Surviving the Messinian Salinity Crisis? Divergence patterns in the genus *Dendropoma* (Gastropoda: Vermetidae) in the Mediterranean Sea

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**ABSTRACT**

Four genetically distinct clades were recently described under the name *Dendropoma petraeum*, a Mediterranean endemic vermetid gastropod. The aim of this work is to date the processes that drove to the diversification within this taxon and to relate them to the corresponding historical events occurred in the Mediterranean Sea. Sequences from mitochondrial and nuclear markers were obtained from specimens collected in 29 localities spanning over 4000 km across the entire distribution range of *D. petraeum* species complex. The phylogenetic and coalescent-based analyses confirmed the four well-supported and largely differentiated lineages of *D. petraeum*, clearly delimited geographically along a west–east axis within the Mediterranean Sea: Western, Tyrrhenian–Sicilian, Ionian–Aegean and Levantine lineages. Divergence time estimates, obtained using a range of known substitution rates for other marine gastropods, indicated two main stages of diversification. In the first period (between 9.5 and 4.5 mya), the ancestral *D. petraeum* diverged into the current four lineages. The most recent period occurred between 3.72 and 0.66 mya in the late Pliocene–early Pleistocene, and included the main within-lineage diversification events. Therefore, if the divergence time between the major lineages of *Dendropoma* in the Mediterranean actually predated or coincided with the Messinian Salinity Crisis, then they should have survived to this dramatic period within the Mediterranean, as supported by Bayes Factors model comparison. Conversely, if the divergence started after the crisis, congruent with the idea that no true marine organism survived the Messinian Salinity Crisis, then our results indicate substitution rates of *Dendropoma* much higher than usual (5.16% per million years for COI, 3.04% for 16S). More recent climate changes seem to have conditioned the demographic history of each lineage differently. While Western and Tyrrhenian–Sicilian lineages both underwent an increase in their effective population sizes from 1.5 to 0.6 mya coinciding with a long interglacial period, the Ionian–Aegean and Levantine lineages showed constant effective population sizes since 2–2.5 mya, suggesting that these eastern lineages might represent small and relict populations surviving the subsequent Quaternary glaciations in isolated refugia.

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1. Introduction

Species distribution is influenced mainly by geological events and by the dispersal capacity of each species. Due to a lack of basic data, however, the complex processes that led to today’s biodiversity patterns of coastal marine taxa are not fully understood. Comparative molecular studies can provide considerable insight into marine biodiversification processes by assigning the genetic structuring of taxa to an inferred historical context (Grosberg and Cunningham, 2001). Phylogeography explicitly integrates micro- and macro-evolution, relating ecology to evolution, current distribution to historical events, the physical environment to genetic structure, and patterns of variation within species to patterns of variation across species (Avise, 2000). It therefore provides a framework to explain and integrate patterns of marine biodiversity at intra- and supra-specific levels (Dawson, 2005).

The Mediterranean Sea has experienced dramatic changes in its configuration and climate over the last ten million years (Taviani, 2002). For instance, the formation of species or major phylogenetic
lineages within species in the Mediterranean has been strongly influenced by modifications to the coastline, intensified during the Pleistocene by repeated glaciations. Hence, the combination of different events, such as the opening and closure of the Strait of Gibraltar, advancing and retreating glaciation and changes in current patterns, have made the Mediterranean region a notably dynamic hotspot of diversity (Pannacciulli et al., 1997).

Our knowledge of species’ phylogeography in the Mediterranean Sea is mainly based on organisms with a high dispersal potential (such as fish, planktonic invertebrates or those with planktotrophic larvae) (Patarello et al., 2007 for a review). This bias may be hindering our understanding of evolutionary patterns within the Mediterranean Sea, since past and contemporary population connectivity may obscure the signal of historical events (Provant and Bennett, 2008). Such phylogeographic studies on organisms with a high dispersal potential have established a common set of processes resulting from fragmentation into glacial refugia, range expansions via postglacial water masses, and/or secondary contact zones among formerly disjunct lineages. However, species with reduced dispersal are likely to have faced ice scouring, local extinctions and isolation in refugia, which may indirectly result in loss of local genetic variability and increased differentiation among populations by promoting the effects of local selection, inbreeding, and drift. Moreover, shallow subtidal/intertidal organisms capable only of short distance dispersal are likely to have faced the many challenges of historical sea level fluctuations, constantly reshaping coastlines and displacing their suitable habitats (Provant and Bennett, 2008). Hence, the study of such species with limited dispersal capacity may reveal different historical patterns of refuges and migration routes, and may thus offer a more complete understanding of relationships between the evolutionary history and contemporary distribution of genetic variation.

In a prior molecular analysis, Calvo et al. (2009) showed that at least four genetically distinct clades exist under the name Dendropoma petraeum. These four cryptic clades seem geographically well-delimited and are highly divergent at both the levels of mitochondrial and nuclear genes. In effect, this may be interpreted as indicative of differentiation at the species-level. This cryptic radiation is characterized by genetic speciation with little or no ecological diversification (‘nonadaptive radiation’ sensu Gittenberger, 1991) and with low morphological differentiation (‘morphostatic radiation’, sensu Davis, 1992). The four cryptic species of the D. petraeum complex are ecological equivalents (occupying the same type of habitat and playing the same ecological role in the different areas they inhabit) (Calvo et al., 2009). A posteriori searching for biological/morphological differences between these cryptic species has revealed some almost unappreciable morphological differences but significant changes in their mode of intra-capsular larval development (Calvo et al., 2009; Usvyatsova and Galil, 2012).

Both ‘nonadaptive radiation’ and ‘morphostatic radiation’ require allopatric speciation. Actually, the allopatric distribution of these cryptic species supports predominantly vicariance-based cladogenetic patterns coupled to restricted post-speciation range expansions. Several lines of indirect evidence support the hypothesis that the allopatric distribution of these four clades persisted over evolutionary time scales in the absence of secondary contacts. Vicariance plays a key role in generating coastal biodiversity and dispersal explains many of the deviations from the phylogeographic hypotheses, but vicariant hypothesis is valid only if temporal congruence between vicariant events and cladogenesis can be established. Additionally, estimating the timing of divergence events is one of the central goals of contemporary evolutionary biology.

The high genetic divergence between the four clades obtained in our previous study (Calvo et al., 2009) could be indicative of an ancient radiation that might have predated the Messinian Salinity Crisis (MSC) between 5.9 and 5.3 Mya (Krijgsman et al., 1999). However, the generally more accepted hypothesis poses that virtually no stenohaline marine organism could have survived to the extreme dryness and salinity levels that existed during this epoch in the Mediterranean basin, and consequently the current fauna and flora need to have subsequently colonized the Mediterranean from the near Atlantic populations (Taviani, 2002). There are nevertheless some data supporting species survival during this period in Mediterranean refugia, probably existing in the deepest areas or near large river delta zones (Por, 2009), but this issue is still under debate.

The main objectives of the present study were: (1) to date the observed divergence events within D. petraeum complex; (2) to relate the vicariance processes driving differentiation to major climatic, oceanographic and geological changes occurring in the Mediterranean Sea; and (3) to infer associated demographic changes within this species-complex in the Mediterranean. Our ultimate goal was therefore to address the questions: (1) could an Atlantic ancestor of Dendropoma have colonized the Mediterranean in an eastward direction after the Messinian or did some Dendropoma species survive the MSC?, and (2) when and where did major cladogenetic events take place among the Mediterranean clades of Dendropoma?

2. Material and methods

2.1. Sampling and laboratory protocols

Specimens were collected from 29 sites spanning over 4000 km across the entire distribution range of D. petraeum species-complex (Fig. 1), from the Atlantic coasts of Morocco and southern Spain, to Israel and Lebanon in the easternmost Mediterranean. Abbreviations and coordinates for the sampling localities (from west to east) are listed in Table 1. All specimens were collected from the lower intertidal zone and were preserved in absolute ethanol.

Total DNA was extracted using ChargeSwitch gDNA Micro Tissue (Invitrogen) or DNeasy (Qiagen) extraction kits. Partial sequences from the mitochondrial cytochrome oxidase subunit I (COI) and 16S rRNA (16S) and from the nuclear internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA, were amplified by polymerase chain reaction (PCR) using the following primers: LCO1490 (Folmer et al., 1994), COI-H (Machordom et al., 2003), 16sar-L-myt (Lydeard et al., 1996), 16SBR (Palumbi et al., 2002), and ITS2.1 and ITS2.2 (Hugall et al., 1999). PCR conditions were the same as in Calvo et al. (2009). The amplified fragments (700 bp for each of the mitochondrial genes and 1200 bp for the ITS region) were purified by ethanol precipitation prior to sequencing both strands using BigDye Terminator in an ABI 3730 genetic analyzer (Applied Biosystems). The quality of the direct sequences obtained for the ITS region was insufficient because of intraindividual variations. Thus, the amplicons were cloned and treated as in Calvo et al. (2009). All sequences were deposited in GenBank under accession numbers: JQ677250–JQ6773012.

2.2. Sequence alignment, phylogenetic analyses and divergence time estimates

Forward and reverse DNA sequences obtained for each specimen were checked and assembled using the program Sequencher 4.8 (Gene Code Corporation) and aligned using Clustal X (Thompson et al., 1997) with a posterior correction by eye. Separate alignments were built for each locus (COI n = 127, 16S
Phylogenetic relationships among samples were assessed using BEAST 1.7.5 (Drummond et al., 2006; Drummond and Rambaut, 2007). All partitions were analyzed with their own substitution model, which according to the Akaiké information criterion implemented in jModelTest 0.1 (Posada, 2008) were: TrN + I for COI, GTR + G for 16S and HKY for ITS. Unlinked uncorrelated lognormal clock models and a common birth–death speciation tree prior for all gene partitions were used. MCMC chains were run for 50 million iterations with trees sampled every 2000 iterations. BEAST runs were repeated four times and their combined results were analyzed with the program Tracer 1.5 (Rambaut and Drummond, 2007) to ensure stability and convergence of the MCMC chains. For this analysis, only individuals amplified for the three loci were included.

Since the fossil record of vermetid gastropods is incomplete and unreliable, and species-specific substitution rates were unavailable, we employed a range of previous estimates of mitochondrial substitution rates for marine gastropods assumed to have evolved at broadly comparable rates as the taxa under study, based on fossil records and/or geological events: 0.85%–1.83% per million years for COI (Wilke, 2003; Kirkendale and Meyer, 2004; Collin, 2005) and 0.4–0.6% per million years for 16S (Kirkendale and Meyer, 2004; Duda and Kohn, 2005).

These rates were used to estimate divergence times and their associated highest posterior density (HPD) intervals between lineages identified in the phylogenetic analysis using the coalescent-based species tree reconstruction method implemented in BEAST (Drummond and Rambaut, 2007; Heled and Drummond, 2010), which incorporates all the data from every individual for every marker. A birth–death species tree and a piecewise linear and constant root population size model were included as priors. Rates were incorporated as priors with a normal distribution encompassing the range of values obtained from the literature within their 95% confidence interval (mean 1.35 ± 0.25% SD for COI, and mean 0.5 ± 0.05% SD for 16S). The substitution rate for the nuclear ITS gene was estimated according to the rates inferred for the mitochondrial genes. Additionally, for comparative purposes, we calibrated two molecular clocks according to two alternative biogeographical scenarios and estimated the substitution rates under each of the following hypotheses: (1) D. petraeum surviving the MSC and its major lineages diverging during this period (normal prior with 5.3 and 5.9 My as its 5% and 95% CI), and (2) D. petraeum not surviving the MSC and recolonizing the Mediterranean after that period (maximum age prior of 5.3 Mya for the most recent common ancestor (MRCA) of all lineages). Finally, we used Bayes factors (BF) to test which of these two biogeographical hypotheses best fitted our data. We used Tracer 1.5 to compare twice the difference in the marginal model posterior likelihoods as estimated from the harmonic mean of the sample of posterior trees for each scenario. Values of 2ln(BF) > 10 were considered as very strong evidence for a given model to be more likely than another (Kass and Raftery, 1995). In both analyses each gene was considered as a different partition with unlinked clock rates and substitution models, which were based on the results by jModelTest described above. Tree topologies were unlinked for the mitochondrial (COI + 16S) and nuclear genes (ITS) using the birth–death speciation tree prior. Each analysis was run four times under the same MCMC parameters described above.

2.3. Historical demography

We used an extended Bayesian skyline plot (EBSP) (Heled and Drummond, 2008) as implemented in BEAST 1.5.4. This method reconstructs changes in effective population size over time by analysing multiple unlinked loci with different modes of inheritance, thus reducing the coalescent error related to estimates based on a single genealogy (Ho and Shapiro, 2011). For each evolutionary lineage of the D. petraeum species-complex, we considered unlinked substitution for the three gene partitions, and unlinked trees for the mitochondrial (COI + 16S) and nuclear genes (ITS). Because of the intra-lineage level of the EBSP analyses, a strict-clock model was used. In shallow or intra-specific level phylogenies rate variation among lineages is mostly attributed to stochastic rather than to evolutionary processes (Drummond et al., 2006). Thus, relaxed-clock models are not recommended as they do not necessarily outperform strict-clocks and may reduce the precision of parameter estimates (Brown and Yang, 2011; Molak et al., 2013). Substitution rates were those used to estimate divergence rates and were included as normal priors. MCMC were run independently four times for 50 million iterations and sampled every 5000 iterations. In this analysis, we included individuals within each lineage amplified for at least one locus. We used the program Tracer 1.5 to construct the final EBSP from the combined runs and to estimate the time to the MRCA for each lineage and their associated HPD intervals.

2.4. Correlation between genetic and geographic distances

We also looked for positive correlation between genetic and geographic distances within each lineage through pairwise
Table 1
Sampling localities. n = number of specimens analyzed.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Geographic area</th>
<th>Code</th>
<th>Latitude</th>
<th>Longitude</th>
<th>n</th>
</tr>
</thead>
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<td>07.22</td>
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<td>DG</td>
<td>35.53 N</td>
<td>41.85 W</td>
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</tr>
<tr>
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<td>DG</td>
<td>36.04 N</td>
<td>05.25 W</td>
<td>4</td>
</tr>
<tr>
<td>Alboran Island, Spain</td>
<td>Alboran Sea AL</td>
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<td>56</td>
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<td>10</td>
<td>02.25</td>
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<tr>
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<td>02.01</td>
<td>3</td>
</tr>
<tr>
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<td>Alboran Sea LA</td>
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<td>52</td>
<td>52.87 W</td>
<td>2</td>
</tr>
<tr>
<td>Calnegre, Murcia, Spain</td>
<td>Alboran Sea LA</td>
<td>36</td>
<td>52</td>
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</tr>
<tr>
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<td>52.87 W</td>
<td>2</td>
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<tr>
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<td>Alboran Sea LA</td>
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<td>52</td>
<td>52.87 W</td>
<td>2</td>
</tr>
<tr>
<td>Portixol, Valencia, Spain</td>
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<td>36</td>
<td>52</td>
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<td>2</td>
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<tr>
<td>Lavezzi Island, Corsica, France</td>
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<tr>
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<td>37</td>
<td>39</td>
<td>09.51</td>
<td>5</td>
</tr>
<tr>
<td>El Haousaria, Cap Bon, Tunisia</td>
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<td>37</td>
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<td>09.51</td>
<td>5</td>
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<tr>
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<tr>
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<td>38.09 N</td>
<td>12.43 E</td>
<td>9</td>
</tr>
<tr>
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<td>14.27 E</td>
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<tr>
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<td>14.27 E</td>
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<tr>
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<td>35</td>
<td>14.27 E</td>
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<tr>
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<td>Tyrrhenian Sea</td>
<td>ES</td>
<td>35.08 N</td>
<td>19.07 E</td>
<td>9</td>
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<tr>
<td>Aci Trezza, Sicily, Italy</td>
<td>Tyrrhenian Sea</td>
<td>ES</td>
<td>35.08 N</td>
<td>19.07 E</td>
<td>9</td>
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<tr>
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<td>36</td>
<td>15.13</td>
<td>6</td>
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<tr>
<td>Torre Squillace, Apulia, Italy</td>
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<td>03.09 N</td>
<td>36.70 E</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>St. Cesarea Term, Apulia, Italy</td>
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<td>03.09 N</td>
<td>36.70 E</td>
<td>2</td>
<td></td>
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<tr>
<td>Falasarna beach, Crete</td>
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<td>Ras Al Bayada, Lebanon</td>
<td>Levantine SL</td>
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<td>6</td>
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<tr>
<td>Batroum, Lebanon</td>
<td>Levantine NL</td>
<td>35</td>
<td>30</td>
<td>19.38</td>
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</table>

A subset of 87 individuals representing the major lineages of the *D. petraeum* species complex were sequenced for the nuclear internal transcribed spacers (ITS1 and ITS2) and 5.8S rRNA genes (ITS; 1113 bp) rendering a combined mitochondrial and nuclear DNA data set of 2375 bp.

The Bayesian phylogenetic analysis identified four well-supported and largely differentiated lineages of *D. petraeum* (all posterior probabilities PP = 1.0, Fig. 2), showing a clearly delimited geographic distribution in a west–east direction as: Western, Tyrrhenian–Sicilian, Ionian–Aegean and Levantine (Figs. 1 and 2). The Western lineage included specimens from the Atlantic coasts of Morocco and Spain to Tunisia and the Spanish Mediterranean coast. Within this lineage, we could distinguish clades from localities in Tunisia (TU and WTU) and the Atlantic Spanish coast (AS). The Tyrrhenian–Sicilian lineage was distributed across Corsica, Gulf of Naples, Sicily and Malta. Specimens from northern Sicily (CNS) appeared in a basal position with respect to the others, which formed two groups, including samples from Malta, Corsica, Naples and north-eastern Sicily (MA1, MA2, IL, CA and SS), and south-eastern and north-western Sicily (NS, ES and SES). The Ionian-Aegean lineage occurred along the Apulian coasts in SE Italy and Crete as two divergent clades, respectively. Finally, the Levantine lineage included specimens from Israel and Lebanon, those from south Lebanon (SL) being clearly differentiated from the others.

### 3.1. Divergence times

The inferred species tree recovered with moderate posterior probability values the same relationships as our phylogenetic hypothesis (PP = 0.89 for the Western and Tyrrhenian clades and 0.80 for the Ionian–Aegean and Levantine clades) (Fig. 3).

Our estimate for the initial divergence time of the two *D. petraeum* complex ancestral lineages ranged from 12.39 to 7.78 mya (mean 10.09 mya, corresponding to the Tortonian period). These lineages subsequently gave rise to the current Ionian–Aegean and Levantine (11.31–4.74 mya, mean 8.14 mya) lineages and Western and Tyrrhenian–Sicilian (10.93–5.04 mya, mean 7.86 mya), respectively (Fig. 3). Highest posterior density interval ranges for these time estimates largely overlap, indicating that these diversification events were coeval.

The most recent stage of diversification includes the main within-lineage diversification events. The mean tMRCA for each of the lineages were estimated between 3.57 and 0.52 mya (Figs. 3 and 4). Again, the overlapping intervals and similar time estimates suggest the existence of a series of global/widespread coeval processes that induced diversification in all lineages.

When calibrating the molecular clock relative to the survival, or not, of *Dendropoma* to the MSC, the estimated substitution rates in the first case for the COI was 1.64% per million years (95% HPD: 1.36–1.95), 0.97% (95% HPD: 0.766–1.17) for 16S and 0.10% (95% HPD: 0.06–0.14) for ITS. On the other hand, if *Dendropoma* had not survived the MSC the inferred rates were: 5.16% per million years (95% HPD: 1.86–10.04) for COI, 3.04% (95% HPD: 1.05–5.95) for 16S and 0.33% (95% HPD: 0.095–0.62) for ITS. Applying this “fast” substitution rates, the tMRCA for each lineage were estimated between 0.81 and 0.21 mya. The BF comparing the MSC survival to the non-survival hypothesis was ln(BF) = 14.04, strongly supporting that *Dendropoma* survived the MSC.

### 3.2. Historical demography

Contrary to the concordant patterns of among-lineage differentiation, patterns of within-lineage demographic changes were strikingly different. Notwithstanding, the demographic histories of the Western and Tyrrhenian-Sicilian lineages were similar and both
reflect an increase in their effective population sizes around 1.5 mya, peaking about 0.6 mya, and thereafter decreasing until the present. In contrast, the Ionian–Aegean and Levantine lineages showed no major demographic changes in their early history, displaying constant effective population sizes since 2.5–2 mya. Current estimated effective population sizes are lowest for the eastern Mediterranean lineage and highest for the Tyrrhenian–Sicilian lineage. To account for possible biases in demographic history reconstruction due to differences in samples sizes among lineages, we repeated the tests with 13 randomly chosen samples four times (i.e. the minimum number of samples available for the Ionian–Aegean lineage). Results were qualitatively similar regardless of sample size and no effect on the relative shapes of the extended Bayesian skyline plots (EBSP) was observed.

3.3. Spatial genetic structure

Spatial genetic structure patterns also differed among lineages. Although direct significant correlation between geographical and genetic distances was observed for the Western lineage localities (Mantel $r = 0.649$, one-tailed $p = 0.0134$), this relationship was not observed for the Tyrrhenian lineage (Mantel $r = 0.166$, one-tailed $p = 0.215$). For the Ionian–Aegean and Levantine lineages, sample sizes ($n = 3$) were too small for valid Mantel test permutations. However, the data suggested positive correlation for the Ionian–Aegean but not for the Levantine lineage (Supplementary Data 1).

Fig. 2. Maximum clade credibility phylogenetic tree recovered from the Bayesian analysis of mitochondrial (COI and 16S) and nuclear (ITS) data of Dendropoma petraeum. Numbers above branches are posterior probability values ($P > 0.95$). Locality codes as indicated in Table 1. Vertical lines represent the main lineages obtained and discussed in this study.
**Fig. 3.** Species tree based on the multispecies coalescent implemented in BEAST and obtained for the complete data set of mitochondrial and nuclear data. Node bars represent 95% highest posterior densities (HPD) of the estimated divergence time between lineages, and bars on branches represent 95% HPD of time estimated tMRCA within lineages. Dashed-lined squares represent the two main periods of diversification discussed in text. The gray square indicates the Messinian Salinity Crisis (MSC).

**Fig. 4.** Extended Bayesian Skyline Plots (EBSP) showing historical demographic trends for the main lineages of *Dendropoma petraeum*. x-axis: time in million years (MY) before present; y-axis: estimated population size [units = Ne(t), the product of effective population size and generation time in years (log transformed)]. Solid lines represent median values for the estimated effective population sizes and dotted lines are associated 95% HPDs.
4. Discussion

Our genetic data confirm the existence of cryptic species within the genus *Dendropoma* in the Mediterranean, some showing surprisingly large genetic differentiation given their morphological similarity (Calvo et al., 2009). The high genetic divergence detected between the different clades is consistent with an ancient split between the four lineages. This suggests a long history of population subdivision, with some clades appearing to be older than of Pleistocene age.

4.1. Miocene radiation

Our results point to two major cladogenetic events in the diversification of this genus in the Mediterranean. The estimates obtained from the substitution rates indicate that the first, and oldest, is an ancient diversification that likely occurred in a period of 10–4.5 mya (HPD interval), in which the four major lineages of *Dendropoma* emerged (inferred Late Miocene radiation). According to the results of the coalescent analysis, this first and older radiation occurred by two successive dichotomies, effectively resulting in three different events. After the first event (about 10 mya), the following two diversification events that subsequently gave rise to the current four major clades occurred in the same period (about 8 mya). By that time, the Mediterranean Sea showed a great geographical complexity, with some consistent landmasses and archipelagos giving rise to some sub-basins and internal seaway connection (sills and corridors) (Harzhauser and Piller, 2007). Also, an intense pattern of thermal zonation (horizontal and vertical) affected the ocean after the drastic climatic and oceanographic changes that characterized the middle Miocene. It is commonly accepted that at the end of this period, during the MSC (5.9–5.3 mya) (Krijgsman et al., 1999), the Strait of Gibraltar was closed for about 630,000 years and the Mediterranean Sea was reduced to a series of brackish-water or hypersaline lakes (the so called “Lago Mare” event that preceded the Zanclean marine flooding). Thus, it is generally assumed that only tolerant euryhaline or brackish-water organisms survived the MSC, and according to Taviani (2002), no robust indication of fully marine trans-Messinian species has been detected so far.

Hence, if the substitution rates for the mitochondrial genes in *Dendropoma* are similar to those in other marine gastropods, our estimates indicate that the divergence time between the different species of *Dendropoma* in the Mediterranean predates or coincides with the MSC suggesting that they would have survived this dramatic period within this sea in isolated refugia, at least the Tyrrenhian–Sicilian, Ionian–Aegean and Levantine species. The westernmost species could have survived in the nearest Atlantic and might have gradually reinvaded the Mediterranean Sea in a stepwise progression following its replenishing. In effect, the genus *Dendropoma* was present in the Canary Islands at least until about 23 mya (González-Delgado et al., 2005).

According to Meijer and Krijgsman (2005), the process of desiccation of the Mediterranean during the MSC was near complete in the western basins, while a significant water column remained in much of the eastern basin. These authors also claimed that the MSC was not a synchronous process, but comprised many cycles of desiccation and re-filling. Similarly, saline conditions in the Messinian period may have been more varied than commonly assumed. Brachert et al. (2007) discussed the possibility of some refugia existing for stenohaline biota to survive and recover after episodes of high salinity. The persistence of a residual body of marine water was also proposed to explain the concomitant occurrence of marine and freshwater faunal assemblages characterizing the Lago Mare scenario of the Sicilian basin (Roveri et al., 2008). It could therefore be that some true marine enclaves persisted in the deeper areas of the Mediterranean and served as refugia for some “Messinian” species (Boudouresque, 2004; Emig and Geistdoerfer, 2004; Popov et al., 2006). The marine angiosperm *Posidonia oceanica* has been claimed as a Tethyan relic surviving the MSC somewhere in the Mediterranean, perhaps the Aegean Sea (Boudouresque, 2004), since it is unlikely that this seagrass has recolonized the Mediterranean from a refuge in the nearby Atlantic. For some taxa it has been suggested that the split between Atlantic and Mediterranean sister species occurred during or even before the Messinian crisis, such as within the species complexes of the sea urchin *Echinocardium cordatum* (Chenuil and Férál, 2003), the prawn *Palaemon elegans* (Reuschel et al., 2010) or within the genus *Maja* (Sotelo et al., 2009). Likewise, Schubart et al. (2001) pointed out that the three Mediterranean endemic crabs of the genus *Brachynotus* probably diverged during the Messinian crisis. In addition, the *Calobius quadricollis* species-complex of moss beetles showed a pattern of geographic diversification very similar to that of the *D. petraeum* complex, with allopatric species mostly occurring in the different Mediterranean sub-basins. The split between the Atlantic and Mediterranean lineages of the *C. quadricollis* species-complex occurred in the Late Miocene, while subsequent radiation in the Mediterranean Sea started in the early Pliocene (Antonini et al., 2010).

Thus, our results drawn from an average distribution of mollusc substitution rates suggest that at least some intertidal marine taxa such as *Dendropoma* spp. could have survived the MSC. Isolation of the eastern Mediterranean refugia may have been accompanied by reduction of their population sizes, enhancing divergence in allopatry of the isolated populations, and in turn resulting in incipient speciation. Hence, these cryptic species of *Dendropoma* could be considered as paleo-endemisms (Miocene, probably of Tethyan origin), contrasting with the neo-endemisms (mainly of Pliocene origin) that constitute most of present-day endemic species in the Mediterranean (Bianchi and Morri, 2000; Boudouresque, 2004). Neo-endemisms arose through in situ speciation after the MSC, while paleo-endemisms (Tethyan relics) are though to have survived MSC in the nearby Atlantic with the subsequent extinction of the Atlantic counterparts after their recolonization of the Mediterranean (Domingues et al., 2005). Our results, which are further supported by the BF comparison, suggest that some paleo-endemisms may have also survived the MSC in true marine enclaves within the Mediterranean itself.

Conversely, if we consider the most accepted hypothesis that no true marine organism could survive the MSC, we must assume that the diversification of the major clades of *Dendropoma* within the Mediterranean happened after the Zanclean re-flooding. If so, the estimated mitochondrial substitution rates in *Dendropoma* would be among the highest ever reported among marine gastropods (between 3 and 6-fold for COI and between 5 and 7-fold for 16S (Wilke, 2003; Kirkendale and Meyer, 2004; Collin, 2005; Duda and Kohn, 2005), or even among other non-marine gastropods (Deliaco et al., 2013, and references therein). It can be argued that the highly dynamic mitochondrial genome of vermetid gastropods pointed out by Rawlings et al. (2001, 2010) may explain these extremely high substitutions rates, and consequently support the non-survival hypothesis. The available fossil record also does not contradict this hypothesis, since the oldest known *Dendropoma* reefs in the Mediterranean were recently described in NE Spain and dated as arising from the maximum flooding conditions after the MSC (Aguirre et al., 2014). Conversely, the most abundant vermetid reef-builder in the Late Miocene belonged to the genus *Petalococonchus* (Brachert et al., 2007; Saint Martin et al., 2007; Vescogni et al., 2008). This could mean that the evolutionary replacement of *Petalococonchus* for *Dendropoma* in the composition
of Mediterranean vermetid reefs started at the end of the Neogene, just after the MSC.

4.2. Pliocene–Pleistocene diversification

The most recent stage of diversification seems to have occurred in the Pliocene (~3.7–1.3 mya). This period corresponds to diversification within each of the lineages, giving rise to groups that do not always show a consistent geographical pattern. This stage also coincides with the cooling down of the region. Lastly, the different clades underwent further diversification during the Pleistocene climatic cycles and eustatic sea-level fluctuations, with the subsequent isolating and fragmentation of their populations.

Except for modified shorelines due to lowering of the sea level, the shape of the Mediterranean basin and adjoining seas did not significantly change after the MSC. The last two million years have been characterized by at least ten ice ages, each with numerous climatic subcycles on scales of thousands and tens of thousands of years (Cronin, 1999). These cycles led to ocean-climate changes and lowered sea levels which greatly influenced the distribution and abundance of marine populations in the Mediterranean and Northeastern Atlantic.

The Mediterranean is subdivided into coastal seas isolated from each other by peninsulas and narrow straits, and this complexity could prompt the isolation of populations by reducing levels of gene flow between regions. For example, several peninsulas partially isolate the Adriatic and Aegean seas from the rest of the Mediterranean, shallow sills in the Bosphorus Strait isolate the Black Sea from the Mediterranean, the sill of the strait of Sicily divides the Mediterranean into the western and eastern basins, and the constriction of the Strait of Gibraltar partially isolates the Mediterranean from the eastern Atlantic. In addition to physical barriers imposed by complex shorelines, the Mediterranean is characterized by local upwelling, current patterns, isotherms and ocean-fronts that reinforce isolation and genetic differentiation of drift-driven populations.

4.3. Time of cladogenetic events in other intertidal gastropods

Within the common intertidal limpets of the genus Patella, Sá-Pinto et al. (2008) obtained a divergence time around 5.7 mya for the Mediterranean P. caerulea versus the other Atlantic species of the same clade. Further, in a recent study on the Northeastern Atlantic/Mediterranean intertidal gastropod genus Phorcus (previously Osilinus), Donald et al. (2012) showed that the main lineages of this genus diverged in the same period suggested for Dendropoma, i.e. prior to the MSC. After this dramatic period (in the Pliocene), the two closest Mediterranean species, P. richardi and P. articulatus, diverged within this basin.

Similar temporal patterns have been also observed for the European brackish-water mud snails Hydrobia s.l. (Wilke and Pfenninger, 2002; Wilke, 2003). The time estimated by these authors for splitting of the main lineages, now considered different genera (Hydrobia, Peringia, Ventrosia, Adriohydrobia and Salenthydrobia), was 14–7 Mya, while diversification within each major lineage occurred during Plio-Pleistocene climatic cycles.

4.4. Historical demography

We observed several patterns of within-lineage demographic changes. The demographic histories of the Western and Tyrrhenian-Sicilian lineages were similar and both revealed an increase in effective population sizes occurring around 1.5 mya, peaking around 0.6 mya, and thereafter declining until the present. This time interval (1.5–0.6 mya) coincides with the most intense and prolonged interglacial period of the Quaternary (Emig and Geistdoerfer, 2004). Since Dendropoma is a warm climate subtropical gastropod, presumably during this long period its effective population sizes gradually increased and expanded along the coasts of the western and central Mediterranean. After this stage of growth, these populations would have markedly declined coinciding with the Günzt Glaciation (around 0.6 mya). From here onwards, they would continue to decline over successive glaciations.

In contrast, the Ionian–Aegean and Levantine lineages underwent no major demographic changes in their early history and have shown constant small effective population sizes since 2–2.5 mya. Thus, these eastern lineages seem to be a series of small relict populations surviving successive Quaternary glaciations in isolated refugia.

4.5. Spatial genetic structure within clades

Inferred gene flow may differ among equidistant localities due to unequal levels of either past or present-day gene flow caused by the presence of corridors (e.g. currents, continuous shorelines) or barriers (e.g. headlands, straits, unsuitable habitats) to dispersal. We detected different spatial genetic structure patterns among the four main lineages of Mediterranean Dendropoma. Although significant positive correlation was observed for the Western lineage localities, this relationship was not observed for the Tyrrhenian–Sicilian lineage.

The positive correlation between genetic and geographic distances within the Western clade may be the result of continuous range expansion along the North African and Spanish Mediterranean coasts following a progression rule through stepwise colonization. This pattern would be expected if the propagule’s vagility is sufficient to reach every subsequent population, but rare enough to maintain integrity of each deme and facilitate diversification. For example, the more or less continuous rocky shoreline from the Strait of Gibraltar to Tunisia or along the Spanish Mediterranean coast has provided a stepping-stone path of expansion over many generations in sessile species without a pelagic larval phase. Conglomerates of the D. petraeum complex seem to be dependent primarily on self-seeding to maintain their populations (most hatching snails act as “aggregators”, settling in the same parental reef or close to it) (Calvo et al., 1998). Thus, in this region, gene flow is not enough to prevent the build-up of fixed genetic differences among zones, as evidenced by the genetically structured clades along the northern coast of Africa (NA, TU, WTU) as well as in the Atlantic coast of Spain (AS). However, under certain conditions, some hatchlings could be dispersed efficiently by drifting through mucous threads (Calvo et al., 1998), acting as “founders” and leading to genetically homogeneous populations, as observed along the Spanish Mediterranean coast.

In contrast to the Western lineage, Tyrrhenian–Sicilian Dendropoma hold isolated populations with restricted gene flow among them. Contrary to a situation of substantial genetic differentiation at a large scale, no clear relationship between geographical distance and genetic divergence was noted at either a regional or local scale. Occasional rafting of post-metamorphic young snails has been suggested for the D. petraeum complex (Calvo et al., 1998), allowing sporadic gene flow among relatively distant populations depending on currents and the rocky coastal configuration. Hence, the apparent geographic/genetic incongruence within this clade may be the outcome of rare dispersal events followed by extensive isolation and random genetic drift.

A further possibility is based on the idea that the eastern Mediterranean basin arose from differential units. Thus, the genetic structure of some species is consistent with the subdivision of the eastern Mediterranean into several sub-basins. In effect,
Bahri-Sfar et al. (2000) suggest that the genetic distinctiveness of each subset probably means that each sub-basin is quite independent from the others in terms of spawning grounds and larval circulation. For the Ionian–Aegean and Levantine lineages of Dendropoma our sample sizes (n = 3 each) were too low to draw any valid conclusions from Mantel test permutations. However, from our data we could deduce positive geographic/genetic correlation for the Ionian–Aegean but not the Levantine lineage. Remarkably, the two extreme Levantine samples (from Israel and north Lebanon) were found to be genetically closer to each other than to the sample from an intermediate locality (south Lebanon). This may have been the result of a sporadic long-distance dispersal event (rare founder event) between otherwise distant and isolated coastal areas.

5. Conclusion

Our data confirm the existence of at least four distinct species of the genus Dendropoma in the Mediterranean and identify two main stages of diversification related to the two main events that marked the history of this sea. One stage took place in the Late Miocene–Early Pliocene and the other stage during the glacial-interglacial cycles in the Plio-Pleistocene. Regarding the first of these stages of diversification, the pattern observed may have resulted from two possible scenarios:

(1) If we assume that substitution rates in the mitochondrial genome of Dendropoma are similar to the ones for other gastropods, our results, as supported by the BF comparison, show that some intertidal truly-marine taxa (tolerant to changes in salinity and temperature) such as Dendropoma spp. could have survived the MSC, and that near marine conditions may have persisted during the MSC, at least somewhere in the eastern Mediterranean basin.

(2) If the hypothesis that no true marine organism could survive the MSC is accepted, and then we assume that the diversification of major Mediterranean clades of Dendropoma were posterior to the Mediterranean desiccation, then these vermetid gastropods would have an unusually high rate of molecular evolution compared to other species.

What we can conclude, is that this species complex and the present genetic structure of its different lineages appear to have arisen from interactions between the biology of these vermetid gastropods and the complex paleohistory, geography and hydrography of the Mediterranean Sea. Moreover, further studies on molecular evolution of vermetids and other groups of gastropods are needed to investigate the possibility of a faster molecular clock. If the no-survival hypothesis is true, then the vicariant events needed for this morphostatic speciation to occur have to be elucidated. Restricted dispersal between the different basins of the Mediterranean could, in this case, be mentioned as possible main factors leading to the genetic diversification of the major clades of Dendropoma.

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Appendix A. Supplementary material

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References


