AQUATIC CONSERVATION: MARINE AND FRESHWATER ECOSYSTEMS

Aquatic Conserv: Mar. Freshw. Ecosyst. **25**: 365–379 (2015)

Published online 2 January 2014 in Wiley Online Library
(wileyonlinelibrary.com). DOI: 10.1002/aqc.2423

Genetic structure and diversity of the endangered bath sponge Spongia lamella

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ABSTRACT

1. Natural populations of Mediterranean commercial sponges have declined substantially over recent decades.

2. The present study explored the distribution of genetic diversity of the endangered bath sponge *Spongia lamella* along the western Mediterranean and the Portuguese coast.

3. 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 performed.

4. Genetic differentiation between populations was large and highly significant (global $F_{ST} = 0.236$, $P < 0.001$). AMOVA and Bayesian analyses showed genetic differentiation among the Atlantic, Balearic, and North Mediterranean areas ($F_{CT} = 0.129$, $P = 0.003$).

5. Restricted gene flow owing to short-distance larval dispersal and hydrographical barriers may be playing an important role in genetic differentiation.

6. Recent bottlenecks were also detected for most populations of this sponge.

7. The high levels of inbreeding, sub-structuring, and modest levels of genetic diversity that characterized populations of *S. lamella* (mean value of genetic diversity 0.512), may compromise its long-term survival. Only one population, from the Gibraltar Strait, presented high levels of genetic diversity (Ceuta, genetic diversity = 0.657), indicating a hotspot of genetic diversity for this species with special relevance for its conservation.

8. Disease outbreaks and overexploitation may be the most important causes of genetic diversity impoverishment of *S. lamella*.

9. Future conservation guidelines should focus on preserving genetic diversity within genetically impoverished populations by limiting exploitation, and increasing population size. Transplanting specimens from areas with high values of genetic diversity to areas with low diversity values or to areas that have recently experienced demographic declines could reverse the local and global recession of this species.

Received 28 May 2013; Revised 23 September 2013; Accepted 01 November 2013

KEY WORDS: 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 affected by human activity, species and populations face stochastic events associated with population size reduction and 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 suffer 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 that 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, 2008; Mariani *et al*., 2006; Uriz and Turon, 2012). Not surprisingly, most studies on population genetics of marine sponges report highly structured populations with limited gene flow (Duran *et al*., 2004a, b, c; 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 behaviour, historical and demographic processes, biotic interactions, or recruitment patterns also affect population connectivity (Barber *et al*., 2002; Vollmer and Palumbi, 2007; Miller and Ayre, 2008).

Sponges are recognized as a natural resource since ancient times as they were traditionally collected for diverse purposes, including body and facial cleaning (bath sponges, Rützler, 1996). The majority of bath sponges belong to the cosmopolitan genus *Spongia* (Demospogiae; Dictyoceratida; Spongiidae) (Pronzato, 1999). Commercial sponge beds in the Mediterranean Sea decreased substantially as a consequence of high demand and decline of bath sponges in other areas of the world (Gaino and Pronzato, 1992; Pronzato, 1999). Also, 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 effects on slow-growing sponge populations leading them to the brink of extinction (Webster, 2007). Factors such as overharvesting, diseases, habitat loss and fragmentation have caused many commercial sponges to practically disappear from the majority of exploited banks (Gaino *et al*., 1992; Pronzato, 1999).

The species *Spongia lamella* is one of the five Mediterranean commercial sponges overexploited in past years (Gaino and Pronzato, 1992; Pronzato and Manconi, 2008). This fan-like (elephant ear) or cup-shaped sponge can reach up to 1.5 m in diameter (Pronzato, 1999). This Mediterranean sponge was previously known as *S. agaricina*, a name that is currently restricted to the Philippine elephant ear (Pronzato and Manconi, 2008).

Populations of *S. lamella*, as with other commercial sponges, are currently scarce and the species was included among marine endangered species in Annex 3 of Bern and Barcelona Conventions (Templado *et al*., 2004). The species is particularly interesting from multiple perspectives; it produces abundant cytotoxic furanoterpenes (Aiello *et al*., 1988; Rueda *et al*., 1998), hosts a diverse community of benthic macrofauna (authors' pers. obsv.), and an abundant and complex bacterial community (Noyer *et al*., 2010). However, the information available on its biology is scarce, which hinders possible conservation measures other than its inclusion in marine endangered species lists.

Genetic descriptors have been applied widely in conservation biology to evaluate the health state and vulnerability of marine species (Hellberg *et al*., 2002; Pearse and Crandall, 2004; Bester-van der Merwe *et al*., 2011; Dailianis *et al*., 2011; Schunter *et al*., 2011). Microsatellite 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 sponges (Duran *et al*., 2004b; Wörheide *et al*., 2005; Uriz and Turon, 2012). Studies on population genetics of native species using microsatellite markers are scarce and available for only three 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).

In this study microsatellite markers specifically designed for *S. lamella* (Noyer *et al*., 2009) were used to investigate the genetic structuring and distribution of genetic diversity, and to infer demographic decline of the sponge *S. lamella* in the Atlanto-Mediterranean area.

METHODS

Samples collection and DNA extraction

In total, 231 individuals of *Spongia lamella* were sampled from nine different locations by scuba diving between 2005 and 2008 (Table 1). Locations were scattered in three distinct areas separated by two major oceanographic discontinuities. The Almeria-Oran front separates Atlantic and western Mediterranean populations (Patarnello *et al*., 2007). The Balearic front further separates western Mediterranean populations from the Balearic Islands and from the north-western Mediterranean (Schunter *et al*., 2011). Within the Atlantic area two populations were sampled, one in the Berlengas archipelago, Portugal (Por), and another one in Ceuta (Ceu), situated in the Gibraltar Strait and strong Atlantic influence. The remaining seven populations were in the western Mediterranean, a single population in the Balearic Islands (Cabrera Island – Cab) and six in the north-western Mediterranean including populations from Catalonia (Arenys (Are), Els Bullents (Bul), and Cap de Creus (Cr)) and the Gulf of Marseille (Pharillon (Far), Plane (Pla), and La Ciotat (Cio)) (Figure 1). For each specimen, a section of the edge of the sponge was cut underwater to minimize damage, placed into a plastic bag containing sea water, and stored in a cool box until further processing (usually 1–2 h after sampling). Sponge tissues were then rinsed in at least two absolute ethanol baths to prevent ethanol dilution and DNA degradation. Then, they were finally preserved in absolute ethanol at −20°C until processed. Samples were cleaned of foreign tissues and DNA was extracted as described in Noyer *et al*. (2009).

Table 1. Samples of *Spongia lamella*. Location and region where samples were collected, population code, geographic 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°70'N 2°57'E	38
Arenys, Catalonia	Are	41°34'N 2°33'E	30
Els Bullents, Catalonia	Bul	41°42'N 2°53'E	21
Cap de Creus, Catalonia	Cr	42°17'N 3°18'E	8
La Ciotat, Marseille Gulf	Cio	43°90'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

Population genotyping

Among the eight microsatellite loci previously developed for this species (Noyer *et al*., 2009), seven microsatellites (SAa, SAc, SAd, SAf, SAg, SAk, and SAn) were selected. One of the eight microsatellites was discarded for this study owing to scoring difficulties. Amplification of fragments were performed by Polymerase Chain Reaction (PCR) in a final volume of 20 µL containing 1 µL of DNA template, 2 µL of 10× Taq polymerase

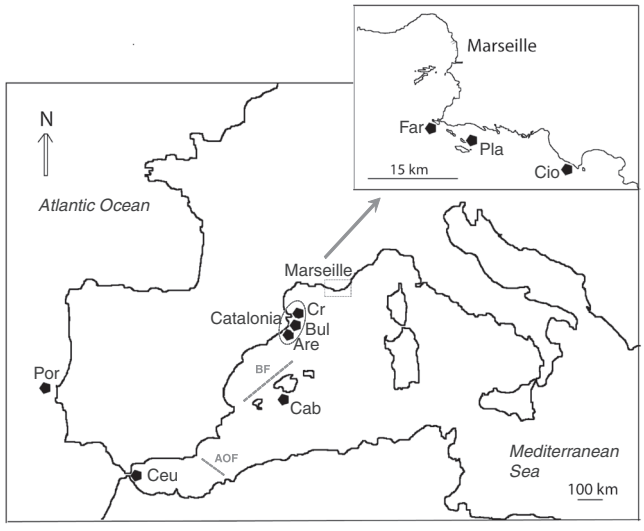

Figure 1. Sampling sites of *Spongia lamella*. AOF: Almeria–Oran Front, and BF: Balearic Front.

buffer, 4 mmol L^{-1} of $MgCl_2$, 0.25 mmol L^{-1} of dNTP, 0.25 μmol L^{-1} of each primer, 350 ng μL^{-1} of bovine serum albumin and 1 U Taq polymerase (Bioron). The forward primer for each locus was labelled with fluorescent dyes (NED, VIC and PET from Applied Biosystems, as previously described in Noyer *et al.*, 2009). PCRs were performed in a MWG primus thermocycler or a Mastercycler® Eppendorf. PCR parameters were: 5 min denaturation step at 95°C, followed by 30 amplification cycles of 55 s at 95°C, 35 s at locus specific annealing temperature (Noyer *et al.*, 2009), and 45 s at 72°C, followed by an extension cycle of 7 min at 72°C. Resulting PCR products were then visualized on a 1.5% agarose gel. Successful amplifications were genotyped using an ABI Prism 3700 automated sequencer (Applied Biosystems, in the Scientific and Technical Services of the University of Barcelona). Re-amplification with decreasing annealing temperature and re-extraction of the DNA were necessary when initial amplification failed. Allele size characterization was performed with internal standard LIZ and Peak Scanner™ software (version 1.0, from Applied Biosystems).

Data analyses

Genetic diversity within localities

The number of alleles per locus and per population, observed heterozygosity (H_o), expected heterozygosity (H_e), genetic diversity, and inbreeding coefficients (F_{IS}) were calculated using Genetix version 4.03 (Belkhir *et al.*, 2004). The exact test for departure from Hardy Weinberg Equilibrium (HWE) was performed using Genepop web version 4.0.10 (Raymond and Rousset, 1995), using a probability test (with level of significance determined by Markov chain parameters: 5000 dememorization steps, 1000 batches, and 5000 interactions per batch). Micro-Checker version 2.2.3 (Van Oosterhout *et al.*, 2004) was used to analyse the potential causes of the observed deficit of heterozygotes. Micro-checker detects large allele dropouts and scores errors due to stuttering and presence of null alleles. The software Genepop was used to test for linkage disequilibrium across all populations using a likelihood ratio test with the level of significance determined by permutation (Markov chain parameters: 5000 dememorization steps, 1000 batches, and 5000 iterations per batch). A false discovery rate (FDR) correction was applied to the *P*-values (B-Y method as described in Narum (2006) to account for multiple tests.

Population differentiation and demographic analyses

Arlequin software (Excoffier *et al.*, 2005) was used to estimate population differentiation with the F_{ST} statistic between pairs of populations based on an allele infinite model. The significance of the values was evaluated by performing 16 000 permutations with the same software and Narum corrections were applied (Narum, 2006). A multidimensional scaling analysis (MDS) was performed to visualize graphically relationships represented by the matrix of genetic distances from the F_{ST} values with the software SYSTAT 9. Isolation by distance (IBD) between populations was tested using a Mantel test implemented in the IBD software (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 distinct groups to test two hypotheses. 'Atlantic' (Por and Ceu) vs. 'Mediterranean' (Cab, Are, Bul, Cr, Far, Pla, and Cio) were used to test for the possible genetic division caused by a basin boundary. Pooling populations within the three biogeographic areas separated by the two major hydrographical fronts allowed testing for the possible effect of the Balearic front: 'Atlantic' (Por and Ceu), 'Balearic' (Cabrera Island, Cab), and 'North-western Mediterranean' (Are, Bul, Cr,Far, Pla and Cio). The significance of the AMOVAs was calculated with 16 000 permutations of the original data. The F_{ST} and AMOVA analyses were performed both including and excluding microsatellites that did not amplify in some populations (SA_a, SA_d and SA_f) to avoid false results due to the absence of data.

The software STRUCTURE version 2.3 (Pritchard *et al.*, 2000) was used to infer population genetic structure and optimal number of homogeneous genetic units (K) based on a Bayesian clustering analyses. 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 run initially with the whole dataset, with a K number from 1 to 16. In a second phase, it was run with several sets of populations; 'Atlantic' (Por and Ceu) and 'North-western Mediterranean' (Are, Bul, Cr, Far, Pla and Cio), and finally the north-western Mediterranean populations in two different groups; 'Catalonia' (Are, Cr, Bul,) and 'Marseille' (Far, Pla, Cio). These follow-up analyses allowed for detection of potential sub-structuring within geographic 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 were determined using the ad hoc statistic ΔK (Evanno *et al.*, 2005). Results were graphically visualized and represented with the same software.

In addition, a discriminant analysis of principal components (DAPC) was applied to assess genetic structure using populations as groups with the 'adegenet package' (Jombart *et al.*, 2008) for the R software. The whole data set was run initially, followed by a second run with populations from the north-western Mediterranean only (Catalonia and Marseille). 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).

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 leaving a genetic signal. Two approaches were used 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) and the two phase model (TPM). The SMM and IAM representing two extreme models of mutation for microsatellites.

RESULTS

Genetic characteristics

The main genetic descriptors of the nine populations investigated are listed in Table 2. All microsatellites were polymorphic although SA_a was monomorphic in the three populations from Marseille and in two populations from Catalonia. SA_a also showed no amplification in Portugal. No specimen from Ceuta amplified for SA_d and none of the three Catalonian populations amplified for SA_f. Unscored microsatellites corresponded to both unsized alleles (although good amplifications were observed and PCR repetitions were performed) and failure in amplification (null allele). It occurred even after various attempts and modification of PCR conditions. Micro-Checker also highlighted the presence of null alleles in SA_n. Although this microsatellite amplified well in all populations, Micro-Checker detected null alleles in four populations. A null allele was also detected in SA_d for three of the nine populations.

Private alleles were found in six loci and in six out of the nine populations. Ceuta, Portugal, and Cabrera showed the highest numbers of overall private alleles per population: seven, six, and four, respectively. Linkage disequilibrium between microsatellite pairs was not detected after Narum corrections ($P \geq 0.0137$). A general excess of homozygotes was detected for all populations and most loci, and inbreeding coefficients (F_{IS}) were significant in all populations except Cr (Table 2). In particular SA_n showed significant inbreeding coefficient in eight out of the nine populations.

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 and per population; F_{IS}: inbreeding coefficients per locus and per population. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; departure from HWE

		Locus							
		SA_a	SA_c	SA_d	SA_f	SA_g	SA_k	SA_n	Total multi-locus
Population		(NED)	(PET)	(PET)	(PET)	(VIC)	(VIC)	(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
	F_{IS}	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
	F_{IS}	-	1.000***	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
	F_{IS}	-	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
	F_{IS}	-	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

(*Continues*)

Table 2. (Continued)

Population		Locus							
		SA_a	SA_c	SA_d	SA_f	SA_g	SA_k	SA_n	Total multi-locus
		(NED)	(PET)	(PET)	(PET)	(VIC)	(VIC)	(NED)	
	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
	F_{IS}	-	−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
	F_{IS}	-	0.713***	0.323	−0.235	0.146	0.178**	0.501***	0.231***

Population differentiation

Multi-locus pairwise comparisons based on F_{ST} tests were, in general, very high (global $F_{ST} = 0.236$, $p < 0.001$) and significant between most population pairs (Table 3). Values of F_{ST} ranged from 0.047 (between Cr and Are) to 0.336 (between Cio and Ceu). The highest values of the F_{ST} statistic were detected between Ceuta and all the other Mediterranean populations. Portugal and Mediterranean populations also presented high levels of differentiation. Cabrera (Cab) displayed moderate levels of differentiation compared with Atlantic and Mediterranean populations. Populations from the Gulf of Marseille and Catalonia were little differentiated from each other but F_{ST} values were significant. Only the comparisons Creus–Els Bullents, and Creus–Arenys were not significant. The MDS plot based on the F_{ST} values showed populations from the Atlantic and the north-western Mediterranean grouped in two clusters at opposite sides of the graph with Cabrera in between (figure not shown).

Results of the AMOVA pooling populations within two or three geographic groups are summarized in Table 4. Both analyses demonstrated that most of the molecular variance occurred within populations (68% and 72%, $P < 0.001$, respectively). Variance among populations within regions (17% and 15%, $P < 0.001$, respectively) and among geographic regions (15% and 13%, $P = 0.02$ and $P = 0.003$, respectively) showed similar and significant values.

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

There was a positive and significant correlation between genetic ($F_{ST}/(1–F_{ST})$) and geographic distances (Mantel test: $r = 0.724$, $P = 0.003$, for the logarithm of geographic distances). This pattern of isolation by distance was particularly strong and highly significant between populations over 200 km apart (Mantel test: $r = 0.849$, $P < 0.001$) while populations separated by less than 200 km showed no IBD (Mantel test: $P = 0.960$) (Figure 2).

Results from STRUCTURE, based on Bayesian clustering, revealed partition of the whole data set

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 (*$P < 0.05$; ** $P < 0.01$, *** $P < 0.001$)

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

Table 4. AMOVA results on *Spongia lamella* microsatellites. Populations were grouped within 'Atlantic' and 'Mediterranean' (two basins), and 'Atlantic', '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 basins						
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 areas						
Among groups	2	122.053	0.33812	12.90	$F_{CT} = 0.12895$	0.003*
Among populations within three 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		$F_{ST} = 0.2357$	

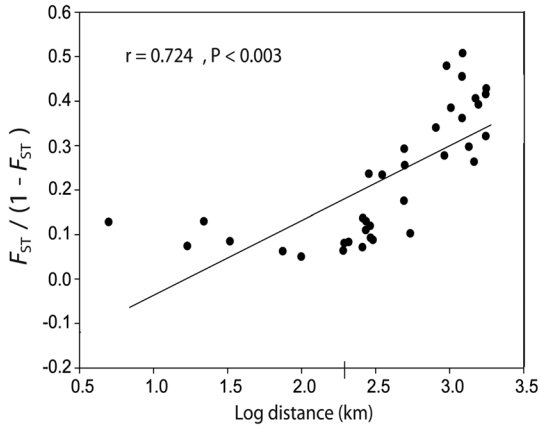

Figure 2. Isolation by distance: relationship between genetic differentiation, F_{ST} / $(1 - F_{ST})$ and the logarithm of geographic distance between *Spongia lamella* populations.

of *S. lamella* within two or three main genetically homogeneous clusters (K), depending on the criterion used to infer optimum K value. The highest likelihood value according to lnP(K) was detected when $K = 3$ whereas the ΔK metric (after Evanno *et al.*, 2005) rapidly decreased after $K = 2$ (Figure 3). When $K = 3$ was fixed, the assignment of individual genotypes separated the Atlantic, Balearic, and north-western Mediterranean pools that had been detected by the AMOVA (Figure 3 (a)). Secondary runs for the Atlantic (Por and Ceu), Catalonia (Are, Cr, Bul), and Gulf of Marseille (Far, Cio, Pla) suggested a segregation between the Catalonian populations and between Far and Pla and Cio (optimal $K = 3$) (Figure 3(c)).

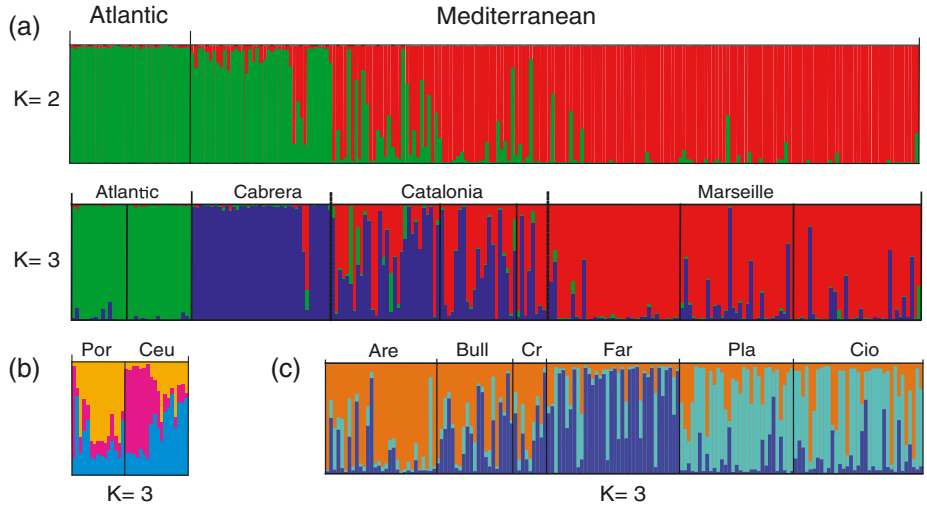

Figure 3. Bayesian analysis results of *Spongia lamella* from STRUCTURE. (a) Results of the assignment of individual genotypes for $K = 2$ and $K = 3$ from the whole data set, and (b) and (c) after defining geographic groups: (b) for the Atlantic area; (c) for north-western Mediterranean. The assignment of individual genotypes is represented by vertical bar partitioned into K-coloured segments that represent its estimated membership fraction in each of the inferred groups.

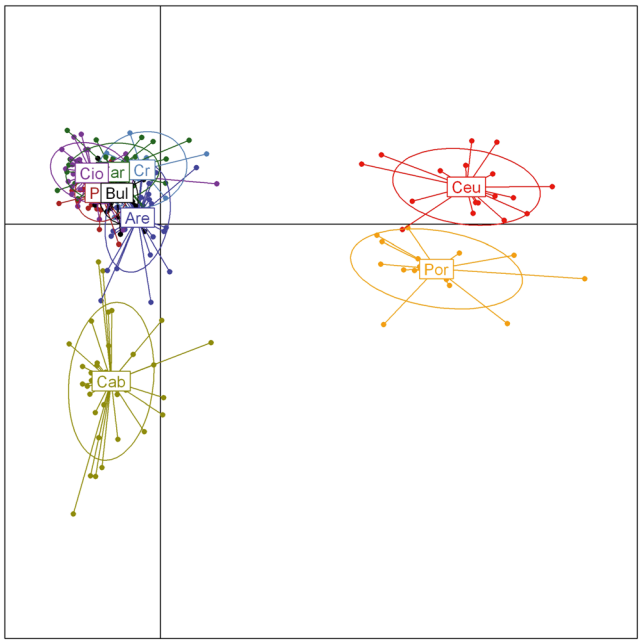

Figure 4. Grouping of *Spongia lamella* populations. DAPC analysis of populations. Sampled geographic locations are indicated with different colours; dots represent individuals, and 95% inertia ellipses are included for each cluster.

Genetic sub-structuring was also detected within localities of the Atlantic (optimal $K = 3$, Figure 3 (b)) and the Marseille area (optimal $K = 4$, graph not shown). The DAPC is in agreement with the previous results (Figure 4). The Atlantic cluster was clearly separated on the first PCA axis, whereas the Balearic population and north-western Mediterranean populations were separated from each other on the second axis. These two first components of the DAPC analysis explained 70% of the observed genetic variability. More than 84% of the individuals were correctly assigned to the source population. No obvious structure was evident within the north-western Mediterranean cluster and the analysis did not reveal a clear pattern of segregation between populations from 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 except Ceuta (Table 5). Populations that have experienced recent bottleneck display a correlative reduction of allele number and heterozygosity. As the allele number reduces faster than heterozygosity, it becomes larger than heterozygosity as expected under a mutation-drift equilibrium (Piry *et al.*, 1999).

DISCUSSION

Nuclear microsatellite loci proved powerful markers to help understand the current status of *Spongia lamella* at both intra- and inter-population levels.

Populations of *S. lamella* were genetically structured at small and large geographic scales displaying high values of genetic differentiation (global $F_{ST=}$ 0.236) compared with other sponge species in the same geographic area such as *Spongia officinalis* (global $F_{ST} = 0.061$) (Dailianis *et al.*, 2011), *Crambe crambe* (global $F_{ST} = 0.18$) (Duran *et al.*, 2004c), and *Scopalina lophyropoda* (global $F_{ST} = 0.122$) (Blanquer and Uriz, 2010). Significant genetic differences were detected between all the population pairs, with the exception of a population from Cap de Creus (Cr). Genetic homogeneity between this locality and the adjacent populations of Arenys and Els Bullents more likely depended on the small population size of Cr than on extensive gene flow between the two localities. Even geographically close populations were highly

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; all values reported are significant

	Wilcoxon test			Graphical descriptor
	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.

differentiated. Within the Gulf of Marseille, Pharillon and Plane displayed significant differences in genetic structure ($F_{ST} = 0.113$) and are less than 5 km apart. The pattern of genetic isolation by distance and very restricted gene flow between nearby populations may be explained by the limited dispersal capability of the lecithotrophic larva and sperm and also by philopatry. It is well known that larval philopatric behaviour, together with short free swimming periods, are prone to restrict dispersal capability and to increase genetic isolation (Palumbi, 1994). Although specific data of dispersal distance and time for the larva and sperm of *S. lamella* are not available, the limited dispersal potential of sponge larvae has been widely reported (Sara and Vacelet, 1973; Uriz *et al.*, 2001, 2008; Mariani *et al.*, 2006; Uriz and Turon, 2012), as has the philopatric behaviour of some sponge species (Uriz *et al.*, 1998; Blanquer and Uriz, 2010). The limited 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 at some Atlantic and Mediterranean localities. The existence of different breeding subunits at scales of metres within what were initially considered populations is therefore possible. The only two studies specifically designed to measure fine genetic structuring in sponges demonstrated genetic clustering at a scale of a few centimetres (Calderon *et al.*, 2007; Blanquer *et al.*, 2009). Unfortunately, the sampling design for *S. lamella* did not allow for testing of the physical scale at which its genetic structure is built.

The strong genetic structuring of *S. lamella* is in concordance with the genetic patterns of other benthic invertebrates with low dispersal potential from the same geographic area (Duran *et al.*, 2004c; Pérez-Portela and Turon, 2008; Blanquer and Uriz, 2010; Costantini *et al.*, 2011). The genetic structure of *S. lamella* surprisingly contrasted with the weak level of differentiation of the congeneric *S. officinalis* at both low and intermediate geographic scales (Dailianis *et al.*, 2011). Accidental translocation of sponge fragments or embryos by fishermen might have enhanced gene flow within the Aegean Sea over the centuries (Dailianis *et al.*, 2011). Hence, in *S. officinalis* the main genetic breaks were found between Mediterranean sub-basins, where hydrographical circulation restricts gene flow between areas.

Despite the general IBD pattern observed in *S. lamella*, genetic isolation was entirely driven by populations located over 200 km apart. This result may be evidence of the effect of major hydrological circulation on the genetic structure of *S. lamella* at large geographic scales. Despite the conservative approach of the Bayesian clustering method to detect homogeneous clusters in *S. lamella*, two or three genetically independent groups were detected. These groups depended on the criterion to select optimum *K* values and corresponded to the distinct geographic areas of the Atlantic, Balearic, and north-western Mediterranean. The AMOVA provided additional support for the differentiation of these three areas. In benthic species major genetic breaks are concordant with biogeographic boundaries and oceanographic discontinuities that generate barriers to gene flow (Fernandez *et al.*, 2005; Mokhtar-Jamaï *et al.*, 2011; Schunter *et al.*, 2011). The Almeria–Oran Front (AOF) and the Balearic Front (BF) seem to prevent connectivity between populations at opposite sides of the fronts. Graphical representation from DAPC and F_{ST} values of *S. lamella* highlighted that the AOF has a greater effect on preventing connectivity between basins than the BF. The Almeria–Oran front is described as the real boundary between the Atlantic and Mediterranean basins (Galarza *et al.*, 2009). It is characterized by strong current regimes, differences in salinity and temperature between water masses (Galarza *et al.*, 2009), hindering dispersion and interchange of propagules of benthic species with short free swimming periods such as *S. lamella*. The lack of gene flow between Atlantic and Mediterranean populations throughout the evolutionary history of the species enhances divergence and adaptation to local conditions on both sides of the Gibraltar Strait. The Balearic population (Cab), although separated from all the other populations, seemed to be genetically more closely related to the Mediterranean than Atlantic populations. Nevertheless, conclusions about causes promoting the genetic differentiation of Cabrera and other

Mediterranean localities should be viewed with caution, since only one population could be included in the biogeographic area of Baleares. Further studies using more nuclear loci and including additional populations from the Balearic Islands and the south-western Mediterranean could clarify the relative contribution of isolation by distance or interruptions of gene flow by the BF on the genetic divergence found. Additionally, other uninvestigated factors might be involved in the population structure observed in *S. lamella*. This species is reported to live in quite deep habitats, occasionally down to 300 m depth (Uriz, 1984). Off the coast of Portofino, it reached its maximum density and largest size between 40 and 100 m depth (Pronzato *et al*., 1998). Limited levels of gene flow may result through short dispersal between unsampled stepping stone populations or from deeper populations scattered throughout the area. Regardless, *S. lamella* displayed a much lower density than *S. officinalis* and *S. virgultosa* and its populations seem to be more patchily distributed than its congeneric sponges (Pronzato *et al*., 1998).

A general deficit of heterozygotes was found within populations of *Spongia lamella* compared with what would be expected for populations at Hardy–Weinberg equilibrium. The deficit of heterozygotes was translated into high positive F_{IS} values. Such heterozygote deficiency seems relatively extended among sponges and other marine invertebrates (Perez-Losada *et al*., 2002; Duran *et al*., 2004c; Pérez-Portela *et al*., 2006; Calderon *et al*., 2008, 2009; Miller and Ayre, 2008; Pérez-Portela and Turon, 2008). Heterozygote deficiency is often explained by various nonexclusive factors such as inbreeding, selection against heterozygotes, the Wahlund effect, technical difficulties with the presence of null alleles, or a combination of all these factors (Pérez-Portela and Turon, 2008). Given the diversity of reproductive strategies among sponges (Whalan *et al*., 2005) it is not surprising that reproductive strategies would differentially influence genetic structure in various species (Duran *et al*., 2004c; Blanquer *et al*., 2009; Blanquer and Uriz, 2010; Dailianis *et al*., 2011). For instance, the sponge *Scopalina lophyropoda* 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 available evidence suggests that this is unlikely in *S. lamella*. Although it is uncertain whether or not *S. lamella* is a gonochoric sponge, Baldacconi *et al*. (2007) found 10 out of 11 specimens of the closely related *S. officinalis* were gonochoric and only one presented hermaphroditism. Hermaphroditism in species of the genus *Spongia*, and hence self-fertilization, seems not to be the most likely factor to explain the heterozygote deficiency observed in *S. lamella*. Clones (identical genotypes) within the samples were absent, suggesting against asexual reproduction as a factor for 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 found 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 favours the existence of breeding subunits such as those revealed by the Bayesian clustering. In addition, 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 data set. 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). Potential fixation of the unamplified alleles and mutations in the microsatellite flanking regions preventing annealing could explain it. DNA re-extraction and PCR reaction in less stringent conditions and PCR program modifications still led to no amplification or unspecific amplifications.

Populations of *S. lamella* displayed moderate levels of genetic diversity scattered across the area studied. Genetic diversity values of *S. lamella* were low compared with other native sponges in the area. Heterozygosity, genetic diversity, and number of private alleles were substantially lower in populations of *S. lamella* than for populations with comparable sample size of the congeneric *S.*

officinalis (Dailianis *et al.*, 2011). Heterozygosity values were also lower in *S. lamella* than in populations of *Crambe crambe* (Duran *et al.*, 2004c) but in the same range as in populations of *Scopalina lophyropoda*, a rare and scarce species with its distribution restricted to the north-western Mediterranean (Blanquer and Uriz, 2010). This particular result might point out a genetic impoverishment of populations in *S. lamella* with future implications for the species' survival.

All populations studied presented clear evidence of recent reduction in size with the exception of Ceuta. The difficult diving conditions in Ceuta may have contributed to the protection of sponge beds from exploitation. This population also displayed the highest gene diversity, the highest mean allele number and private alleles compared with the other populations. Ceuta has a particular situation in the Gibraltar Strait and receives both Mediterranean and Atlantic waters. However, no other populations of *S. lamella* have been identified around the Gibraltar Strait, and a limited connectivity with other populations might threaten the maintenance of genetic diversity in this area. Along the north-western Mediterranean coast, recent disease outbreaks have caused mass mortality of sponges (Perez *et al.*, 2000; Coma *et al.*, 2009), particularly horny sponges with a fiber skeleton (Gaino *et al.*, 1992; Perez *et al.*, 2000; Webster, 2007). These mass mortality events are consistent with the low values of genetic diversity, the absence of private alleles in three populations (Cr, Bul and Cio), and the recent bottlenecks observed in the area for *S. lamella*. Because *S. lamella* also lives in deep zones beyond the typical recreational diving depth, the observed bottleneck could be due to sampling on the species' upper distribution depth limit, thus only affecting a marginal subunit of the putative population. 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*. To date, published information on commercial species' abundance and decline is scarce and fragmented (Pronzato and Manconi, 2008). Available studies evaluating the status of commercial populations have treated all species uniformly within 'commercial types' without further estimations at specific levels (Voultsiadou *et al.*, 2011). Harvesting and fragmentation of *S. lamella* populations are likely to have caused population size reduction, converting once larger populations into small and discrete units.

Identifying how genetic diversity is spatially distributed is fundamental to understanding ecological threats and to developing appropriate recovery strategies for endangered species. *Spongia lamella* is a slow-growing organism with limited dispersal potential, which makes natural recovery of reduced populations quite difficult. Further exploitation of this bath sponge could weaken its genetic diversity further, risking its potential to cope with environmental changes. Reduction of population size increases the risk of genetic diversity loss by genetic drift in very short periods of time, and the high levels of inbreeding may finally drive populations into inbreeding depression (Amos and Balmford, 2001; Brook *et al.*, 2002; Frankham *et al.*, 2002; Frankham, 2005b). Maintaining genetic diversity is crucial for species in the context of global warming or when exposed to disease outbreaks. In addition, *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 and Becerro, 2012). Future management conservation strategies on *S. lamella* should focus on designing marine protected reserves, where exploitation of marine resources and recreation activities such as fishing and diving are limited, in an effort to protect population densities and genetic diversity of the species. These limitations would potentially increase longevity of large adult sponges, reproduction chances and the potential for new recruitments within populations. Increasing population size by natural recruitment reduces the likelihood of genetic diversity loss by genetic drift. Furthermore, transplantations of sponge specimens or fragments of individuals from 'source populations' after careful genotyping to genetically compatible areas affected by strong

bottlenecks and high levels of inbreeding could also facilitate population recovery by increasing population size and genetic diversity. This technical approach based on sponge transplantation has been applied successfully for restocking populations of the bath sponge *S. officinalis* (Baldacconi *et al*., 2007), and may be appropriate for *S. lamella* in the future. Microsatellite loci and population genetic studies are then invaluable tools for population monitoring after restocking to evaluate the success of any conservation measures.

Despite the limitations of this study, mainly imposed by the difference in number of populations between geographic areas, the reduced size of some populations, and the presence of null alleles, it highlights the need for continuing population genetic studies of this endangered species. It also provides an overall status of the current situation of this species, pointing to areas with high genetic diversity or strong bottleneck episodes, whose protection could benefit the species tremendously. Complementing this genetic information with a better understanding of the biology and ecology of the sponge (Becerro, 2008), including physiological traits, may be critical for its survival in a changing environment (UNEP-MAP-RAC/SPA 2008). Studies exploring larval dispersion and survival, recruitment success under different environmental conditions, long-time monitoring of genetic diversity and densities of shallow and deep populations as well as the prevalence of diseases in different geographic areas are all important.

ACKNOWLEDGEMENTS

We thank Isabel Calderón and Gemma Agell for technical support. We also thank Thierry Perez for the various samples in the area of Marseille, Joanna Xavier for the sponge specimens from Portugal, and Josep Carreras Carbonell, Andrea Blanquer, and Xavier Turon for their help during field sampling in Spain. We also thank Xavier Turon for his assistance with the DAPC analyses. Research funded by grants from the Agence Nationale de la Recherche (ECIMAR), a 'Beatriu de Pinós' contract (Comissionat per a Universitats i Recerca from Departament d'Innovació, Universitats i Empresa, Generalitat de Catalunya) and a 'Juan de la Cierva' contract (Ministry of Science from the Spanish Government) to R. P-P. Spanish Ministry of Education and Science (projects BENTHOMICS: CTM2010-22218 and SOLID: CTM2010-17755), and the BIOCAPITAL project (MRTN-CT-2004-512301) of the European Union. This is a contribution of the Consolidated Research Group 'Grupo de Ecología Bentónica', SGR2009-655 of Generalitat de Catalunya.

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