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► **To cite this version:**

Gwilherm Penant, D. Aurelle, Jean-Pierre Feral, Anne Chenuil. Planktonic larvae do not ensure gene flow in the edible sea urchin *Paracentrotus lividus*. *Marine Ecology Progress Series*, 2013, 480, pp.155-170. 10.3354/meps10194 . hal-01631351

**HAL Id: hal-01631351**

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Submitted on 24 Aug 2019

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# Planktonic larvae do not ensure gene flow in the edible sea urchin *Paracentrotus lividus*

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**ABSTRACT:** Previous studies of the commercially harvested sea urchin *Paracentrotus lividus* revealed genetic differentiation between the Atlantic Ocean and the Mediterranean Sea and between the Adriatic and other Mediterranean basins, and reported an absence of genetic structure within basins. These studies used mitochondrial markers. We augmented these data with new mitochondrial (COI) and nuclear (calpain exon-primed intron crossing) sequences and re-analyzed them. We found within-basin and within-region differentiation with each genetic marker, providing, for this species, the first report of a significant and consistent genetic structure within regions in which no stable or identified oceanographic barriers had ever been reported. This was unexpected given the long planktonic larval phase. With the mitochondrial marker, the easternmost population from Lebanon appeared strongly differentiated from other populations, with  $\Phi_{ST}$  values of the same order as those between Atlantic and Mediterranean basins, and a differentiation of the same magnitude was found for the northernmost population (Galway, Ireland). Among basins, gene flow appeared unidirectional, from the Atlantic to the Mediterranean Sea, and we found an admixture area between Adriatic and other Mediterranean populations. The divergence between Atlantic and Mediterranean basins was estimated to have started between 270 000 and 370 000 yr ago. Chaotic genetic patchiness appears unlikely to be the single factor responsible for such a differentiation, and some environmental or hydrological factors that are relatively stable over years, are probably involved. Methodological aspects such as sample sizes and the choice of statistics ( $F_{ST}$  versus  $\Phi_{ST}$ ) contributed to the increased detection power of our study.

**KEY WORDS:** Phylogeography · Mitochondrial DNA · Nuclear EPIC · Divergence time · Genetic structure ·  $F_{ST}$  ·  $\Phi_{ST}$

## INTRODUCTION

The Mediterranean Sea is a biodiversity hotspot, sheltering 6.27% of the total marine biodiversity in less than 1% of the global ocean surface. The Mediterranean Sea is subjected to high anthropogenic pressures, such as increasing human population density along the coasts, harvesting, maritime traffic, and aquaculture (Coll et al. 2010). Thus, knowledge of marine biodiversity, including intraspecific genetic diversity, is required for effective management of this environment. The marine realm was traditionally considered to be a highly dispersive environment,

where genetic differentiation is lower and barriers to gene flow are less obvious than in terrestrial environments (Avisé 1998). Indeed, large population sizes and free larval stages theoretically induce much lower differentiation levels than those observed for related freshwater species (Avisé 2000). Nevertheless, studies of larval dispersal have highlighted the fact that pelagic larval duration may be poorly correlated with genetic structure (Weersing & Toonen 2009), and that larvae are not simply passive particles. Population genetics studies using variable markers also reported genetic structure in highly mobile species (Ruzzante et al. 2006, Chaoui et al. 2009).

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These results agree with the observation that the marine environment is heterogeneous and composed of different water masses that do not readily mix. Relationships between water masses, which are strongly associated with differences in temperature and salinity, influence marine circulation and thus dispersal between populations. This means that the main oceanographic barriers constitute phylogeographical breaks for various species, which are often congruent with biogeographical breaks, as predicted by theory (Avice 2000). For the majority of species investigated, a discontinuity in allele frequencies is observed between the Atlantic and the western basin of the Mediterranean Sea. This break is often located at the Almeria-Oran front, about 200 km east of the strait of Gibraltar (Borsa et al. 1997a, Patarnello et al. 2007). The less studied transition between western and eastern basins of the Mediterranean Sea, often associated with the Siculo-Tunisian sill, also constitutes a common discontinuity in allele frequencies within the Mediterranean Sea. This was revealed by genetic analyses in macrophytes (Arnaud Haond et al. 2007), fishes (Borsa et al. 1997a, Bahri-Sfar et al. 2000), and invertebrates (Zitari-Chatti et al. 2009, Zulliger et al. 2009). A break was also reported within the Aegean Sea, as seen in fishes (Borsa et al. 1997b, Domingues et al. 2005, Magoulas et al. 2006, Zitari-Chatti et al. 2009, Zulliger et al. 2009), in a bivalve (Tarnowska et al. 2010) and in an ophiuroid species complex (Boissin et al. 2011). A discontinuity between Adriatic and Mediterranean populations was also observed in fishes (Maggio et al. 2009) and the sea urchin *Paracentrotus lividus* (Maltagliati et al. 2010), and is suspected in other marine invertebrates (e.g. Peijnenburg et al. 2006, Aurelle et al. 2011). Analyzing the genetic structure of marine species is important for understanding their evolution in this dispersive environment, and is also a prerequisite for fisheries management and conservation (Avice 1998).

The commercially harvested sea urchin *Paracentrotus lividus* is considered to be a key species of the infralittoral rocky shore of the Mediterranean Sea and northeastern Atlantic Ocean (Sala et al. 1998, Pinnegar et al. 2000, Micheli et al. 2005, Guidetti 2006). It is found from the Canary Islands and the coast of Mauritania to the western coast of Ireland in the Atlantic Ocean, and throughout the entire Mediterranean Sea. Although numerous studies have dealt with the biology and the ecology of this species (e.g. Pedrotti 1993, Lozano et al. 1995, Boudouresque & Verlaque 2007, Sellem & Guillou 2007), phylogeography and population genetics approaches using DNA markers are recent. The first studies on these topics

were based on allozymes (Arculeo et al. 1998), random amplification of polymorphic DNA (Rizzo et al. 2009), or suggested genetic determinants for some morphological characteristics (Louise & Benard 1993, 1995a,b). However, their spatial scale and the characteristics of the markers were of limited relevance for phylogeographical inference. In *P. lividus*, only mitochondrial-based phylogeographic studies have evidenced discontinuities between major ocean basins (i.e. between the Mediterranean and the Atlantic: Duran et al. 2004, Calderón et al. 2008, Maltagliati et al. 2010; between the Mediterranean and the Adriatic: Maltagliati et al. 2010). Within basins, spatial genetic structure has been observed with the mitochondrial cytochrome *c* oxidase subunit I (COI) marker along the Mediterranean Spanish coast (Calderón et al. 2012). Nevertheless, such a structure was not observed in all years and was not consistent among all populations, and this differentiation disappeared when cohorts were pooled. At a local scale, Iuri et al. (2007) did not find any genetic structure in the region of Naples, Italy. For temporal comparisons, differentiation among cohorts was demonstrated using the *bindin* gene (Calderón & Turon 2010), appeared occasionally with COI (Calderón et al. 2012), and was not observed with microsatellites (Calderón et al. 2009a).

One potential limitation of previous studies could be the use of a single locus. The high stochasticity of the coalescent process makes inferences based on a single marker less reliable (Hudson & Turelli 2003). Moreover, mitochondrial DNA, which is the most used in these cases, might be misleading because of its uniparental inheritance and the potential impact of selective effects (Galtier et al. 2009). In our study, we combined published and novel genetic data to investigate the population structure of *Paracentrotus lividus* at different spatial scales at several loci. Specifically, we re-analyzed the data from the 3 published large-scale phylogeographic studies (Duran et al. 2004, Calderón et al. 2008, Maltagliati et al. 2010) and combined this with new mitochondrial (COI) and nuclear (calpain exon-primed intron crossing, EPIC) datasets. Both distance-based and frequency-based metrics were used for 2 reasons: (1) in order to compare new to previous data and (2) because the frequency-based metric, which was not used in previous phylogeographic studies, appeared more appropriate to detect subtle genetic differentiation caused by relatively recent and contemporary processes, where migration-drift dominates over mutation. We did not use data from the ANT locus (Calderón et al. 2008) because of the uncertainty in the reconstruction of diploid sequence genotypes for this highly variable marker. We paid special

attention to differentiation among populations within the previously defined major geographical groups (i.e. Atlantic basin, Mediterranean basin, and Adriatic basin), because increasing knowledge at this scale appeared essential for the protection of this exploited species. We also characterized the direction and intensity of gene flow between the main groups of populations, using a coalescence analysis of isolation with migration processes to improve our knowledge on the evolutionary history of this species.

## MATERIALS AND METHODS

### Sampling

Between 2007 and 2010, we collected 370 *Paracentrotus lividus* from 15 locations across most of the distribution range of this species (Table 1 and Fig. S1 in the Supplement at [www.int-res.com/articles/suppl/m480p155\\_supp.pdf](http://www.int-res.com/articles/suppl/m480p155_supp.pdf)). One specimen of *Psammechinus miliaris* was collected from the Roscoff location. All specimens were collected from depths of between 0 and 1 m. Samples of spines or gonads were preserved in 95% ethanol.

### Extraction, amplification, and sequencing of DNA

Different extraction protocols were chosen depending on available tissues. A Chelex protocol was used for DNA extraction from gonad tissue. Tissues were submerged in a 10% Chelex solution and heated at 95°C for 45 min. The Wizard SV Genomic DNA purification system (Promega) was used for DNA extraction from spine samples, according to the manufacturer's instructions. DNA sequences from 3 previous studies (Duran et al. 2004, Calderón et al. 2008, Maltagliati et al. 2010) were retrieved from GenBank (See Table 1 for dataset details).

Amplifications of a 609 bp fragment of the mitochondrial COI gene were performed using the primers COIe-F and COIe-R (Arndt et al. 1996), with the following cycling conditions: 94°C for 2 min, 30 cycles of 94°C for 30 s, 48°C for 15 s, and 72°C for 1 min; and a final 4 min elongation at 72°C. Reactions were performed in a 25 µl volume containing 1 U of FlexiGo *Taq* polymerase (Promega), 1.5 mM of MgCl<sub>2</sub>, 200 µM of dNTPs, 0.25 µM of each primer, and 2.5 µl of DNA template. Amplicons were then purified and sequenced using an ABI automated sequencer by the Genomer platform (Roscoff Marine Station Sequencing Core Facility, Roscoff, France).

Amplifications of ~650 bp fragments from *calpain 7* intron i21 (Chenuil et al. 2010) were performed using primers specific for *Paracentrotus lividus* (i21-F1: 5'-GAG TCA AGA GAA AGG TAT GAG C-3'; i21-R2: 5'-CGA TAC CCA GAA TTC ATT GCG G-3'). Reactions were carried out using the following cycling conditions: 94°C for 2 min; 12 'touch-down' cycles of 94°C for 30 s, annealing between 60 and 50°C with a 2°C decrease every 2 cycles for 15 s, and 1 min at 72°C; and a final 4 min elongation at 72°C. Reactions were performed in a 25 µl volume containing 1 U of FlexiGo *Taq* polymerase (Promega), 2 mM of MgCl<sub>2</sub>, 200 µM of dNTPs, 0.25 µM of each primer, and 2.5 µl of DNA template.

### Cloning of i21 amplicons

The i21 marker PCR products were cloned into pGEM-T easy (Promega) following the manufacturer's instructions. Four clones for each individual were sequenced by AGOWA, Germany. Direct sequencing from PCR products was performed to check for homozygosity when 4 identical clones were retrieved. For confirmed homozygotes, 2 identical sequences were retained in the dataset for further analysis. For heterozygotes, the 2 different sequences were retained in the dataset. In rare cases, more than 2 different sequences were obtained for the same individual, and it was always due to the appearance of singletons, which were removed.

### Data analysis

The COI dataset produced in the present study was analyzed along with the COI dataset retrieved from Duran et al. (2004). The 3 other datasets (i21, this study; 16S, Calderón et al. 2008; cytochrome *b* [Cyt *b*], Maltagliati et al. 2010) were analyzed separately. For the i21 intron, a fragment of 200 to 250 bp, including a poly-A sequence, was rejected because of a potential error introduced by PCR, cloning, or sequencing. Two fragments of the i21 marker were used for further analyses; a 110 bp coding fragment (exon; without indel) and a non-coding fragment (intron) of 300 to 310 bp. Insertion/deletion positions in this second fragment were re-coded using the simple gap coding algorithm implemented in SeqState (Muller 2005), resulting in a 310 bp alignment.

Haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ) were computed for each marker using DNAsp version 5.1 (Librado & Rozas 2009). A rarefaction

Table 1. *Paracentrotus lividus*. Location of sampling sites and number of individual samples per marker. -: Unavailable data. Superscripts indicate data sources: <sup>a</sup>this study, <sup>b</sup>Duran et al. (2004), <sup>c</sup>Calderón et al. (2008), <sup>d</sup>Maltagliati et al. (2010). Collection years of the new data are given next to the population name. Codes are the same as in Fig. S1 in the Supplement at [www.int-res.com/articles/suppl/m480p155\\_supp.pdf](http://www.int-res.com/articles/suppl/m480p155_supp.pdf)

Code	Population	COI <sup>a</sup>	COI <sup>b</sup>	i21 <sup>a</sup>	16S <sup>c</sup>	Cyt b <sup>d</sup>	Region
1	Galway	-	-	-	-	10	Atlantic
2	Roscoff (2007)	25	10	13	9	10	Atlantic
3	Santander	-	11	-	10	-	Atlantic
4	Ferrol (2007)	20	11	14	10	-	Atlantic
5	Baiona	-	-	-	-	10	Atlantic
6	Cascais / Lisbon	-	11	-	11	-	Atlantic
7	Mosteiros	-	-	-	-	10	Atlantic
8	Tenerife	-	11	-	8	-	Atlantic
9	Morocco (2008)	26	-	14	-	-	Atlantic
10	Cadiz	-	-	-	11	-	Atlantic
11	Tarifa	-	11	-	10	-	Gibraltar
12	Ceuta	-	-	-	10	-	Gibraltar
13	La Herradura	-	9	-	-	-	Alboran Sea
14	Cabo de Gata	-	10	-	10	-	Western Mediterranean
15	Cabo de Palos	-	10	-	-	-	Western Mediterranean
16	Nao	-	-	-	7	-	Western Mediterranean
17	Valencia (2010)	21	-	15	-	-	Western Mediterranean
18	Eivissa	-	11	-	-	-	Western Mediterranean
19	Cabrera	-	-	-	9	-	Western Mediterranean
20	Columbretes	-	-	-	12	-	Western Mediterranean
21	Blanes	-	12	-	-	-	Western Mediterranean
22	Tossa de Mare	-	-	-	13	-	Western Mediterranean
23	Palamos	-	-	-	-	10	Western Mediterranean
24	Medes	-	10	-	-	10	Western Mediterranean
25	Cadaques	-	-	-	8	-	Western Mediterranean
26	Carro (2010)	15	-	-	-	-	Western Mediterranean
27	Marseille (2010)	34	-	12	-	-	Western Mediterranean
28	Porquerolles (2010)	27	-	-	-	-	Western Mediterranean
29	Saint-Raphaël (2010)	29	-	-	-	-	Western Mediterranean
30	Villefranche / Mer (2010)	25	-	-	-	-	Western Mediterranean
31	Corsica (2010)	39	-	13	8	-	Western Mediterranean
32	Quercianella	-	-	-	-	10	Western Mediterranean
33	Pittulongu	-	-	-	-	10	Western Mediterranean
34	Alghero	-	-	-	-	10	Western Mediterranean
35	Costa degli Dei	-	-	-	-	10	Western Mediterranean
36	Ustica	-	-	-	-	10	Western Mediterranean
37	Annaba (2008)	19	-	15	-	-	Western Mediterranean
38	Tunis	-	-	-	-	10	Western Mediterranean
39	Marsaskala	-	-	-	-	10	Eastern Mediterranean
40	Siracusa	-	-	-	-	10	Eastern Mediterranean
41	Giardini Naxos	-	-	-	-	10	Eastern Mediterranean
42	Santa Catarina di Nardo	-	-	-	-	10	Eastern Mediterranean
43	Brindisi	-	-	-	-	10	Adriatic
44	Manfredonia	-	-	-	-	10	Adriatic
45	Lesina	-	-	-	-	10	Adriatic
46	Termoli (2007)	16	-	15	-	-	Adriatic
47	Ancona	-	-	-	-	10	Adriatic
48	Mljet	-	-	-	-	10	Adriatic
49	Miramare	-	-	-	-	10	Adriatic
50	Iraklion (2009)	29	-	14	-	10	Eastern Mediterranean
51	Matala (2009)	20	-	15	-	-	Eastern Mediterranean
52	Greece	-	-	-	12	-	Eastern Mediterranean
53	Epanomi	-	-	-	-	10	Eastern Mediterranean
54	Rhodes	-	-	-	-	10	Eastern Mediterranean
55	Kyrenia	-	-	-	-	10	Eastern Mediterranean
56	Lebanon (2008)	25	-	14	-	-	Eastern Mediterranean



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analysis was performed for allelic richness in COI using the Contrib software (Petit et al. 1998) to estimate the potential effects of differences in sample sizes between sites. This analysis was not performed for the other markers because sample sizes were homogeneous.

Haplotype networks based on the most parsimonious connections of haplotypes at the 95% probability level were reconstructed using the program TCS 1.21 (Clement et al. 2000). Based on these networks, we defined haplogroups for COI, Cyt b, and i21. Haplogroups could not be defined for the 16S marker, because of the low level of polymorphism. Haplogroup frequencies per population were then recorded on geographical maps.

Selective neutrality tests were used to test for departure from mutation-drift equilibrium due to changes in effective size or selective effects. We used Fu's  $F_s$  test (Fu 1997), Tajima's  $D$  test (Tajima 1989), and Ramos-Onsins and Rozas'  $R^2$  test (Ramos-Onsins & Rozas 2002) implemented in DNAsp 5.1 (Librado & Rozas 2009). Significance levels were calculated using 10 000 coalescent simulations without recombination.

Among-population differentiations were estimated by the  $\theta$  estimator of  $F_{ST}$  (Weir & Cockerham 1984) and the  $\Phi_{ST}$  statistics using the proportion of differences between haplotypes (Excoffier et al. 1992). These parameters were calculated using ARLEQUIN version 3.5 (Excoffier & Lischer 2010). The null hypothesis of no differentiation was tested by performing 10 000 permutations among individuals between populations. The Benjamini and Hochberg correction (Benjamini & Hochberg 1995) for multiple tests was applied to the p-values calculated for the  $F$  statistics. ARLEQUIN was used to study the hierarchical population structure for each molecular marker, with an analysis of molecular variance (AMOVA) based on haplotype frequencies. Different hypotheses on the position of the transition between the Atlantic and the Mediterranean basins, and following an east-west transect, were tested for the COI and the 16S markers, but not for Cyt b and i21 markers. The density of sampled sites around this transition zone was too weak to perform such an analysis. Different hypotheses on the position of the transition between the Adriatic and the Mediterranean basins were tested for the Cyt b marker, which displayed a sufficient density of samples in this area (see Fig. 2, and Tables S8 to S10 in the Supplement for details). Isolation by distance (IBD) was studied at different scales using the correlation between  $F_{ST}/(1 - F_{ST})$  as the genetic distance (i.e. based on haplotype fre-

quencies) and the logarithm of shoreline distance between sampled sites following the sea current direction as the geographic distance. The correlation was tested using a Mantel test based on 10 000 permutations realized with the Genepop software (Raymond & Rousset 1995).

To estimate demographic parameters using the IMA2 software (Nielsen & Wakeley 2001), we first had to estimate the rate of sequence evolution for the markers used. Previous studies on *Paracentrotus lividus* used a range of nucleotide sequence divergence, consisting of 1.6 to 3.5% Myr<sup>-1</sup> for the COI marker (Lessios et al. 1999, McCartney et al. 2000). Because of the high variability in the evolutionary rate reported for some sea urchins (Chenuil et al. 2008), we attempted to calibrate the COI molecular clock. Data for the coding fragment of the i21 marker were not available in the literature, but we obtained sequences for the same fragment from *Strongylocentrotus purpuratus* and *Psammechinus miliaris*. For Cyt b, as no molecular clock references were found, we also attempted to calibrate its molecular clock with species from the genus *Strongylocentrotus*. The age of the most recent common ancestor for *S. purpuratus* and the Echinidae (to which *P. miliaris* and *P. lividus* belong) was set at 30 Myr on the basis of paleontological records (Smith et al. 2006). The jModelTest software (Posada 2008) was used to choose the optimal model of sequence evolution. The HKY model of nucleotide substitution (Hasegawa et al. 1985) was retained, and the rate of evolution was calibrated using a Bayesian Markov chain Monte Carlo (MCMC) framework implemented in BEAST v1.61 (Drummond & Rambaut 2007). Rates were estimated under the assumption of both strict clock and relaxed clock models. Following  $5 \times 10^5$  steps of burn-in, posterior probabilities of parameter estimates were calculated with  $5 \times 10^6$  cycles of data collection. Five runs were performed and combined to detect and avoid biased estimations due to MCMC chains blocked at local maxima.

The time of splitting (from a single population) between pairs of populations (or groups of populations), effective population sizes, and migration rates in both directions were estimated using IMA2 (Hey 2010). This analysis provides estimators of a split time and migration rates under an island model. A generation time of 3 yr was used for the conversion of generation times into years (Lozano et al. 1995). Following  $5 \times 10^5$  steps of burn-in, posterior probabilities of parameter estimates were calculated with  $10 \times 10^6$  cycles of data collection. Five runs with different starting positions were performed and combined. We

used uniform priors and 20 heated chains with the heating terms suggested by the IMA2 user's guide for small to medium size dataset and medium heating, in order to obtain high swap rates between adjacent pairs of chains.

The PowSim application (Ryman & Palm 2006) was used to assess the influence of sample sizes on the probability of a given  $F_{ST}$  value to appear significant, for a given allele frequency distribution, which was the one we obtained for the COI data set. The program simulates a population fission which occurred a given number of generations ago from a population of a given effective size, and this pair of parameters determines the resulting  $F_{ST}$  value between populations.

## RESULTS

### Marker polymorphism

**Mitochondrial markers.** The complete COI dataset comprised 497 sequences, with 370 new sequences and 127 previously obtained by Duran et al. (2004).

These sequences corresponded to 179 different haplotypes (Table 2). Thirty-four percent of the sequences corresponded to the 4 most common haplotypes found in western and eastern Mediterranean and Atlantic samples, and 30% were unique sequences. The Lebanese sample did not share any haplotype with Atlantic populations. Hd was high in all populations (0.91–1.0), the highest being observed in Atlantic and Lebanese populations (Table 2). Nucleotide diversity ranged from 0.005 to 0.010 with an overall mean of 0.007 (Table 2). Rarefaction analyses did not indicate any influence of sample size differences on allelic richness. Previous studies indicated nucleotide diversity of 0.001 to 0.005 for 16S and 0.003 to 0.012 for Cyt b (Calderón et al. 2008, Maltagliati et al. 2010).

**Nuclear marker.** The amplification and cloning of the i21 locus for 154 individuals from 11 populations resulted in 296 sequences and 97 alleles for the non-coding part of this locus. Similar results were obtained when considering the coding part but with less diversity and larger sample size, as we did not succeed in sequencing the non-coding part for 6 individuals (data not shown). Haplotype diversity and

Table 2. *Paracentrotus lividus*. Diversity measures for the studied populations. n: number of individuals; Nh: number of haplotypes; Hd: haplotype diversity;  $\pi$ : nucleotide diversity; -: data unavailable. Superscripts indicate data sources: <sup>a</sup>this study, <sup>b</sup>Duran et al. (2004). Codes are the same as in Fig. S1 in the Supplement

Code	Population	COI				i21 (coding)				i21 (non-coding)			
		n	Nh	Hd	$\pi$	n	Nh	Hd	$\pi$	n	Nh	Hd	$\pi$
2	Roscoff <sup>ab</sup>	35	19	0.96	0.008	13	13	0.92	0.042	12	16	0.96	0.056
3	Santander <sup>b</sup>	11	10	0.98	0.007	-	-	-	-	-	-	-	-
4	Ferrol <sup>ab</sup>	31	18	0.96	0.007	14	12	0.88	0.039	11	17	0.98	0.063
6	Cascais <sup>b</sup>	11	7	0.91	0.006	-	-	-	-	-	-	-	-
8	Tenerife <sup>b</sup>	11	9	0.95	0.008	-	-	-	-	-	-	-	-
9	Morocco <sup>a</sup>	26	22	0.99	0.006	14	12	0.92	0.039	12	15	0.96	0.061
11	Tarifa <sup>b</sup>	11	8	0.95	0.005	-	-	-	-	-	-	-	-
13	La Herradura <sup>b</sup>	9	8	0.97	0.007	-	-	-	-	-	-	-	-
14	Cabo de Gata <sup>b</sup>	10	6	0.84	0.004	-	-	-	-	-	-	-	-
15	Cabo des Palos <sup>b</sup>	10	10	1.00	0.010	-	-	-	-	-	-	-	-
17	Valencia <sup>a</sup>	21	15	0.96	0.008	15	12	0.72	0.013	15	10	0.68	0.014
18	Eivissa <sup>b</sup>	11	10	0.98	0.007	-	-	-	-	-	-	-	-
21	Blanes <sup>b</sup>	12	8	0.92	0.005	-	-	-	-	-	-	-	-
24	Medes <sup>b</sup>	10	9	0.98	0.006	-	-	-	-	-	-	-	-
26	Carro <sup>a</sup>	15	9	0.91	0.009	-	-	-	-	-	-	-	-
27	Marseille <sup>a</sup>	34	20	0.92	0.006	12	9	0.66	0.018	12	11	0.82	0.015
28	Porquerolles <sup>a</sup>	27	17	0.96	0.007	-	-	-	-	-	-	-	-
29	Saint-Raphaël <sup>a</sup>	29	19	0.92	0.007	-	-	-	-	-	-	-	-
30	Villefranche / Mer <sup>a</sup>	25	15	0.91	0.008	-	-	-	-	-	-	-	-
31	Corsica <sup>a</sup>	39	25	0.96	0.007	13	13	0.79	0.024	13	15	0.90	0.037
37	Annaba <sup>a</sup>	19	15	0.97	0.007	15	6	0.58	0.017	15	17	0.84	0.027
46	Termoli <sup>a</sup>	16	11	0.91	0.006	15	10	0.56	0.014	15	10	0.71	0.015
50	Iraklion <sup>a</sup>	29	19	0.92	0.006	14	12	0.73	0.022	15	13	0.85	0.031
51	Matala <sup>a</sup>	20	15	0.96	0.005	15	9	0.47	0.010	14	13	0.75	0.015
56	Lebanon <sup>a</sup>	25	20	0.98	0.009	14	9	0.67	0.019	14	14	0.78	0.028
Total		497	179	0.96	0.007	154	73	0.80	0.027	148	97	0.88	0.035

nucleotide diversity appeared to be higher for the Atlantic populations than for the Mediterranean ones (Table 2). One i21 allele was found only in the Mediterranean basin, where its frequency reached ~30% of all alleles. Atlantic and Mediterranean populations only shared 4 alleles for this marker.

**Statistical parsimony network and haplogroup mapping.** The haplotype networks for COI and i21 were characterized by the presence of star-like patterns centered on the most frequent haplotypes (Fig. 1). The Cyt b haplotype network displayed a higher level of polymorphism than COI and i21, and

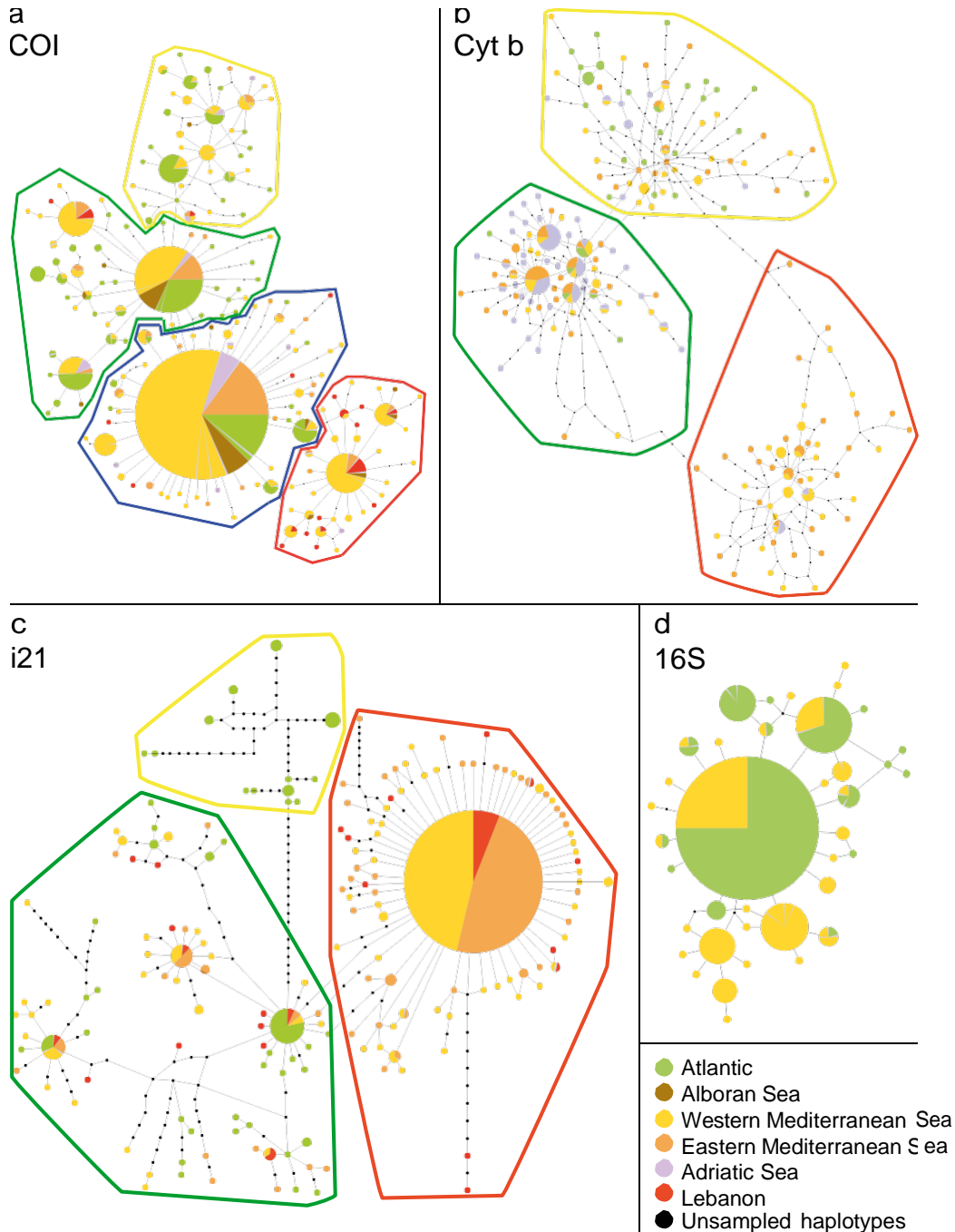


Fig. 1. *Paracentrotus lividus*. Statistical parsimony networks of haplotype relationships for (a) COI, (b) Cyt b, (c) the non-coding fragment of the nuclear intron i21, and (d) 16S. Each branch represents 1 mutation between the 2 adjacent sequences and black circles represent unsampled haplotypes. Circle sizes for each network are proportional to the number of individuals.

Yellow, green, blue, and red polygons enclose the different haplogroups used for mapping in Fig. 2



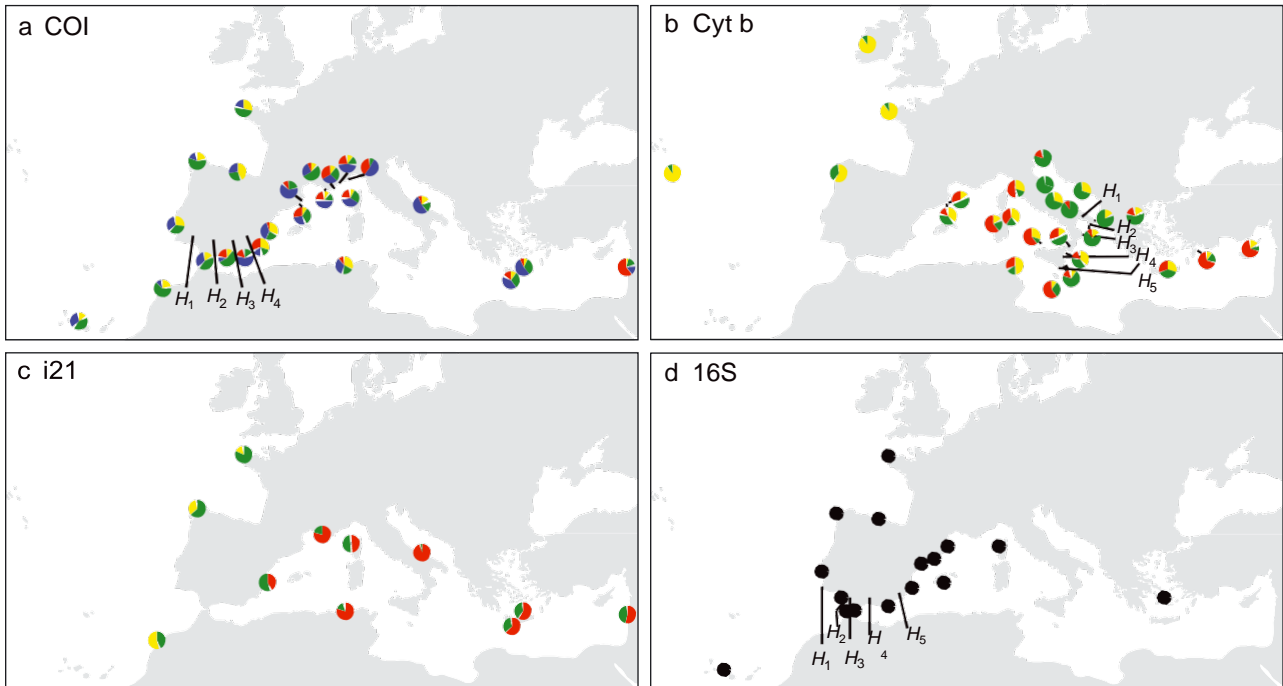


Fig. 2. *Paracentrotus lividus*. Mapping of haplogroups based on statistical parsimony network reconstruction for (a) COI, (b) Cyt b, and (c) i21. Pie chart colors (same as for Fig. 1 polygons) correspond to haplogroup frequencies per sampled site. Hypotheses tested by AMOVAs (see Table 3) are given as  $H_x$  for the 3 previously cited markers and (d) 16S

consisted of 3 major haplogroups (Fig. 1). For each of these 3 markers, 1 group of haplotypes was absent from Atlantic samples. This group represented half of all Mediterranean sequences for i21 (red group in Figs. 1 & 2). This Mediterranean haplogroup was dominant in eastern Mediterranean samples, where it reached its maximum frequency. A second group comprised COI and Cyt b haplotypes mostly from Atlantic samples, as well as i21 haplotypes unique to Atlantic specimens (yellow group in Figs. 1 & 2). For Cyt b and i21 markers, a third group contained haplotypes shared between all regions (green group in

Figs. 1 & 2). A shared haplogroup for COI corresponded to 2 subgroups around 2 main haplotypes, 1 with a majority of unique haplotypes from Atlantic samples (green group in Figs. 1 & 2), and a second mainly comprised of unique haplotypes from Mediterranean samples.

### Population differentiation

Pairwise population comparisons based on  $F_{ST}$  were significant for most tests using the mitochondr-

Table 3. *Paracentrotus lividus*. AMOVA results between 2 different transition zones: between the Atlantic (ATL) and Mediterranean (MED) basins (COI and 16S) and between the Adriatic (ADR) and Mediterranean basins (Cyt b). Only hypotheses that maximized differentiation between groups and minimized differentiation within groups are presented. All hypotheses tested are presented in Tables S8 to S10 in the Supplement.  $F_{SC}$ : fixation index within groups (p-values in parentheses);  $F_{CT}$ : fixation index among groups (p-values in parentheses)

Transition	Marker	Transition position hypothesis	Within groups		Among groups	
			$F_{SC}$	% of variation	$F_{CT}$	% of variation
ATL / MED	COI	$H_{2COI}$	0.028 (<0.001)	2.65	0.059 (<0.001)	5.92
	16S	$H_{416s}$	0.006 (0.337)	0.54	0.135 (<0.001)	13.54
ADR / MED	Cyt b	$H_{3cytb}$	0.034 (<0.05)	2.86	0.159 (<0.001)	15.88
		$H_{4cytb}$	0.033 (<0.05)	2.81	0.152 (<0.001)	15.18
		$H_{5cytb}$	0.025 (0.073)	2.12	0.159 (<0.001)	15.89

ial markers (Tables S1 to S3 in the Supplement). The few non-significant  $F_{ST}$  values for Cyt b were among Adriatic samples. For the non-coding region of i21, all pairwise  $F_{ST}$  values were significant, except among Atlantic populations (Table S4). The  $F_{ST}$  between Atlantic and Mediterranean Sea populations was the only significant comparison for the coding region of i21.

Pairwise  $\Phi_{ST}$  for COI showed significant differentiation between Atlantic and Mediterranean populations and between the Lebanese and other populations. The highest values were found between Lebanese and other populations. For the Cyt b marker, significant pairwise  $\Phi_{ST}$  values were observed between Atlantic and Mediterranean populations, between Adriatic and Mediterranean populations, and between the northernmost population of Galway, Ireland, and all other populations. Pairwise  $\Phi_{ST}$  for intron i21 showed significant differentiation only between Atlantic and Mediterranean populations. Details of the values are given in Supplementary materials (Tables S1 to S4).

AMOVA on the COI marker showed a maximum differentiation between groups and a minimum differentiation within groups when the transition between Atlantic and Mediterranean Sea was set between Tarifa and La Herradura, Spain (hypothesis  $H_2$  in Fig. 2a;  $F_{CT} = 0.059$ , Table 3; for exact site locations, see Fig. S1 in the Supplement). AMOVA on the 16S marker showed a maximum differentiation between groups and a minimum differentiation within groups when setting the transition between Atlantic and Mediterranean Sea between Ceuta and Cabo de Gata, Spain (hypothesis  $H_4$  in Fig. 2d;  $F_{CT} = 0.135$ , Table 3). AMOVA on the Cyt b marker revealed a maximum differentiation between groups and a minimum differentiation within groups for different hypotheses when setting the transition between groups between Brindisi and Giardini Naxos, Italy (hypotheses  $H_3$  to  $H_5$  in Fig. 2c;  $F_{CT} = 0.152$ – $0.159$ , Table 3). Details of the alternate hypotheses are given in Tables S8 to S10.

The  $F_{ST}/(1-F_{ST})$  values for mitochondrial COI were positively correlated with the geographical distance over the whole sample range (Mantel test  $r = 0.3617$ ,  $p = 0.017$ ) and within the western basin of the Mediterranean Sea (Mantel test  $r = 0.657$ ,  $p = 0.009$ ). The nuclear genetic distance for i21,  $F_{ST}/(1-F_{ST})$ , was significantly positively correlated with geographical distance over the whole sample range (Mantel test  $r = 0.644$ ,  $p = 0.002$ ), but not at other scales. Intra-basin pairwise comparisons  $F_{ST}/(1-F_{ST})$  were lower than inter-basin pairwise comparisons, independent of geographic distance.

### Demographic and selective neutrality tests

**Mitochondrial markers.** For COI, we obtained significant values from Fu's  $F_s$  test for the majority of samples. Results of the  $R^2$  test partially supported these results (4 out of 9 Atlantic samples and 6 out of 17 Mediterranean samples showing non-significant p-values). Tajima's  $D$  test was significant for 1 sample from the Atlantic, 5 from the Mediterranean Sea, and 1 from the Adriatic Sea. For 16S, we obtained significant values from Fu's  $F_s$  test for 1 of the 8 Atlantic samples and 6 out of 8 Mediterranean samples. Results of the  $R^2$  test supported Fu's test results for the Atlantic sample and for 3 Mediterranean samples. Tajima's  $D$  test was significant for 2 Atlantic samples and 1 Mediterranean sample. For Cyt b, we obtained significant values from Fu's  $F_s$  test for 1 out of 4 Atlantic samples, 10 out of 16 Mediterranean samples, and 4 out of 6 Adriatic samples. Results of the  $R^2$  test partially confirmed these results for Atlantic and Adriatic samples. Tajima's  $D$  test was significant for a single Adriatic sample. For all 3 markers, pooling samples by basin resulted in significant values for all tests (Tables S5 to S7).

**Nuclear marker.** We obtained significant values for the 3 tests for Mediterranean and Adriatic samples, but never for the Atlantic samples. All tests were significant when samples were pooled by basin.

Table 4. *Paracentrotus lividus*. Results of the estimation of parameters of the isolation with migration model (IMA2) at the inter-basin level between Atlantic (ATL) and Mediterranean (MED) populations, according to different evolution rates for the COI marker.  $t_0$ : Splitting time from an ancestral population in million years; Ne: effective population sizes in millions of individuals; 2NM: population migration rate in number of gene copies per generation. Confidence intervals are in parentheses

COI sub. rate (%)	$t_0$	Ne ATL	Ne MED	Ne ancestral population	$2NM_{ATL \rightarrow MED}$	$2NM_{MED \rightarrow ATL}$
0.49	0.37 (0.11–0.22)	1.24 (0.60–2.20)	11.9 (8.20–28.00)	0.54 (0.20–1.00)	31.17 (17.11–50.02)	0.01 (0.00–10.59)
1.60	0.27 (0.22–0.34)	2.05 (1.41–2.77)	3.95 (2.61–5.34)	0.29 (0.15–0.60)	31.17 (17.11–50.02)	0.01 (0.00–10.59)

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## Demographic parameter estimates

The results obtained with the strict clock and relaxed clock models were similar. Therefore, the COI marker, the Cyt b marker, and the exon fragment of the i21 marker were considered to follow a strict clock model. The nucleotide sequence divergence rate was estimated to be 0.98% Myr<sup>-1</sup> (95% highest posterior density, HPD: 0.62–1.42% Myr<sup>-1</sup>) for COI, 1.64% Myr<sup>-1</sup> (95% HPD: 1.14–2.39% Myr<sup>-1</sup>) for Cyt b, and 0.72% Myr<sup>-1</sup> (95% HPD: 0.38–1.10% Myr<sup>-1</sup>) for the exon fragment of i21.

Results based on a single marker led to poor precision in parameter estimation or even failed to estimate parameters for the Cyt b marker (Table S11). We estimated that the mean splitting times based on a dataset comprising all available markers (COI, 16S, Cyt b, and i21<sub>exon</sub>) ranged from 0.27 to 0.37 Myr according to the substitution rate used for the COI marker (0.49 and 1.60% Myr<sup>-1</sup>, respectively). Gene flow appeared asymmetric, actually unidirectional, with about 30 gene copies per generation from the Atlantic Ocean to the Mediterranean Sea, and not significantly different from 0 in the reverse direction. Details and confidence intervals are given in Table 4.

Gene flow also appeared asymmetric and higher from Lebanon to the other Mediterranean populations, with about 60 gene copies per generation, and did not appear different from 0 between Lebanon and the Atlantic populations, based on a dataset comprising sequences from COI and the i21 exon.

### Influence of sample size

The PowSim results confirmed that sample sizes used in previous studies for the COI marker had poor chances of detecting significant differentiation: between 25% (with a chi-squared test) and 52% (with Fisher's exact test) of  $F_{ST}$  values appeared significant when sample sizes equaled 12, the maximum size used by Duran et al. (2004), whereas more than 95% of  $F_{ST}$  values were significant when sample size reached 30 individuals.

## DISCUSSION

### Intra-basin genetic structure

Our results provide a new perspective on the genetic structure among *Paracentrotus lividus* populations. While previous studies based on mitochon-

drial markers and  $\Phi_{ST}$  statistics highlighted differentiation between basins, namely the Atlantic–Mediterranean transition (Duran et al. 2004, Calderón et al. 2008, Maltagliati et al. 2010) and the Adriatic–Mediterranean transition (Maltagliati et al. 2010), our study reveals significant genetic differentiation at a much more local scale. The statistic used ( $\Phi_{ST}$  instead of  $F_{ST}$  in our case) and the low number of individuals within population samples probably account for the failure of previous studies to detect the differentiation within basins and within regions that our results unambiguously established. The lower polymorphism of the 16S marker may also explain previous results based on this marker.

Intra-basin  $F_{ST}$  revealed significant differentiation between nearly all population pairs within the Mediterranean and Adriatic basins, some of which were geographically close (about 40 to 60 km between our populations from the French Mediterranean coast). Non-significant  $F_{ST}$  values for the COI markers were obtained only for populations retrieved from Duran et al. (2004), which presented the smallest sample size for this marker ( $9 < N < 12$ ). We believe that these small sample sizes prevented the detection of regional genetic structure (Ryman et al. 2006) as supported by our simulation results. Discrepancy between  $\Phi_{ST}$  and  $F_{ST}$  can be explained by the nature of those statistics. While the  $\Phi_{ST}$  statistic is strongly dependent on the accumulation of mutations between the observed haplotypes, the  $F_{ST}$  statistic is only dependent on the haplotype frequency distribution, because it does not consider the distances among haplotypes. Significant  $F_{ST}$  might thus be observed after relatively few generations of genetic drift, while significant  $\Phi_{ST}$  will be observed after the appearance of some mutant haplotypes.  $F_{ST}$  is, however, expected to be more powerful than  $\Phi_{ST}$  to detect significant differentiation, when allele divergence does not contain any relevant information since it gives an equal weight to all alleles and since the  $F_{ST}$  statistics have a lower variance than the  $\Phi_{ST}$ . However, the regional genetic differentiation revealed in this study was unexpected for a species dispersing via a planktotrophic larval stage lasting about 1 mo (Pedrotti 1993, Lozano et al. 1995). The characteristics of the reproductive biology of various marine invertebrates (e.g. variance in reproductive success, stochastic nature of larval connectivity, temporal fluctuations in selection pressures) may generate spatial or spatio-temporal patterns of genetic structure (Johnson & Black 1984, Siegel et al. 2008). This is often called ‘chaotic genetic patchiness’ and refers to patterns of genetic structure which are not

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stable in time, because they are caused by neither stable oceanographic or physical barriers, nor by distance. In *Paracentrotus lividus*, cases of differentiation were observed among cohorts in locations of the Iberian coast but not among locations when cohorts were pooled (Calderón et al. 2012), a pattern which is typical of chaotic genetic patchiness. Our results strongly suggest that a totally different mechanism also creates genetic differentiation among locations in this species for 2 reasons: (1) genetic differentiation within regions was revealed among samples composed of various cohorts, and (2) we also found a significant pattern of IBD within the Western Mediterranean basin (with the COI marker). This strongly suggests that gene flow is not only limited by fluctuating events susceptible to generate chaotic genetic patchiness, but also by distance and by environmental factors which are relatively stable in time and remain to be determined. Such factors may either affect dispersal (hydrological factors) or fitness (differential selection). It is not likely, however, that the process of isolation by distance generates genetic differentiation among the closest locations of our survey (about 40 km) since they represent the smallest distances we used in the IBD test (thus they were associated with low differentiation values, since IBD appears significant, with COI).

The observation of genetic differentiation within basins refutes the definition of panmictic units proposed in previous studies (Duran et al. 2004, Calderón et al. 2008). Moreover, the analysis of the gene coding for the gamete recognition protein binding suggested a role for prezygotic processes in the temporal genetic structuring of *Paracentrotus lividus*, and a differential fertilization success according to the origin of the gametes in the northwestern Mediterranean Sea (Calderón et al. 2009b, Calderón & Turon 2010). This may contribute to the genetic structure shown here. Several studies investigating Atlantic and Mediterranean genetic population structure in echinoderms did not evidence differentiation within regions for species with planktotrophic or lecithotrophic larvae (Borrero-Pérez et al. 2011, So et al. 2011, Chatti et al. 2012), whereas some cases of differentiation at smaller distances have been reported for species without a dispersing larva (Baus et al. 2005, Boissin et al. 2008a,b). This reflects the expected influence of life history traits on genetic structure (Tarnowska et al. 2012), although it is possible that in some of those species, larger sample sizes and refined data analyses (e.g. using  $F_{ST}$  instead of  $\Phi_{ST}$ ) could reveal other cases of genetic differentiation within region, even for species that dis-

perse via a larva. This is supported by our results and by the finding, in the Pacific Ocean, of genetic structure at small distances (<10 km) in a highly fecund larva-producing echinoderm, the crown-of-thorns starfish (Timmers et al. 2012).

### The case of Lebanon

The second major finding of our study is the differentiation of the population of the Levantine basin, as highlighted by the COI marker, for which the most common haplotype was absent in Lebanon (Figs. 1 & 2), and by high  $F_{ST}$  values (Table S1). The distinction between the easternmost populations and other Mediterranean populations is rarely investigated in marine species. As an example, a Levantine clade has been discovered within the cryptic species complex of the gastropod *Dendropoma petraeum*, but this species does not present a pelagic larval stage (Calvo et al. 2009), and a separate clade within the Levantine basin is also suspected in the sea bass *Dicentrarchus labrax* (Castilho & Ciftci 2005).

A possible explanation for the differentiation of the Lebanese population may involve the low planktonic blooms within the oligotrophic Levantine basin (D'Ortenzio & Ribera 2008). Larval survival and recruitment in *Paracentrotus lividus* are highly dependent on spring planktonic blooms (López et al. 1998). The reduction of this bloom might decrease larval survival time, and thus gene flow in this area. The absence of differentiation with the nuclear marker may be explained by the lower genetic drift affecting nuclear genomes relative to mitochondrial ones (due to effective size differences), causing slower differentiation (Ballard & Whitlock 2004). An alternative hypothesis assumes that selection on the mitochondrial marker would be responsible for the differentiation observed in the Lebanese sample. Selection was already suggested in a study of stressed populations from Greece (Rizzo et al. 2009). The Lebanese population is located in the region with the most oligotrophic waters in the distribution range of *P. lividus*, as well as the highest mean seawater temperatures. It is possible that natural selection eliminates the larvae containing the very common COI haplotypes found in other populations (including the most common haplotype). A correlation between sea surface temperature and mitochondrial DNA haplotypes was reported for the North Pacific walleye pollock (Grant et al. 2006), and a relation between chlorophyll *a* concentration and the relative abundance of mitochondrial lineages of an ophiuroid species complex was reported in



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Mediterranean basins (Boissin et al. 2011). This hypothesis implies that a migration load affects these sea urchin populations, with maladapted mitochondrial genotypes being introduced by gene flow (as suggested by nuclear markers) and eliminated continuously by selection.

### Position of transition zones and asymmetric gene flow

At the inter-basin level, the AMOVA tests favor the hypothesis that the transition zone between the Mediterranean and Atlantic population groups occurs within the Alboran Sea, west of the Almeria-Oran front. The transition zone between Adriatic and other Mediterranean populations remains unclear, but is likely to be between the south of the Adriatic Sea and Sicily. Moreover, the multilocus analyses of gene flow under a model of isolation with migration (IM model) unambiguously established the unidirectionality of gene flow from the Atlantic Ocean to the Mediterranean Sea, confirming previous studies which suggested asymmetric gene flow (Calderón et al. 2008, Maltagliati et al. 2010). Strasburg & Riesberg (2010) demonstrated that population structure within species had little effect on parameter estimates using the IM model, even for fairly high levels of structure. Thus, despite within-basin population structure, the unambiguously asymmetric pattern we found can be considered a robust result. The results of the Bayesian analysis (IMa2) are clearly illustrated by the fact that some frequent haplotypes and even some haplogroups of the COI, Cyt b, and i21 markers are not found outside the Mediterranean Sea (Fig. 2). Such asymmetrical gene flow was previously suggested for a cuttlefish (Pérez-Losada et al. 1999), a seabream (Bargelloni et al. 2005), an oyster (Saavedra et al. 1993), and a crustacean (Pannacciulli et al. 1997). Such observations are consistent with the current circulation pattern across the Strait of Gibraltar (Millot 1999), i.e. superficial waters flowing from the Atlantic to the Mediterranean Sea.

We estimated that the Atlantic and Mediterranean Sea populations diverged from an ancestral population between 0.27 and 0.3 Myr ago, depending on the mutation rate considered. This places the divergence after the transition during the middle Pleistocene that started between 0.7 and 0.8 Myr ago. This period corresponds to an increase in long-term global ice volume and a strong reduction of the North Atlantic thermohaline circulation that influenced global oceanic circulation (Head et al. 2008). Cycles

of climate fluctuation starting from the transition between early and middle Pleistocene were characterized by more severe glaciations, which may have initiated the differentiation between Atlantic and Mediterranean populations (Almogi-Labin 2011). However, we may have underestimated the substitution rate of our markers, due to calibration using an old divergence time, and thus overestimated divergence times (e.g. Crandall et al. 2012). This would strengthen the conclusion that the ‘Atlanto-Mediterranean’ divergence and the demographic expansion did not predate the middle Pleistocene. The differentiation between Mediterranean and Atlantic basins may have been maintained because of hydrographic barriers to dispersal, differential selective pressures or the evolution of mechanisms of reproductive isolation (Bierne et al. 2011). Nevertheless, the Quaternary sea level and sea temperature fluctuations do not appear to have strongly reduced the genetic diversity of this species, which appears to have undergone demographic expansions much before the last glaciation for both the Atlantic and the Mediterranean basins (always more, sometimes much more, than 100 000 yr ago for all basins and all markers, Penant 2012). This observation is in line with the present distribution of *Paracentrotus lividus*, which may be observed as far north as the Irish coasts. *P. lividus* populations likely persisted in the Mediterranean Sea and in the southern part of the North Atlantic during glacial events, as sea surface temperatures for the Mediterranean during the Last Glacial Maximum (LGM) were estimated at around 7°C in winter and 9 to 13°C in summer (Thiede 1978, Hayes et al. 2005). Published studies analyzing models of isolation with migration between the Atlantic and Mediterranean basins are not common, but many studies estimated divergence times between pairs of Atlantic and Mediterranean sister species, finding both pre-Messinian (Sotelo et al. 2009, Xavier et al. 2012) and post-Messinian events (Luttikhuisen et al. 2008, Egea 2011). Population expansion times are more often estimated (Patarnello et al. 2007), giving expansions as recent as the LGM (Palero et al. 2008) to expansions older than 500 000 yr, most of them being between 50 000 and 200 000 yr. *P. lividus* thus appears in the range of divergence and expansion times inferred for other Atlanto-Mediterranean taxa.

### CONCLUSIONS

Beyond its paradoxical nature and fundamental interest, our finding that genetic differentiation



within regions is the rule in the benthopelagic sea urchin *Paracentrotus lividus* raises important questions for the management of this exploited species. The local depletions reported by fishermen on the French Mediterranean coast where this species is traditionally consumed may be explained by limited gene flow among localities; moreover, replenishment from migrants appears less likely than previously thought, and the sustainability of this level of harvesting may become an issue. Determining whether population differentiation is due to limited dispersal or to local adaptation will be the next step. The use of more nuclear markers should help to separate demographic and selective components in the observed patterns of genetic structure (within regions, and also to test whether the discrepancy observed for Lebanon between i21 and COI is due to stochasticity or to selection). Experimental approaches or methods based on genome scanning may help to detect local adaptation.

*Acknowledgements.* We thank E. Egea, K. Gérard, C. Abed, F. Zuberer, J. Garrabou, and J. Vierna who kindly provided specimens for this work. This work is part of G.P.'s PhD thesis funded by Région Provence-Alpes-Côte-d'Azur.

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