**Genetic structure and diversity of the endangered bath
sponge *Spongia lamella* in Occidental Europe**

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Running head: Genetics of an endangered bath sponge

ABSTRACT

The present study explored population structuring, connectivity, and distribution of genetic diversity of the endangered bath sponge *Spongia lamella* along the Western Mediterranean and the Portuguese coast. Seven microsatellite markers were used to genotype 231 individuals scattered over nine populations. Basic genetic descriptors and population genetic analyses based on F_{ST} test, analyses of the molecular variance (AMOVA), Bayesian clustering, dissimilarity analysis of principal components, and demographic analyses were computed from microsatellite data. Results demonstrated large genetic differentiation between populations (global $F_{ST}=0.236$, $p=0.000$), and AMOVA and Bayesian analyses showed genetic divergence among the Atlantic, Balearic, and North Mediterranean areas ($F_{CT}=0.129$, $p=0.003$). Restricted gene flow due to short-distance dispersal of the larvae and hydrographical barriers are surely playing an important role on genetic differentiation in the species. The high levels of inbreeding, sub-structuring and modest levels of genetic diversity, which characterized populations *S. lamella* (mean value of genetic diversity 0.512), may compromise the long time survival of the species. Recent bottlenecks were also detected for most populations of this sponge. Only one population from the Gibraltar Strait, presented high levels of genetic diversity (Ceuta, genetic diversity = 0.657), being a hotspots of genetic diversity for this species with special relevance for its conservation. Disease outbreaks and overexploitation may be the most important causes of genetic diversity impoverishment of *S. lamella*. Future conservation guidelines for *S. lamella* should focus on: a) preserving genetic diversity within genetically impoverished populations by limiting exploitation and b) increasing population size by transplantation of specimens

48 from source areas with high values of genetic diversity to those which have recently
49 passed through a demographic decline and present low values of diversity.

50 **Keywords:** Subtidal, sublittoral. genetics, biodiversity, dispersal, benthos, invertebrates

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INTRODUCTION

Genetic diversity, defined as one of the three major levels of biodiversity, plays a crucial role in the long term survival of species because it is the raw material on which selection acts (Frankham, 2005a). In natural habitats impacted by anthropogenic activity, species and populations face not only stochastic events associated with population size reduction but also threats from deterministic factors such as habitat loss, overexploitation, pollution, or introduced species among others (Brook *et al.*, 2002). Threatened species with isolated, small, or declining populations may lead to fast loss of genetic diversity by genetic drift and inbreeding depression, which jeopardize species survival (Frankham, *et al.*, 2002; DiBattista, 2008). Enhancing genetic fitness and connectivity between populations of endangered species should be one of the pivotal points in marine conservation policies (Uriz and Turon, 2012).

Marine sponges (Porifera) are mainly benthic sessile filter-feeders which confer stability and structure to benthic assemblages (Uriz and Turon, 2012). Sponges exhibit a diversity of reproductive patterns, including various forms of asexual and sexual strategies (Whalan *et al.* 2005). Sponge larvae are commonly classified among the lecithotrophic meroplankton with limited swimming and dispersal abilities (Sara and Vacelet, 1973; Uriz *et al.*, 2001; Mariani *et al.*, 2006; Uriz *et al.*, 2008; Uriz and Turon, 2012) and most studies on population genetics of marine sponges demonstrated highly structured populations with limited gene flow (Duran *et al.*, 2004a; Duran *et al.*, 2004b; Duran *et al.*, 2004c; Nichols and Barnes, 2005; Calderon *et al.*, 2007; Blanquer *et al.*, 2009; López-Legentil and Pawlik, 2009; Blanquer and Uriz, 2010; Dailianis *et al.*, 2011). However other factors such as ocean currents, philopatric larval behavior,

historical and demographic processes, biotic interactions, or recruitment patterns also affect on connectivity among populations, as reported for other marine groups with similar larval development (some examples in Barber *et al.*, 2002; Sponer and Roy, 2002; Vollmer and Palumbi, 2007; Miller and Ayre, 2008; Perez-Portela and Turon, 2008; Yasuda *et al.*, 2009).

Sponges are recognized as a natural resource since ancient times as they were traditionally collected for diverse purposes (Rützler, 1996). The majority of bath sponges belong to the cosmopolitan genus *Spongia* (Demospogiae; Dyctyoceratida; Spongiidae) (Pronzato, 1999). Their characteristics reside in their lack of mineral spicules and the presence of a spongine fiber skeleton with a remarkable elasticity and water holding capacity (Rützler, 1996). Commercial sponge beds in the Mediterranean Sea decreased substantially as a consequence of high demand and declined of bath sponges in other areas of the world (Gaino and Pronzato, 1992; Pronzato, 1999). An increasing number of studies have also reported diseases in marine invertebrates, often associated with environmental factors such as temperature anomalies, pollution, and introduction of species including pathogens (Webster, 2007; Garrabou *et al.*, 2009). Sponges seem particularly vulnerable to disease outbreaks (Gaino *et al.*, 1992; Pronzato, 1999; Perez *et al.*, 2000; Webster, 2007) which can have serious long-term effect on long-lived, slow-growing sponge populations (Webster, 2007). The combination of harvesting, diseases, loss of habitat quality and fragmentation has driven numerous commercial sponges practically disappeared from the majority of exploited banks (Gaino *et al.*, 1992; Pronzato, 1999).

The species *Spongia lamella* (previously known as *S. agaricina*) is one of the five Mediterranean commercial sponges (Gaino and Pronzato, 1992; Pronzato and

Manconi, 2008) overexploited during the past years. This fan-like (elephant ear) or cup-shaped sponge can reach spectacular diameters up to 1.5 m (Pronzato, 1999). Although the Mediterranean elephant ear is known as *S. agaricina*, recent studies have reopened the debate concerning the Mediterranean and Philippine elephant ears showing significant differences between these two species (Castritsi-Catharios *et al.*, 2007; Pronzato and Manconi, 2008). Furthermore, *S. agaricina* was first described in the Indian Ocean. For these reasons, Pronzato and Manconi (2008) suggested keeping *S. agaricina* Pallas 1766 for the Philippine elephant ear and using *S. lamella* Schulze 1879 for the Mediterranean elephant ear. Thus in this article, the reader will find *S. lamella* to refer to the Mediterranean elephant ear although previous references used *S. agaricina* to deal with the same Mediterranean sponge.

Populations of *S. lamella*, as other commercial sponges, are currently scarce and the species was included among marine endangered species in the Annex 3 of Bern and Barcelona Conventions (Templado *et al.*, 2004). The species is particularly interesting in the maintenance of marine diversity from different perspectives; it produces abundant cytotoxic furanoterpenes (Aiello *et al.*, 1988; Rueda *et al.*, 1998), host a diverse community of benthic macrofauna (authors' pers. obsv.), and an abundant and complex bacterial community (Noyer *et al.*, 2010). However, we have a very limited data on its biology and the available information for conservation purposes is extremely scarce.

Genetic descriptors have been widely applied in conservation biology to evaluate the health state and vulnerability of marine species (see Hellberg *et al.*, 2002; Pearse and Crandall, 2004; and some examples in Bester *et al.*, 2011; Dialianis *et al.*, 2011; Schunter *et al.*, 2011 among others). Microsatellites nuclear markers are one of the most efficient genetic tools to investigate population genetics, demography, and

clonality in sponges since mitochondrial sequences have shown little variability, and insufficient resolution for population genetics approaches in most cases (Duran *et al.*, 2004b; Wörheide *et al.*, 2005; Uriz and Turon, 2012). Microsatellites have been isolated for only 7 marine sponges up to now (Blanquer *et al.*, 2005; Duran *et al.*, 2002; Hoshino and Fujita, 2006; Knowlton *et al.*, 2003; Noyer *et al.*, 2009; Dailianis and Tsigenopoulos, 2010; Guardiola *et al.*, 2012) but studies on population genetics of native species using these markers are scarce and available for only 3 marine sponges: *Crambe crambe* (Duran *et al.*, 2004c; Calderon *et al.*, 2007), *Scopalina lophyropoda* (Blanquer *et al.*, 2009; Blanquer and Uriz, 2010) and *Spongia officinalis* (Dailianis *et al.*, 2011).

Here, in this study we sought to understand genetic structuring, connectivity, and distribution of genetic diversity of the sponge *S. lamella* along the Atlanto-Mediterranean area. To accomplish these objectives seven microsatellite loci specifically designed for *S. lamella* (Noyer *et al.*, 2009) were used to: a) explore genetic structuring and distribution of the genetic diversity along the Atlantic- Western Mediterranean area, b) identify potential barriers to gene flow, and c) infer demographic decline of populations.

METHODS

Samples collection and DNA extraction

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4 146 A total of 231 individuals of *Spongia lamella* were sampled from nine different
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6 147 localities by SCUBA diving between 2005 and 2008 (see Table 1). Locations were
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8 148 distributed within three "a priori" different areas. Within the "Atlantic" area two
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10 149 populations were collected, one in the Berlengas archipelago in Portugal (Por) and
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12 150 another in Ceuta (Ce), which is situated at the Gibraltar Strait with strong Atlantic
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14 151 influence. Within the northwestern Mediterranean we defined two areas; "Cabrera
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16 152 Island" (Cab) in the Balearic archipelago, and the "Northwestern Mediterranean"
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18 153 including populations from Catalonia [Cap de Creus (Cr), Els Bullents (Bul) and Arenys
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20 154 (Are)], and the Gulf of Marseille [Plane (Pla), Pharillon (Far) and La Ciotat (Cio)] (see
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22 155 Fig. 1). For each specimen, a fraction on one edge of the sponge was cut underwater to
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24 156 minimize damages, placed into plastic bag containing sea water, and stored in cool box
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26 157 until further processing (usually 1 – 2 h after sampling). Sponge tissues were then rinsed
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28 158 in, at least, two absolute ethanol baths to prevent ethanol dilution and DNA degradation,
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30 159 ant they were finally preserved in absolute ethanol at –20°C until processed. Samples
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32 160 were cleaned of foreign tissues and DNA was extracted as described in Noyer *et al.*,
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34 161 (2009).
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163 **Population genotyping**

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46 165 Among the 8 microsatellite loci previously developed for this species (Noyer *et al.*,
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48 166 2009), seven microsatellites (SAa, SAc, SAd, SAf, SAg, SAK, and SAn) were selected.
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50 167 One of the eight microsatellites was discarded for this study due to scoring difficulties.
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52 168 Amplification of fragments were performed by Polymerase Chain Reaction (PCR) in a
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54 169 final volume of 20µl containing 1 µl of DNA template, 2µl of 10X Taq polymerase
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170 buffer, 4mM of MgCl₂, 0.25 mM of dNTP, 0.25 µM of each primer, 350ng/µl of Bovine
171 Serum Albumin and 1U Taq polymerase (Bioron). The forward primer for each locus
172 was labeled with fluorescent dyes (NED, VIC and PET from Applied Biosystems, as
173 previously described in Noyer *et al.*, 2009). PCRs were performed in a MWG primus
174 thermocycler or a Mastercycler® Eppendorf. PCR parameters were: 5 min denaturation
175 step at 95°C, followed by 30 amplification cycles of 55 sec at 95°C, 35 sec at locus
176 specific annealing temperature (Noyer *et al.*, 2009), and 45 sec at 72°C, followed by an
177 extension cycle of 7 min at 72°C. Resulting PCR products were then visualized on a
178 1.5% agarose gel. Successful amplifications were genotyped using an ABI Prism 3700
179 automated sequencer (Applied Biosystems, in the Scientific and Technical Services of
180 the University of Barcelona). Reamplification with decreasing annealing temperature
181 and reextraction of the DNA were necessary when initial amplification failed. Allele
182 size characterization was performed with internal standard LIZ and Peak Scanner™
183 software (version 1.0, from Applied Biosystems).

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185 **Data analyses**

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187 Analyses within localities

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189 The number of alleles per locus and per populations, observed heterozygosity (H_o),
190 expected heterozygosity (H_e), genetic diversity and inbreeding coefficients (F_{IS}) were
191 calculated using Genetix version 4.03 (Belkhir *et al.*, 2004). The exact test for departure
192 from Hardy Weinberg Equilibrium (HWE) was performed using Genepop web version
193 4.0.10 (Raymond and Rousset, 1995), using a probability test (with level of significance

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4 194 determined by Markov chain parameters: 5,000 dememorization steps, 1,000 batches,
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6 195 and 5,000 iterations per batch). Micro-Checker version 2.2.3 (Van Oosterhout *et al.*,
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8 196 2004) was used to analyze the potential causes of the observed deficit of heterozygotes.
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10 197 Micro-checker detects large allele dropouts and scores errors due to shuttering and
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12 198 presence of null alleles. The software Genepop was used to test for linkage
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14 199 disequilibrium across all populations using a likelihood ratio test with the level of
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16 200 significance determined by permutation (Markov chain parameters: 5,000
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18 201 dememorization steps, 1,000 batches, and 5,000 iterations per batch). A false discovery
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20 202 rate (FDR) correction was applied to the p-values (B-Y method as described in Narum,
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22 203 2006) to account for multiple tests.
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28 205 Population differentiation and demographic analyses

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33 207 Arlequin software (Excoffier *et al.*, 2005) was used to estimate population
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35 208 differentiation with the F_{ST} statistic between pairwise populations based on an allele
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37 209 infinite model. Since the F_{ST} has lower variance and performs better than the R_{ST}
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39 210 statistic, which assumes a stepwise mutation model, when sampled size is under 50
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41 211 individuals per sites or the number of loci is under 20 (Gaggiotti *et al.*, 1999) we only
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43 212 used the F_{ST} in this study. The significance of the values was evaluated by performing
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45 213 16,000 permutations with the same software, and the Narum corrections were applied
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47 214 (Narum, 2006). A multidimensional scaling analysis (MDS) was performed to
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49 215 graphically visualise interrelationships represented by the matrix of genetic distances
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51 216 from the F_{ST} values with the software Sigmaplot 10.0. Isolation by distance (IBD)
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53 217 between populations was tested using a Mantel test implemented in the IBD software
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(Bohonak, 2002). Geographic distances were estimated as the marine linear distance between pairs of populations, and genetic distances were calculated as $F_{ST} / (1 - F_{ST})$, using F_{ST} value obtained from Arlequin. Significance of the test was assessed using 50,000 permutations of the logarithm of geographic distances. Arlequin software was also used to perform analyses of molecular variance (AMOVA) based on the number of different alleles (F_{ST}). Populations were grouped within two and three different groups: Atlantic (Por and Ceu) and Mediterranean (Bul, Are, Cr, Pla, Far, Cio, Cab), following our prior expectation of a genetic division caused by “basin boundary”, and pooling populations within three geographical areas: Atlantic (Por and Ceu), Northwestern Mediterranean (Bul, Are, Cr, Pla, Far, Cio) and Balearic (Cabrera Island, Cab). The significance of AMOVA was calculated with 16,000 permutations of the original data. The F_{ST} and AMOVA analyses were performed both including and excluding microsatellites which did not amplified in some populations (SA_a, SA_d and SA_f) to avoid false results due to the absence of data.

To infer population genetic structure and optimal number of homogeneous genetic units (K) based on a Bayesian clustering analyses the software STRUCTURE version 2.3 (Pritchard *et al.*, 2000) was used. The program also allows detecting the occurrence of different subpopulations within a locality, the so-called Wahlund effect, when several subpopulations are sampled as one. The software was initially run with the whole dataset, with a K number from 1 to 16. In a second phase, we separately run several sets of populations; Atlantic (Por and Ceu), Catalonia (Cr, Bul, Are), and Gulf of Marseille (Pla, Far, Cio) in order to obtain a more detailed analysis of potential sub-structuring within geographical areas with simulations from $K= 1$ to 6. Ten independent replicates

with one million of MCMC (Markov Chain Monte Carlo) were performed for each run, and 100,000 burn-in period under the "admixture model" implemented within the software. The most likely value of "real" clusters was identified comparing the rate of change in the likelihood of K ($L(K)$). The optimal K values was determined using the ad hoc statistic ΔK (Evanno *et al.*, 2005). Results were graphically visualized and represented with the same software.

Additionally, a discriminant analysis of principal components (DAPC) was ran to assess genetic structure using populations as groups with the "adeigenet package" (Jombart *et al.*, 2008) for the R software. We initially ran the whole dataset, and in a second run only populations from Catalonia and Gulf of Marseille were included. DAPC is a multivariate analysis that integrates principal component analysis (PCA) with discriminant analysis to summarize genetic differentiation between groups. This kind of analysis can outperform more computer-intensive Bayesian clustering approaches in detecting genetic structure (Jombart *et al.*, 2010).

Because we empirically know that some marine sponges have suffered from disease outbreaks during the last decades in the Mediterranean (Pronzato, 1999), the program Bottleneck version 1.2.02 (Cornuet and Luikart, 1996; Piry *et al.*, 1999) was used to test if *Spongia lamella* has passed through recent population bottlenecks leading a genetic signal. We used two approaches to detect transient heterozygosity excess: the Wilcoxon test (the most powerful and robust test when used with few polymorphic loci, Piry *et al.*, 1999) and the "mode-shift" indicator (a graphical descriptor of the shape of allele frequency distribution; Luikart *et al.*, 1998; Piry *et al.*, 1999). The Wilcoxon test was conducted under the stepwise mutation model (SMM), the infinite allele model (IAM)

266 and the two phase model (TPM). The SMM and IAM representing two extreme models
267 of mutation for microsatellites (Luikart and Cornuet, 1998).

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269 **RESULTS**

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271 **Genetic characteristics**

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273 The main genetic descriptors of the nine populations investigated are listed in Table 2.

274 All microsatellites were polymorphic although SAa was monomorphic in the three

275 populations of the area of Marseille, and in two populations of Catalonia. SAa also

276 showed no amplification in Portugal. None individual amplified in Ceuta for SAd, and

277 none of the three populations of Catalonia amplified for SAf. Unsourced microsatellites

278 corresponded to both unsized alleles (although good amplifications were observed and

279 PCR repetitions were performed) and failure in amplification (null allele). It occurred

280 even after various attempts and modifications of PCR reactions. Micro-Checker also

281 highlighted the presence of null alleles in SAn. Although this microsatellite amplified

282 well in all populations, presence of null alleles was detected with Micro-Checker in 4

283 sponge populations. Null allele was also detected in SAd for 3 of the 9 populations.

284 Total number of alleles ranged from six for SAa, SAc and SAf to 24 for SAk. The

285 average number of alleles per locus across the seven loci varied from 3.0 in Cap de

286 Creus to 5.67 in Ceuta. Private alleles were found in six loci and for six out of the nine

287 sponge populations. Populations of Ceuta, Portugal, and Cabrera showed the highest

288 numbers of overall private alleles per population: seven, six, and four, respectively.

289 Among the 21 pairs of microsatellite comparison across all sampled, linkage

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4 290 disequilibrium was not detected after Narum corrections ($p \geq 0.0137$). We detected a
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6 291 general excess of homozygotes for most allele size classes. A deficit of heterozygotes
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8 292 was detected for the seven loci, and inbreeding coefficients (F_{IS}) were significant in
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10 293 populations, excepting Cr (Table 2). In particular SAn showed significant inbreeding
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12 294 coefficient for eight out of the nine sponge populations.
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16 17 296 **Population differentiation**

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21 298 Multi-locus pairwise comparisons based on F_{ST} tests were, in general, very high (global
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23 299 $F_{ST}=0.236$, $p=0.000$) and significant between most populations' pairs (see Table 3).
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25 300 Values of the F_{ST} ranged from 0.047 to 0.336, between Cap de Creus and Arenys, and
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27 301 La Ciotat and Ceuta, respectively. More detailed analyses of the F_{ST} values highlighted
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29 302 that the highest values of the statistic were detected between Ceuta and all the other
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31 303 populations, excluding Portugal. Also Portugal presented very high levels of divergence
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33 304 from all the other Mediterranean populations. The only population from the Balears,
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35 305 Cabrera (Cab), displayed moderate levels of differentiation from other populations.
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37 306 Populations from Gulf of Marseille and Catalonia had the lowest differentiation values,
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39 307 and only the differences between Creus and Els Bullents and Arenys were not
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41 308 significant. The MDS plot based on the F_{ST} values showed divergence between Atlantic
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43 309 populations, on one side, Mediterranean locations, on the opposite, and Cabrera on an
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45 310 intermediate position (Fig. 2, A).
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47 311 Results of the AMOVA pooling populations within two and three different groups are
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49 312 summarized in Table 4. The analyses in two and three different areas demonstrated that
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51 313 most of the molecular variance occurred within populations (68% and 72%, $p < 0.001$,
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314 respectively). Variance among populations within regions (17% and 15%, $p < 0.001$,
315 respectively), and among geographical regions (15% and 13%, $p = 0.02$ and $p=0.003$,
316 respectively) showed similar and significant values in both cases.

317 The significance values of the F_{ST} and the AMOVA analyses did not differ when only
318 four microsatellites were considered (data not shown). Hence all seven microsatellites
319 were finally considered for further analyses.

320 A general positive and significant correlation between genetic ($F_{ST}/(1-F_{ST})$) and
321 geographic distances (Mantel test: $r = 0.724$, $p = 0.003$, for the logarithm of
322 geographical distances) was obtained. This pattern of isolation by distance is
323 particularly strong and highly significant between populations over 200 km apart
324 (Mantel test: $r = 0.849$, $p < 0.001$) (see Fig. 3).

325 Results from STRUCTURE, based on Bayesian clustering, revealed partition of
326 the whole dataset of *S. lamella* within two or three main genetically homogeneous
327 clusters (K), depending on the criterion to select optimum K value. The highest
328 likelihood value was detected when $K=3$ whereas the ΔK (1248) as Evanno *et al.*,
329 (2005) rapidly decreased after $K=2$ (see Fig. 4 and 5). The assignment of individual
330 genotypes separated the Atlantic, Balearic, and Northwestern Mediterranean pools
331 already detected by the AMOVA when we fixed $K=3$ (see Fig. 4 A). Secondary runs for
332 Atlantic (Por and Ceu), Catalonia (Are, Cr, Bul) and Gulf of Marseille (Far, Cio, Pla)
333 populations suggested the existence of genetic sub-structuring within localities of the
334 Atlantic and Marseille area (optimal $K=3$ and 4, respectively from both criterion to
335 select optimum K) (see Fig. 4 B, C and D).

336 The DAPC analysis graphically depicts the inter-population structure for *S.*
337 *lamella*. The pattern of population divergence within three different clusters or groups is

in agreement with the results of other analyses (Fig. 2, B). The Atlantic cluster (Por and Ceu) was clearly separated on the first PCA axis, whereas the Balearic population (Cab) and Northwestern Mediterranean populations (Are, Cr, Bul, Far, Cio, Pla) were separated from each other on the second axis. These two first components of the DAPC analysis explained a 70% of the genetic variability observed, and more than an 84% of the individuals were correctly assigned to the source population. No obvious structure was evident within the Northwestern Mediterranean cluster and the analysis did not reveal a clear pattern of segregation between populations of Catalonia and the Gulf of Marseille (graph not shown).

Population bottleneck

Bottlenecks were detected in all populations under the IAM but only in three populations (Cr, Pla and Por) under the SMM. However under the Two Phase model (TPM), an intermediate model between the Step-wise Mutation Model (SMM) and the Infinite Allele Model (IAM), bottlenecks were detected for all populations with the exception of Ceuta (Table 5). The bottleneck test presented a mode shift in allele frequency classes attributed a normal L-shaped distribution (consistent with normal frequency class distribution) for 6 of the 9 sponge populations, and a shifted mode for Cap de Creus, Pharillon and Portugal (Table 5). Populations that have experienced recent bottleneck display a correlative reduction of the allele number and the heterozygosity. As the allele number reduced faster than heterozygosity, it becomes larger than heterozygosity expected under a mutation-drift equilibrium (Piry *et al.*, 1999).

363 **DISCUSSION**

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365 Nuclear microsatellite loci were powerful markers to provide information at both intra
366 and inter population level in *Spongia lamella*. Genetic statistics used revealed that
367 genetic structure and diversity was spatially structured over multiple spatial scales (as
368 shown by F_{ST} test and AMOVA).

369 Populations of *S. lamella* were genetically structured at small, medium and large
370 geographical scales displaying high values of genetic differentiation (global F_{ST} = 0.236)
371 compared to other sponges species on the same geographical area such as *Spongia*
372 *officinalis* (global F_{ST} = 0.061) (Dailianis *et al.*, 2011), *Crambe crambe* (global F_{ST} =
373 0.18) (Duran *et al.*, 2004c) and *Scopalina lophyropoda* (global F_{ST} = 0.122) (Blanquer
374 and Uriz, 2010). We detected significant genetic differences between all the
375 populations' pairs, with the exception of a population from Cap de Creus (Cr). Genetic
376 homogeneity between this locality and the nearest populations of Arenys and Els
377 Bullents, more likely depended on the small population size of Cr than an extensive
378 gene flow between localities. Even geographically close populations, separated by few
379 kilometers to each other, were highly differentiated. Within the Gulf of Marseille,
380 Pharillon and Plane, which are less than five kilometers apart, displayed significant
381 differences in genetic structure (F_{ST} = 0.113). The positive regression between genetic
382 differentiation and geographic distance between populations highlighted a short-range
383 effective dispersal in *S. lamella*. This pattern of genetic isolation by distance and very
384 restricted gene flow between nearby populations may be explained by a limited
385 dispersal capability of a lecithotrophic larva and sperm, and also philopatry. It is well

known that the philopatric behavior of the larvae, together with short free swimming periods, are prone to restrict the dispersal capability and to increase genetic isolation (Palumbi, 1994). Although specific data of dispersal distance and time for the larva and sperm in *S. lamella* are not available, the limited dispersal potential of sponge larvae have been largely reported (Sara and Vacelet, 1973; Uriz *et al.*, 2001; Mariani *et al.*, 2006; Uriz *et al.*, 2008; Uriz and Turon, 2012), as well as the philopatric behavior in some species (Uriz *et al.*, 1998; Blanquer and Uriz, 2010). The absence of connectivity among populations indicated that populations of *S. lamella* are probably maintained by local recruitment with sporadic exchanges of propagules through stochastic dispersal events. The Bayesian approach (STRUCTURE) detected sub-structuring in some Atlantic and Mediterranean localities, which suggested the existence of different breeding subunits at scales of meters within what we initially considered as "populations". The only two studies specifically designed to measure fine genetic structuring in sponges demonstrated genetic clustering at a scales of few centimeters (Calderon *et al.*, 2007; Blanquer *et al.*, 2009). Unfortunately, the sampling design in *S. lamella* did not allow testing the physical scale at which genetic structure is built for the species.

The strong genetic structuring of *S. lamella* is in concordance with genetic patterns observed along the same geographical area in other benthic invertebrates with low dispersal potential (Duran *et al.*, 2004c; Pérez-Portela and Turon, 2007; Blanquer and Uriz, 2010; Costantini *et al.*, 2011). Nevertheless, genetic structure of *S. lamella* surprisingly contrasted with a weak level of differentiation at both low and intermediated geographic scales in the congeneric *S. officinalis* (Dailianis *et al.*, 2011). In that case, authors hypothesized that accidental translocation of sponge fragments or

embryos by fishermen might have been enhanced gene flow within the Aegean Sea during centuries (Dailianis *et al.*, 2011). Hence, in *S. officinalis* main genetic breaks were found between Mediterranean sub-basins, where hydrographical circulation restricts gene flow between areas.

The results also evidenced the importance of major hydrological circulation on the genetic structure of *S. lamella* at large geographical scales. The Bayesian clustering method was very conservative for detecting homogeneous clusters in *S. lamella* but even so two, or three (depending on the criterion to select optimum *K* value) genetically independent groups could be detected, corresponding to different geographical areas: Atlantic, Balearic and North Mediterranean. The AMOVA also supported that divergence into three areas. In benthic species major genetic breaks are concordant with biogeographic boundaries and oceanographic discontinuities such as the Alboran-Oran Front (AOF) and the Balearic Front (BF) generate barriers to gene flow (Fernández *et al.*, 2005; Mokhtar-Jamaï *et al.*, 2011; Schunter *et al.*, 2011), preventing connectivity among NW Mediterranean, Balearic and Atlantic populations. Graphical representation from DAPC and F_{ST} values of *S. lamella* highlighted that the AOF has a deeper effect on preventing connectivity between basins than the BF. The Balearic population (Cab), although separated from all the other populations, seemed to be genetically closer related to other Mediterranean populations. The Alboran-Oran front, the real boundary between the Atlantic and Mediterranean basins (Galarza *et al.*, 2009) characterized by strong current regimes, differences in salinity and temperature between water masses, hinders dispersion and propagules interchange in benthic species with short free swimming periods such as *S. lamella*. Lacking gene flow between Atlantic and

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4 433 Mediterranean populations along the evolutionary history of the species enhance
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6 434 divergence and adaptation to local conditions at both sides of the Gibraltar Strait.
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8 435 Additionally, other uninvestigated factors might be involved in the population structure
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10 436 observed in *S.lamella*. This species is reported to live in quite deep habitats,
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12 437 occasionally until 300 m depth (Uriz, 1984). In the coast of Portofino, the species
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14 438 reached its maximum density and largest size between 40 and 100 m depth (Pronzato *et*
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16 439 *al.*, 1998). Limited levels of gene flow by short dispersal might occur through stepping
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18 440 stones populations not sampled or deeper populations scattered in the area. Nonetheless,
19
20 441 compared with *S. officinalis* and *S. virgultosa*, *S. lamella* displayed a much lower
21
22 442 density than its congeneric sponges (Pronzato *et al.*, 1998), and populations of *S.*
23
24 443 *lamella* seems to be much more patchily distributed.
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29 445 We observed a general deficit of heterozygotes within populations of *Spongia lamella*
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31 446 compared to what would be expected for populations at Hardy–Weinberg equilibrium.
32
33 447 The deficit of heterozygotes was translated into high positive F_{IS} values. Such
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35 448 heterozygote deficiency seems relatively extended not only among sponges but also on
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37 449 other marine invertebrates (Calderon *et al.*, 2008; Calderon *et al.*, 2009; Duran *et al.*,
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39 450 2004c; Miller and Ayre, 2008; Perez-Losada *et al.*, 2002; Perez-Portela *et al.*, 2006;
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41 451 Perez-Portela and Turon, 2008), and it is often explained by various nonexclusive
42
43 452 factors such as inbreeding, selection against heterozygotes, Wahlund effect, and
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45 453 technical difficulties as presence of null alleles, or a combination of all these factors.
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47 454 Given the diversity of reproductive strategies among sponges (Whalan *et al.*, 2005) it is
48
49 455 not surprising that reproductive strategies would differentially influence genetic
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51 456 structure in various species (see Duran *et al.*, 2004c; Blanquer *et al.*, 2009; Blanquer
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53 457 and Uriz, 2010; Dailianis *et al.*, 2011). For instance, the sponge *Scopalina lophyropoda*
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maintains genetic diversity in isolated populations by predominant outcrossing and/or selection against homozygotes (Blanquer and Uriz, 2010). In hermaphrodite species, self-fertilization could explain heterozygote deficiency (Duran *et al.*, 2004c) but it seems unlikely in *S. lamella*. We do not know the extent to which *S. lamella* is a gonochoric sponge but studies carried out in the closely related species *S. officinalis*, showed that on 11 specimens, 10 were gonochoric and only one presented hermaphroditism (Baldacconi *et al.*, 2007). Hermaphroditism in species of the genus *Spongia*, and hence self-fertilization, seemed not be the most likely factor to explain the heterozygote deficiency observed in *S. lamella*. We did not either identify presence of clones (equal genotypes) within our samples, which discarded asexual reproduction as relevant on the maintenance of populations in this species. A high level of inbreeding and the Wahlund effect (admixture of different genetic units) may be the most plausible explanations for the HWE deviations in *S. lamella*. Mating among relatives in isolated and discrete populations of *S. lamella* may not only increase levels of inbreeding within populations, but also favors the existence of breeding subunits as those unraveled by the Bayesian clustering. Additionally, when dealing with slow-growing, long-living organisms, the deficit in heterozygotes might also be the result of a temporal Wahlund effect by sampling different age cohorts.

Evidence of null allele was detected in the dataset. Null alleles with failed amplifications also occurred in other studies of marine sponges but they did not prevent population genetic analyses (Duran *et al.*, 2004c; Calderon *et al.*, 2007; Dailianis *et al.*, 2011). Mutations in the microsatellite flanking regions preventing annealing, and potential fixation of the unamplified alleles would explain it. DNA re-extraction and

481 PCR reaction in less stringent conditions and PCR program modifications still led to no
482 amplification or unspecific amplifications.

483 Populations of *S. lamella* displayed moderate levels of genetic diversity,
484 scattered across the area studied. Genetic diversity values of *S. lamella* were low
485 compared to other native sponges in the area. Heterozygosity, genetic diversity and
486 number of total and private alleles were substantially lower in populations of *S. lamella*
487 than those assessed from the congeneric *S. officinalis* (Dailianis *et al.*, 2011).
488 Heterozygosity and number of alleles were also lower in *S. lamella* than in *Crambe*
489 *crambe* (see Duran *et al.*, 2004c), but at the same range as *Scopalina lophyropoda*, a
490 rare and scarce species, with restricted distribution to the Northwestern Mediterranean
491 (Blanquer and Uriz, 2010). This particular result might point out a genetic
492 impoverishment of populations in *S. lamella* with future implications for the species'
493 survival.

494 Moreover, all populations here analyzed presented clear evidences of recent reduction
495 size with the exception of Ceuta. The difficult diving conditions in Ceuta may have
496 contributed to the protection of sponge beds from exploitation. This population also
497 displayed the highest gene diversity, the highest mean allele number and private alleles
498 compared to the other populations. Ceuta has a particular situation in the Gibraltar Strait
499 and receives both Mediterranean and Atlantic waters. Nevertheless, other populations of
500 *S. lamella* have not been identified around the Gibraltar Strait, and a limited
501 connectivity with other populations might threat the maintenance of genetic diversity in
502 this area. In the Northwestern Mediterranean coast, recent mortality outbreak events
503 have particularly affected sponges (Coma *et al.*, 2009; Perez *et al.*, 2000), especially
504 horny sponges with a fiber skeleton (Gaino *et al.*, 1992; Perez *et al.*, 2000; Webster,

2007) and it is consistent with low values of genetic diversity and the absence of private alleles in three populations (Cr, Bul and Cio), and the recent bottlenecks observed of the area for *S. lamella*. Additionally, in Cap de Creus (Cr), only eight specimens could be found and collected on the area. Nevertheless, as *S. lamella* also lives in quite deep zones, bottleneck observed on our sampled could be due to sampling on the species' distribution limit, and would affect only marginal subunit of the putative population. *S. lamella* has been first reported in a study in the 1980s (Lopes and Boury-Esnault, 1981). Although this species does not seem to be very abundant in Portugal the genetic diversity of this population obtained from the Berlengas archipelago was higher than Mediterranean populations.

Field monitoring would be necessary to assess population demography and dynamics, and to unravel the extent of population size reduction in *S. lamella* since, to date published information on commercial species' abundance and decline is scarce and fragmented. Available studies evaluating the status of commercial populations have treated all species uniformly within "commercial types" without further estimations at specific level (Voultsiadou *et al.*, 2011). Harvesting and fragmentation of *S. lamella* populations have likely caused population size reduction, turning out these populations as small and discrete units.

Our work provides the second population genetics study on a commercial sponge (see Dailianis *et al.*, 2011). Identifying how genetic diversity is spatially distributed is fundamental to understand ecological threats and to develop appropriate recovery strategies for endangered species. *Spongia lamella* is a slow-growing organism with limited dispersal potential which makes natural recovery of extinct population quite

difficult. Further exploitation of this bath sponge could weaken its genetic diversity, risking its potential to cope with environmental changes. The high levels of inbreeding may finally drive populations into inbreeding depression, and reduction of population size increases the risk of genetic diversity loss by genetic drift in very short periods of time (Amos and Balmford, 2001; Brook *et al.*, 2002; Frankham *et al.*, 2002; Frankham, 2005b). Maintaining genetic diversity is crucial for species in a context of global warming or when exposed to disease outbreaks. Additionally, *S. lamella* hosts a remarkable diversity of macro and microfauna (Noyer *et al.*, 2010; authors' unpublished data) so loss of sponge populations will lead to habitat fragmentation and loss for the associated organisms as well, affecting biodiversity at several levels (Noyer *et al.*, 2012). Future management conservation strategies on *S. lamella* should focus on designing marine protected reserves in an effort for preserving populations and genetic diversity of the species. Transplantations of sponge specimens or fragments of individuals from "source populations" after a carefully genotyping to genetically compatible areas affected by strong bottlenecks and high levels of inbreeding could also facilitate populations recovery increasing population size and genetic diversity. This technical approach based on sponges transplantation has been already applied successfully for restocking populations of the bath sponge *S. officinalis* (Baldacconi *et al.*, 2010), and may be also worth to consider it for *S. lamella* in a near future. Microsatellite loci and population genetic studies are then invaluable tools for populations monitoring after restocking and to evaluate the success of the conservation strategies. However, managing this bath sponge species also require increasing our knowledge about its biology.

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Table 1. Samples of *Spongia lamella*. Location and region where samples were collected, population code, geographical coordinates and sample size (N).

Location, region	Code	Coordinates	N
Berlengas, Central Portugal	Por	39°26' N 9°30'W	15
Ceuta, Gibraltar Strait	Ceu	35°53'N 5°17'W	18
Cabrera Island, Balearic	Cab	39°7'N 2°57'E	38
Arenys, Catalanian	Are	41°34'N 2°33'E	30
Els Bullents, Catalanian	Bul	41°42'N 2°53'E	21
Cap de Creus, Catalanian	Cr	42°17'N 3°18'E	8
La Ciotat, Marseille Gulf	Cio	43°9'N 5°35'E	35
Plane, Marseille Gulf	Pla	43°11'N 5°23'E	31
Pharillon, Marseille Gulf	Far	43°12'N 5°20'E	35
TOTAL			231

Table 2. Summary of genetic characteristics for seven loci at nine populations of *Spongia lamella*. Ns: number of individuals scored per locus; A: total number of alleles per locus and mean per population (Total multilocus); pA: number of private alleles per locus and mean per population (Total multilocus); H_o and H_E : observed and expected heterozygosity per locus and per population; G. div: gene diversity per locus a population; F_{IS} : inbreeding coefficients per locus and per population. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$: departure from HWE

		Locus							Total Multilocus
Population		SA_a (NED)	SA_c (PET)	SA_d (PET)	SA_f (PET)	SA_g (VIC)	SA_k (VIC)	SA_n (NED)	
Por	Ns	0	15	12	15	13	15	14	
	A	0	1	3	6	4	4	8	4.33
	pA	0	0	2	0	0	1	3	0.86
	H_o	-	0	0.333	0.733	0.571	0.533	0.429	0.433
	H_E	-	0	0.670	0.807	0.759	0.646	0.804	0.619
	G. div.	-	0	0.686	0.810	0.750	0.650	0.846	0.611
	F_{IS}	-	-	0.514*	0.094	0.254	0.179	0.494**	0.308**
CeU	Ns	17	18	0	18	18	17	17	
	A	4	3	0	6	6	9	6	5.67
	pA	2	0	0	0	0	3	2	1
	H_o	0.176	0.389	-	0.722	0.333	0.471	0.3529	0.398
	H_E	0.722	0.338	-	0.806	0.575	0.870	0.715	0.662
	G. div.	0.739	0.337	-	0.809	0.582	0.882	0.673	0.657
	F_{IS}	0.761***	-0.155	-	0.107	0.427**	0.467***	0.563***	0.407***
Cab	Ns	34	38	13	37	35	35	17	
	A	3	4	3	2	4	12	5	4.71
	pA	1	1	1	0	0	1	0	0.57
	H_o	0.618	0.421	0.077	0.434	0.600	0.686	0.059	0.416
	H_E	0.578	0.416	0.532	0.513	0.566	0.725	0.801	0.574
	G. div.	0.578	0.416	0.551	0.433	0.566	0.725	0.791	0.580
	F_{IS}	-0.069 **	-0.012 *	0.860 ***	-0.185	-0.061	0.054	1.000***	0.282***
Are	Ns	19	30	27	0	27	29	22	
	A	3	3	6	0	6	8	5	5.17
	pA	0	0	2	0	0	1	0	0.43

	H _o	0.263	0.100	0.593	-	0.518	0.621	0.227	0.385
	H _E	0.472	0.159	0.640	-	0.666	0.736	0.690	0.552
	G. div.	0.478	0.160	0.641	-	0.669	0.738	0.650	0.539
	FIS	0.450	0.374*	0.076	-	0.225*	0.159***	0.666***	0.307***
Bul	Ns	21	19	18	0	20	21	16	
	A	1	3	3	0	6	7	4	4
	pA	0	0	0	0	0	0	0	0
	H _o	0	0.000	0.111	-	0.350	0.571	0.187	0.203
	H _E	0	0.199	0.209	-	0.637	0.546	0.544	0.356
	G. div.	0	0.205	0.212	-	0.645	0.545	0.556	0.390
	FIS	-	1.00***	0.477	-	0.457**	0.048	0.663**	0.436***
Cr	Ns	7	7	8	0	8	8	7	
	A	1	3	3	0	4	4	4	3
	pA	0	0	0	0	0	0	0	0
	H _o	0	0.429	0.250	-	0.375	0.625	0.1429	0.307
	H _E	0	0.560	0.242	-	0.592	0.642	0.582	0.413
	G. div.	0	0.571	0.241	-	0.607	0.643	0.467	0.458
	FIS	-	0.250	-0.037	-	0.382	0.028	0.643	0.270
Cio	Ns	29	35	27	33	30	35	30	
	A	1	3	3	2	6	7	7	4.14
	pA	0	0	0	0	0	0	0	0
	H _o	0	0.171	0.259	0.000	0.733	0.714	0.200	0.297
	H _E	0	0.429	0.540	0.058	0.660	0.631	0.766	0.444
	G. div.	0	0.433	0.546	0.061	0.659	0.630	0.797	0.430
	FIS	-	0.604***	0.525**	1.00*	-0.113*	-0.134	0.749***	0.335***
Pla	Ns	22	31	30	28	31	31	23	
	A	1	4	6	3	5	10	6	5
	pA	0	1	2	0	0	0	0	0.48
	H _o	0	0.645	0.467	0.250	0.838	0.548	0.174	0.424
	H _E	0	0.628	0.686	0.408	0.730	0.732	0.751	0.551
	G. div.	0	0.627	0.690	0.411	0.728	0.735	0.681	0.530
	FIS	-	-0.028	0.323**	0.392*	-0.151	0.254	0.681***	0.234***
Far	Ns	30	35	35	35	34	35	35	
	A	1	2	3	3	6	8	5	4
	pA	0	0	1	0	1	1	0	0.48
	H _o	0	0.108	0.171	0.722	0.647	0.343	0.286	0.325
	H _E	0	0.373	0.252	0.587	0.756	0.416	0.603	0.422
	G. div.	0	0.377	0.253	0.585	0.558	0.417	0.572	0.418
	FIS	-	0.713***	0.323	-0.235	0.146	0.178**	0.501***	0.231***

Table 3. Multi-locus F_{ST} values for pairwise comparisons between *Spongia lamella* populations. P -values for significance were set at 0.0122 following corrections (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$).

	Cab	Ceu	Cr	Are	Pla	Far	Cio	Bul
Ceu	0.253***							
Cr	0.189***	0.278***						
Are	0.114***	0.217***	0.047					
Pla	0.149***	0.265***	0.074***	0.084***				
Far	0.226***	0.312***	0.059***	0.106***	0.113***			
Cio	0.203***	0.336***	0.076***	0.080***	0.068***	0.114***		
Bul	0.191***	0.323***	0.058	0.077***	0.120***	0.066***	0.098***	
Por	0.229***	0.092***	0.281***	0.208***	0.243***	0.293***	0.300***	0.288***

Table 4. AMOVA results on *Spongia lamella* microsatellites. Populations were grouped within "Atlantic" and "Mediterranean groups" (two basins), and "Atlantic", and "Balearic" and "Northwestern Mediterranean" (three areas) (*: $p < 0.05$; ***: $p < 0.001$)

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	Fixation indices	P-value
TWO GROUPS						
Among groups	1	65.434	0.40531	14.69	$F_{CT} = 0.14693$	0.02*
Among populations within two basins	6	185.882	0.46839	16.98	$F_{SC} = 0.19905$	0.000***
Within populations	457	861.325	1.88474	68.33	$F_{ST} = 0.31674$	0.000***
THREE GROUPS						
Among groups	2	122.053	0.33812	12.90	$F_{CT} = 0.12895$	0.003*
Among populations within 3 areas	6	129.263	0.39918	15.22	$F_{SC} = 0.17478$	0.000***
Within populations	457	861.325	1.88474	71.88	$F_{ST} = 0.28119$	0.000***
TOTAL	465	1112.642	2.62204		$F_{ST} = 0.2357$	

Table 5. Bottleneck outcomes for populations of *Spongia lamella* following Wilcoxon test and a mode-shift indicator of allele frequencies. Results under the three different mutation models are displayed (SMM: stepwise mutation model; IAM: infinite allele mutation; and TPM: two phase model). Ns: Non significant

	Wilcoxon test			graphical descriptor ⁹³⁹
	IAM	SMM	TPM	"mode-shift indicator"
Portugal	0.0078	0.0269	0.0078	shifted mode
Ceuta	0.0391	Ns	Ns	Normal L-shaped
Cabrera	0.0039	Ns	0.0273	Normal L-shaped
Arenys	0.0078	Ns	0.0156	Normal L-shaped
Els Bullents	0.0078	Ns	0.0156	Normal L-shaped
Cap Creus	0.0078	0.0156	0.0078	shifted mode
La Ciotat	0.0039	Ns	0.0078	Normal L-shaped
Plane	0.0039	0.0273	0.0039	Normal L-shaped
Pharillon	0.0039	Ns	0.0078	shifted mode

One tail for H excess Wilcoxon tests

FIGURE CAPTATIONS

943

944 **Fig. 1** Sampling sites of *Spongia lamella*. AOF: Alboran-Oran Front, and BF: Balearic
945 Front

946

947 **Fig. 2** Grouping of *Spongia lamella* populations. (a) Multidimensional scaling (MDS)
948 plot based on multi-locus F_{ST} values between populations, and (b) DAPC analyses of
949 populations. For the DAPC, sampled geographic locations are indicated with different
950 colours; dots represent individuals, and 95% inertia ellipses are included for each cluster

951

952 **Fig. 3** Isolation by distance: Relationship between genetic differentiation, $F_{ST} / (1 - F_{ST})$
953 and the logarithm of geographical distance between *Spongia lamella* populations

954

955 **Fig. 4** Bayesian analysis results of *Spongia lamella* from STRUCTURE. (a) Results of
956 the assignment of individual genotypes for $K=2$ and $K=3$ from the whole dataset, and
957 (b), (c) and (d) results after define geographical groups: (b) for the Atlantic area; (c) for
958 Catalonia, and (d) for the Gulf of Marseille. The assignment of individual genotypes is
959 represented by vertical bar partitioned into K -coloured segments that represent its
960 estimated membership fraction in each of the inferred groups

961

962 **Fig. 5** Values of ΔK for each number of genetically homogeneous cluster (K) including
963 the whole dataset of *Spongia lamella*

190x142mm (300 x 300 DPI)

184x108mm (300 x 300 DPI)

FIGURE 3

92x81mm (300 x 300 DPI)

FIGURE 4

175x157mm (300 x 300 DPI)

FIGURE 5

Values of ΔK for each number of genetically homogeneous cluster (K) including the whole dataset of *Spongia* lamella
132x109mm (300 x 300 DPI)