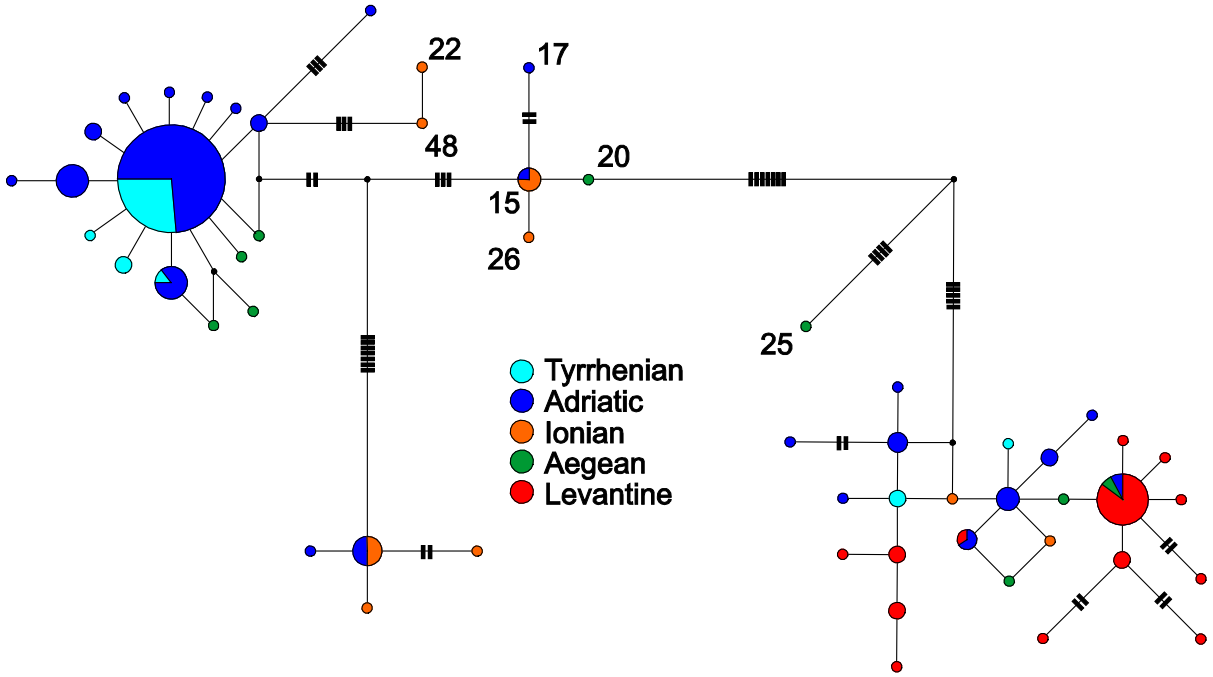
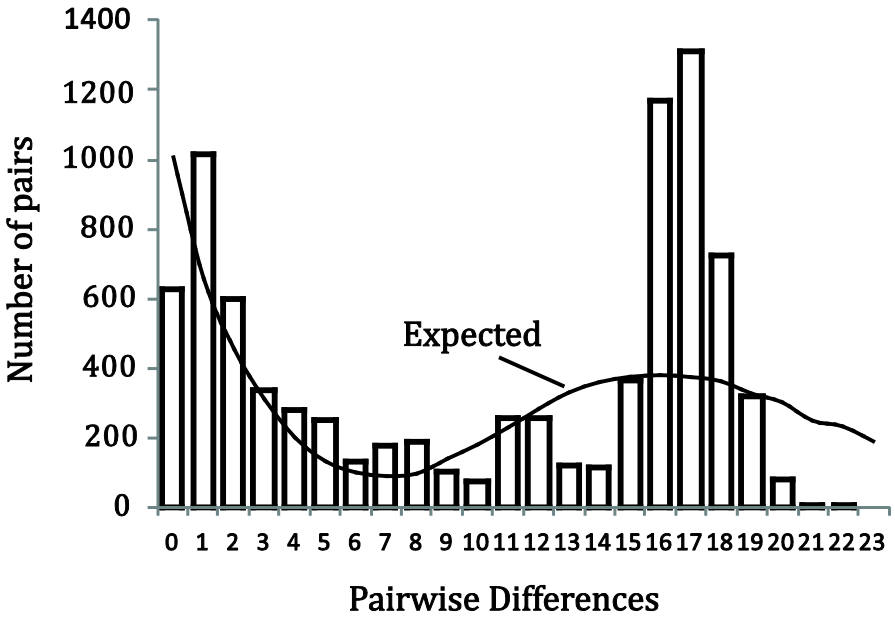

1 - Tyrrhenian
2 - Adriatic
3 - Ionian
4 - Aegean
5 - Levantine
Can 2, 9.72%
Can 1, 83.14%
−4
−2
0
2
4

Figure 3


22
17
48
20
15
26
25
Tyrrhenian
Adriatic
Ionian
Aegean
Levantine

1 **Figure 4**


1400
1200
1000
800
600
400
200
0
Number of pairs
Expected
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Pairwise Differences

2