# Microsatellite markers reveal shallow genetic differentiation between cohorts of the common sea urchin *Paracentrotus lividus* (Lamarck) in NW Mediterranean

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Keywords: *Paracentrotus lividus*; sea urchins; microsatellite loci; cohorts; temporal genetic structure; effective population size.

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Running title: Temporal genetic structure in *P. lividus*

**Abstract**

Temporal variability was studied in the common sea urchin *Paracentrotus lividus* through the analyses of the genetic composition of three yearly cohorts sampled over two consecutive springs in a locality in North-Western Mediterranean. Individuals were aged using growth ring patterns observed in tests and samples were genotyped for five microsatellite loci. No reduction of genetic diversity was observed relative to a sample of the adult population from the same location or within cohorts across years. Significant differentiation was found in one intra-cohort comparison and a few inter-cohort comparisons with contingency table analysis, although $F_{ST}$ and AMOVA results indicated that the differentiation is rather shallow, as most variability is found within samples and within individuals. This mild differentiation translated into estimates of effective population size of ca. 100 individuals. Given our restricted sampling area and the known small-scale heterogeneity in recruitment in this species, our results suggest that at stretches of a few km of shoreline, large numbers of progenitors are likely to contribute to the larval pool at each reproduction event. Inter-cohort variation in our samples is seven times smaller than spatial variation between adults of four localities in Western Mediterranean. Our results indicate that, notwithstanding the stochastic events that take place during the long planktonic phase and during the settlement and recruitment processes, reproductive success in this species is high enough to produce cohorts genetically diverse and with little differentiation between them. Further research is needed before the link between genetic structure and underlying physical and biological processes can be well established.

## Introduction

The study of both spatial and temporal variation of population structure is essential to fully understand factors that affect genetic variability and demographic processes within species. Spatial patterns have been extensively studied in the last years (reviewed in Avise 2000; Palumbi 2004; Cowen *et al*. 2006). For these studies, populations are often sampled without any regard for their cohort structure, thus mixing individuals of different ages collected at a given time. Comparatively, the temporal component of genetic variability has received considerably less attention. Most genetic studies show the net result, averaged over time, of dispersal patterns. Therefore, they yield little information concerning temporal structure of larval dispersal (Bossart & Prowell 1998), its impact on marine populations being still poorly understood (e.g., Caley *et al*. 1996).

The hypothesis of "sweepstake reproductive success" suggests that chance determines how many and which adults actually contribute to the demographic continuity of marine species at each reproductive event (Hedgecock 1994a). High fecundity and juvenile mortality create potential for large variance in reproductive success. This random variation may generate, among other consequences, chaotic patchiness in the genetic composition of each new generation arriving at a population, leading to genetic heterogeneity among local populations on a small spatial scale (Hedgecock 1994b; Edmands *et al*. 1996). These temporal changes in allele frequencies can be used to infer the genetically effective population size ($N_e$) of natural and managed populations (Pollack 1983; Waples 1989; Jorde & Ryman 1995). Large stochastic variability in reproductive success may be explained by the small effective size frequently detected in marine organisms relative to census sizes, which are sometimes several orders of magnitude larger than $N_e$ (Avise *et al*. 1988; Hedgecock 1994a; Avise 2000; Turner *et al*. 2002; Hedgecock *et al*. 2007). If this is true, then recruits should have a reduced genetic diversity relative to the adult population. Besides, if only a subset of adults from a population contribute to reproduction at each spawning event, this may result in changes in allelic frequencies from one generation to the next, resulting in high differentiation among cohorts (even exceeding spatial differentiation among populations at broad geographic scales; Watts *et al*. 1990; Hedgecock *et al*. 1992; Hedgecock 1994b; Edmands *et al*. 1996). Some studies on marine invertebrates confirm these predictions (e.g., Li & Hedgecock 1998; Moberg & Burton 2000; Planes & Lenfant 2002; Pujolar *et al*. 2006; Hedgecock *et al*. 2007; Lee &

Boulding 2007). On the contrary, other studies do not detect evidence of sweepstake reproduction when comparing adult and recruit genetic make-up in invertebrates and fish (Flowers *et al*. 2002; Bernal-Ramírez *et al*. 2003; Poulsen *et al*. 2006).

Sea urchins' larvae can disperse over scales of hundreds of km and therefore it is reasonable to think that their larval pool is well mixed over large spatial scales. However, echinoids feature high interannual variation in settlement and recruitment for reasons not fully understood (Ebert 1983; Pearse & Hines 1987; Schroeter *et al*. 1992; López *et al*. 1998; Sala *et al*. 1998; Hereu *et al*. 2004; Tomas *et al*. 2004), which may indicate heterogeneity in the larval pool at small scales. Indeed, many factors can determine the actual pool of larvae arriving at a given location, which will determine the genetic composition of adult populations. Hydrological features, phytoplankton availability, predator abundances or water temperature are among the multiple factors that can determine survival of larval batches. Since these factors vary in space and time, remarkable genetic variation between different groups of age has been observed in several studies concerning sea urchins. Edmands *et al*. (1996) found evidence for significant differentiation among subpopulations of recruits and between adults and recruits of *Strongylocentrotus purpuratus* from the same location based on allozymes, but Flowers *et al*. (2002) did not find a temporal structure in the same species using mitochondrial DNA. Similarly, Moberg & Burton (2000) acknowledged extensive between-year variation in the genetic structure of populations of *S. franciscanus*, suggesting that larval pool is not well homogenized during the long planktonic larval phase. Other studies have suggested that selection upon larvae may cause differentiation in *Echinometra mathaei* and that forces causing genetic differentiation can act locally and occur in a single generation (Watts *et al*. 1990). A fine scale spatial patchiness in recruitment within localities has been detected in *Paracentrotus lividus* (Hereu *et al*. 2004; Tomas *et al*. 2004), reinforcing the idea of a non-homogeneous pool of larvae in the water column.

Temporal genetic processes can be examined by sequential long-term sampling through time or by evaluating genetic data with respect to the age structure of the population sampled at a single point in time. The aim of the present study was to obtain the first insights of temporal genetic variation of the common sea urchin *Paracentrotus lividus* (Lamarck, 1816) in Tossa de Mar (North-Western Mediterranean; Fig. 1). *P. lividus* is a keystone species in benthic

sublittoral communities of the Mediterranean, as its browsing activity is one of the main factors regulating algal abundance (Palacín *et al*. 1998; Boudouresque & Verlaque 2001). In addition, this commercially important species is heavily harvested for its roe in some areas, which can lead to overfishing and population depletion along its distribution range (e.g., Guidetti *et al*. 2004). *P. lividus* is a long-lived free-spawning species with a long planktonic larval phase. Population dynamics of this species in the study area has been previously analyzed by Lozano *et al*. (1995), Turon *et al*. (1995) and López *et al*. (1998). Despite the initial controversy on the subject, it now seems well established that a main spawning event occurs in spring and smaller recruitment events take place in autumn (López *et al*. 1998; Tomas *et al*. 2004). Taking this into account, we used microsatellite markers developed for this species (Calderón *et al*. 2009) to analyse temporal genetic variability of cohorts of individuals arrived at this locality in three consecutive springs (2004, 2005 and 2006) sampled over two years (2006 and 2007).

## Material and methods

### Sampling and age estimation

In June 2006 and June 2007, samples of *P. lividus* were collected by SCUBA at the same location in Tossa de Mar (41º 43.26'N, 2º 56.41'E; Fig. 1). The particular site sampled was very restricted spatially, comprising an area of ca. 10*20 m of a bottom dominated by boulders at 15 m of depth. At this spot, small-sized sea urchins were abundant under the boulders. A total of 374 sea urchins of between 10 and 40 mm in diameter were sampled and kept in 96% ethanol at –20ºC until processed. Maximum diameter of adults was measured to the nearest 0.1 mm in the laboratory with callipers and gonads were extracted and preserved in absolute ethanol. When specimens were too small to have gonads, whole Aristotle's lanterns were preserved. Tests were carefully cleaned to remove spines and dried at 90ºC for at least 72 h.

Individuals were aged using growth ring counts in interambulacral series of plates (Jensen 1969; Allain 1978; Azzolina 1988; Gage 1991) as described in Turon *et al*. (1995). In short, dried tests were immersed in xylene, which penetrates the calcite mesh (stereom) that constitutes the sea urchin test. Denser stereom corresponds to periods of active growth and appears as opaque rings, while looser stereom corresponds to periods of slow growth, visible

as more translucent bands once embedded in xylene. The alternance of opaque and translucent bands is interpreted as yearly growth rings (see below). The pattern of these rings was determined under the binocular preferentially using the older plates, from the coronal (i.e., corresponding to the maximum diameter) to the peristomial ones. A whole oral-aboral series of plates was also examined to discern true bands from smaller, supernumerary bands that may occur in some individuals due, for instance, to periods of stress. These supernumerary bands fade away when they approach the nucleus of the plates as younger plates are observed. The pattern of translucent/opaque bands was then transformed into age of individuals.

Turon *et al*. (1995) provided evidence of the annual formation of growth bands in *Paracentrotus lividus* in this area. To further validate the method, more than 115 individuals were tagged with tetracycline in November 2005 and collected in January 2007 from the same location in Tossa de Mar. Tetracycline is an antibiotic that chelates with $CaCO_3$ and is thus incorporated into the tests. This tagging technique has proved to be an effective method to follow growth in sea urchins (Gage 1992a, b; Lamare & Mladenov 2000). After 14 months, all individuals were collected at the particular spot where the tagging was conducted and 34% of the marked individuals were recovered. Tests were cleaned and kept at –20ºC until observed under UV light. The position of the band of tetracycline (indicating the moment of tagging) was marked on the test with a scalpel. Growth during that period corresponds to the marginal deposition of calcite between the tetracycline mark and the distal end of the plates. Growth bands observed in that area were then examined under the binocular to confirm the annual formation of rings in *P. lividus*.

We use the term cohort to define the group of individuals that are assumed to have arrived at Tossa de Mar within a single recruitment event. Individuals belonging to cohorts arrived in spring 2004, 2005 and 2006 were used in this study, and these data were compared to an adult population (N=27, larger than 40 mm in diameter) collected at the same location in 2005. Since samples were collected over two consecutive years (2006 and 2007), data on variation among and within cohorts are available. It should be noted that recruits arrived on the same year of collection were not included in this study. Therefore, for the cohort recruited in 2004, we have samples collected in 2006 (when they were 2 years old) and 2007 (3 years old). For the cohort recruited in 2005, samples were collected at one (2006) and two (2007) years of

age. Finally, for the cohort recruited in spring 2006 we have data of the one-year-old juveniles (collected in 2007). These data can be pooled in different ways for analysis: as age-classes (one, two and three-year-old individuals), as cohorts (2004, 2005 and 2006), and as cohorts-by-year, referring to individuals of each cohort collected at both sampling years (cohorts-by-year will be designated with the cohort year first and the collection year second: Spring04-06, Spring04-07, Spring05-06, Spring05-07 and Spring06-07). Additionally and in order to compare temporal with spatial variation, we collected 44 individuals from three localities of the Western Mediterranean: Cabrera Island (Balearic Archipelago), Cabo de Gata and Tarifa, located between 320 and over 1000 km away from Tossa de Mar (Fig. 1).

DNA extraction and genotyping

DNA was extracted from gonads (or Aristotle´s lantern of small individuals) using REALPURE extraction kit (Durviz, Spain). Microsatellites are highly variable markers that have proven to be suitable for analyses of biogeographic processes operating over relatively localized spatial and short temporal scales (Estoup & Angers 1998). Thus, five polymorphic microsatellites were genotyped in this study: Pl-B, Pl-C, Pl-T, Pl-Hist and Pl-28F (Calderón *et al*. 2009). PCRs were performed in a final volume of 25 µL containing 3 mM $MgCl_2$, 0.12 µM of each primer and 1 U of *Taq* polymerase (Promega). The forward primer for each locus was labelled with fluorescent dyes (6-Fam and Hex from Sigma-Genosys or Ned from Applied Biosystems; Table 1). Alleles were sized on an ABI3700 automated sequencer relative to the internal standard ROX 70-500 (Ecogen) using Peak Scanner software (Applied Biosystems).

Genetic and statistic analyses

Standard population genetic parameters were used to describe genetic variability within and among cohorts. The genetic diversity of each cohort-by-year was calculated as number of alleles and allelic richness per locus and combined over loci using the software FSTAT 2.9.3 (Goudet 2001). This software was also used to calculate linkage disequilibrium among loci. GENETIX version 4.05.2 (Belkhir *et al*. 2004) was used to estimate observed and expected heterozygosities, as well as to calculate $F_{IS}$ coefficients and test their significance (using 10000 permutations). We used Micro-checker v2.2.3 (Van Oosterhout *et al*. 2004) to further analyze potential causes of the deficit of heterozygotes observed. The genetic differentiation among cohorts-by-year was analyzed by contingency table analysis using traditional chi-

square tests with summation over loci with the program CHIFISH (Ryman 2006). There has been considerable debate about the performance of methods for testing differentiation among populations, either using permutation tests or contingency table analyses (chi-square, G test or Fisher's exact test) and, in the latter case, about methods to combine the results for different loci (Raymond & Rousset 1995; Ryman & Jorde 2001). Simulation studies showed that the traditional chi-square method provides the best results in terms of type I errors and power (Ryman & Jorde 2001; Ryman 2006). The program GenAlex (Peakall & Smouse 2006) was used to calculate $F_{ST}$ statistics (based on variance of allele frequencies, following Weir & Cockerham 1984) and analyses of molecular variance (AMOVA, Excoffier *et al*. 1992). The significance of AMOVA was calculated with 10000 permutations of the original data.

Estimates of effective population sizes

We used two different methods for estimating effective population sizes (*Ne*) from allele frequency changes among cohorts. The first method was analytical: the so-called temporal method (Nei & Tajima 1981; Pollack 1983; Waples 1989), as modified by Jorde & Ryman (1995, 1996) for overlapping generations. The implicit assumption is that shifts in allele frequencies between consecutive cohorts are due to random genetic drift (plus sampling error). We further assumed that removal of some individuals for analyses had no effect on the allelic frequencies of the following cohorts and that the number of newborns in each generation is large (Jorde & Ryman 1996).

To measure changes in allele frequencies we used the unbiased estimator $F_s$ proposed by Jorde & Ryman (2007). The formula of the estimator (our sampling scheme corresponds to Plan II; Waples 1989; Jorde & Ryman 2007) is:

$$F_s = \frac{\sum_{i=1}^{a}(x_i - y_i)}{\sum_{i=1}^{a} z_i(1 - z_i)}$$

Where $a$ is the number of alleles at a given locus; $x_i$ is the observed frequency of the $i$th allele in the sample of individuals of the first cohort; $y_i$ is the corresponding frequency in the sample drawn from the second cohort, and $z_i$ is the mean of $x_i$ and $y_i$.

The estimator $F_s$ was corrected for the expected effect of sampling as in Jorde & Ryman (2007):

$$F_s' = \frac{F_s(1-1/4\tilde{n}) - 1/\tilde{n}}{(1+F_s/4)(1-1/2n_y)}$$

Where $\tilde{n}$ is the harmonic mean of the sample sizes taken from the two cohorts ($n_x$ and $n_y$). The $F_s'$ values were obtained for each locus and averaged using TempoFs (Jorde & Ryman 2007), which also calculates 95% confidence intervals by jackknifing over loci.

In the case of overlapping generations, the amount of temporal change in allele frequencies depends not only on *Ne* but also on demographic characteristics of the population. These are incorporated in the estimation of *Ne* through a correction factor (*C*) and an estimate of the generation interval (*G*; as defined in Jorde & Ryman 1995, 1996). For the computation of these correction factors, we need to estimate age-class specific survival rates ($l_i$) and birth rates ($b_i$). We used previous biological information on *Paracentrotus lividus* (Lozano *et al*. 1995; Turon *et al*. 1995) to calculate *C* and *G*. The details of these estimations are presented in Supporting material 1.

Finally, the effective population size *Ne* was calculated from the formula (Jorde & Ryman 1995, 1996):

$$Ne = \frac{C}{2G\overline{F}_s'}$$

Where $\overline{F}_s'$ is the average of $F_s'$ values across loci. We pooled the data by cohort (Palm *et al*. 2003) to obtain a more robust estimate and we compared pairs of consecutive cohorts: 2004 with 2005, and 2005 with 2006.

The second method used was based on a simulation approach. The rationale behind it is that in species with long-lived larvae it is hard to define a "parental" population, as larvae may come from a wide geographic range. In our case, there seems to be enough gene flow among populations of the Iberian Mediterranean shores as to prevent significant genetic divergence

between them (Duran *et al*. 2004). We can therefore think of a "source" meta-population, much bigger than the population under study. Our best approach to the allele frequencies of this meta-population can be obtained by pooling all adult samples from the four localities studied in the area (Fig. 1).

We simulated the genetic differentiation expectable from cohorts deriving from an increasing number of progenitors drawn from this source meta-population. For this purpose, we simulated a sample of $N$ reproductive individuals from the meta-population using the pooled allele frequencies of the adults, and drew a sample of their offspring of size equal to the first cohort sample being compared ($n_x$). We then repeated the sampling of reproductive individuals and of their offspring, this time with size equal to the second cohort sample ($n_y$), and computed the uncorrected $F_s$ value between cohorts. Repeating this process a large number of times (1000 replicates) we obtained the expected value of $F_s$ between samples of size ($n_x$) and ($n_y$) from consecutive cohorts deriving from a source meta-population if the number of progenitors (i.e., effective population size) was $N$. The process can be repeated for increasing values of $N$ to generate a distribution of expected $F_s$ values and to determine the number of progenitors that corresponds to the observed value of $F_s$.

The assumptions of this procedure are: a) that our pooled allele frequencies are representative of the allele structure of the source meta-population, b) that this allelic structure will not change appreciably, at least at the scale of a few generations, c) that in the two generations compared the number of progenitors was the same and d) that the contribution of these progenitors to the recruited individuals was the same. Although these assumptions make our model somewhat simplistic, it provides an estimate of *Ne* that can be compared to that obtained with the analytical method above. The simulation routines were written in Turbo Pascal.

## Results

### Age assignments

Growth rings were successfully counted for every individual. Our tagging experiment confirmed the annual formation of growth bands, as suggested by Turon *et al*. (1995) and as corroborated by results from adults labelled with tetracycline (all tagged individuals presented

one translucent and one opaque band between the mark corresponding to the incorporation of tetracycline and the margin of the plates).

Based on this annual formation of growth rings, we classified all the sampled individuals in different age-classes and we selected the subset of those individuals estimated to have recruited at Tossa de Mar in spring 2004, spring 2005 and spring 2006 (a total of 121 individuals). Grouping these samples in age-classes, we have a representation of sea urchins estimated to be one year old: the cohort recruited in 2005 sampled in 2006 (N=15) and the cohort recruited in 2006 sampled in 2007 (N=22); two years old: the cohort recruited in 2004 sampled in 2006 (N=31) and the cohort recruited in 2005 sampled in 2007 (N=29); and three years old: the cohort recruited in 2004 sampled in 2007 (N=24). Figure 2 shows the size-frequency distribution of these three age-groups. It is apparent how size may vary considerably among individuals of the same age-class. In particular, the size interval becomes wider as the individuals become older. We therefore confirm that band pattern is a better method for estimating age than size.

Genetic characteristics

The main genetic characteristics of the different cohorts-by-year studied, as well as those of the adult population at the same locality, are listed in Table 1. LD was not detected between any of the loci analyzed, as previously observed by Calderón *et al*. (2009).

A deficit of heterozygotes was detected for the 5 loci, with significant inbreeding coefficients for all loci and cohorts-by-year (Table 1), with the exception of locus C in two and locus T in three cohorts-by-year. When considering all cohorts-by-year together, all loci showed significant inbreeding coefficients. According to Micro-checker, null alleles may be present at all loci, as suggested by the general excess of homozygotes for most allele size classes. However, the lack of failed amplifications (homozygote individuals for null alleles) and the coincident result with other nuclear markers (Calderón *et al*. 2008) leave place for alternative explanations (see Discussion). Most alleles found in the adult population were recovered in the juveniles analyzed, with the exception of 1 (out of 19) for locus B, 1 (out of 29) for locus C, four (out of 45) for locus Hist and four (out of 32) for locus 28F (See Supporting material 2).

There was no observable reduction in genetic diversity (in terms of allelic richness or expected heterozygosity) between successive years of a single cohort (compare in Table 1 the cohort recruited in 2004 sampled in 2006 and 2007, and the 2005 cohort sampled in 2006 and 2007) nor relative to the adult population from Tossa de Mar. Pooling data in age-groups to increase our power, no pattern of decline was evident either (Fig. 3).

Population differentiation analysis considering the cohorts separated by year was performed using Chi-square tests on allele frequency tables (Table 2). Some of the tests were significant (after Bonferroni correction). Of these, one value corresponded to intra-cohort comparison (cohort of 2004 sampled at two consecutive years) and the remaining corresponded to inter-cohort comparisons. $F_{ST}$ values between cohorts-by-year were generally low, both for intra- and inter-cohort comparisons (ranging from -0.002 to 0.008; Table 2). Additionally, $F_{ST}$ values among cohorts only showed significant differences between the cohort of 2004 (samples collected in 2006 and 2007 pooled together) and the cohort of 2006 ($F_{ST}$=0.006; $P$<0.05).

When comparing each cohort-by-year with the adult population collected in Tossa de Mar, significant differences (after Bonferroni correction) in allele frequency (contingency table analysis) were found with the cohort of 2004 sampled in 2007, and with the cohort of 2005 sampled in 2007. In all cases, $F_{ST}$ values were low, ranging between -0.006 and 0.005. Nevertheless, these differences disappeared when each cohort (2004 and 2005, respectively, data from the two years pooled together) was compared to the adult population.

Concerning differentiation between 4 localities of the Western Mediterranean (including the adult population of Tossa de Mar), pairwise $F_{ST}$ values were 0.027±0.007 (mean±SE), while the average values obtained in our dataset (Table 2) were 0.004±0.001. Thus, spatial variation was almost seven-fold the observed temporal variation (among and within cohorts), and the difference was significant ($t$-test, $P$=0.001).

An AMOVA analysis on the cohort-by-year data showed that most variation was found within individuals and among individuals within cohorts-by-year (ca. 70% and 30%,

respectively), with only a minor component (0.42%) associated with differences between cohorts-by-year, which was nevertheless significant ($P$=0.012). This small variance component, however, was not due to differences among the three cohorts: when we introduced another hierarchical level grouping the samples by cohort (2004, 2005 and 2006), this grouping level explained only 0.083% of the total variance.

Effective population sizes

Table 4 summarizes the observed values of corrected ($F_s'$) and uncorrected ($F_s$) allele differentiation between cohorts and the results of the two approaches used to estimate *Ne*. The temporal method provided an estimate of 132 individuals for the comparison between cohorts of 2004 and 2005, and 81 individuals when comparing the cohorts of 2005 and 2006. The simulation method provided estimates of $F_s$ that rapidly flattened out as the number of reproductive individuals ($N$) increased (Fig. 4). The asymptote of the curves corresponds to the allele differentiation expectable from the sampling effect alone. The number of progenitors (and hence the effective population size) for which $F_s$ value was the same as the observed value was found by interpolation and corresponded to 117 individuals (comparison 2004-2005) and 66 individuals (comparison 2005-2006). The jackknife estimation of confidence intervals on $F_s'$ and $F_s$ provided by the program TempoFs allowed us to establish 95% confidence intervals for the effective population sizes. These confidence intervals were wide for the 2004-2005 comparison, and much narrower for the 2005-2006 comparison. The estimates of the two methods were quite consistent, and both point towards a somewhat lower *Ne* in the comparison of the second pair of cohorts. Combining the four estimates we obtained a mean value for the effective population size of 99 individuals.

**Discussion**

Only in recent years the importance of temporal genetic structure in marine organisms has become widely acknowledged. Among other aspects, this structure has profound implications is species conservation (Turner *et al*. 2002; Palm *et al*. 2003). Unfortunately, temporal data are particularly scarce due to the difficulties in making reliable estimates of age, and our knowledge of temporal patterns of genetic structure lags much behind that of spatial patterns. This study provides the first insights on temporal genetic structure of populations of *Paracentrotus lividus*, an ecologically and commercially important species, showing a shallow variability between the cohorts analyzed.

As already detected for other species of sea urchin (Gage 1991 and references therein), our results confirm that the use of growth bands on skeletal plates is a reliable method for estimating growth and thus, for inferring age in *P. lividus* (Turon *et al*. 1995). Indeed, the band pattern observed in the interambulacral plates corresponds to processes of seasonal growth. However, the appearance of supplementary translucent rings likely due to events of stress rendered the reading difficult, especially in older specimens that may have undergone several such episodes of stress. The reading of whole series of plates from the oral to the aboral end allowed us to discern true growth bands from smaller, artefactual bands.

Our data on microsatellite markers show a high genetic diversity within cohorts, as already detected with these same markers for adult populations of Tossa de Mar and Cabrera (Calderón *et al*. 2009) and for geographically distant populations, based on nuclear and mitochondrial markers (Duran *et al*. 2004; Calderón *et al*. 2008). Additionally, our results do not show a reduction in diversity of cohorts relative to adult populations (Table 1). Furthermore, levels of differentiation detected between cohorts were seven times lower than spatial differentiation found between localities located within 1000 km of shoreline.

Besides the high variability associated to settlement both at temporal and spatial scales, recruitment and other post-settlement events may also play a very important role in shaping genetic composition of cohorts in this species (Hereu *et al*. 2004; Tomas *et al*. 2004). We found a significant differentiation within the same cohort sampled over two consecutive years (Spring04-06 and Spring04-07; Table 2), which could be taken as evidence of high mortality coupled with selection between the two sampling years. However, no reduction in genetic diversity was observed in the cohorts for which data were available for the two sampling years, and no differentiation was detected between samples from the cohort 2005 collected in 2006 and 2007. Thus, given our small sample size, we favour the idea that stochastic sampling error may explain this significant outcome (Waples 1998). Annual mortality rate in *Paracentrotus lividus* is high over the first year and lower as individuals grow older (Turon *et al*. 1995; Verling *et al*. 1995; López *et al*. 1998). It could thus be expected to observe a reduction in genetic diversity during the first year of benthic life of a given cohort, as commonly detected for many marine invertebrates with dispersing larvae (Gosselin & Qian

1997; Hunt & Scheibling 1997). In our case, however, as individuals from our cohort of 2004 were between 2 and 3 years old by the time we sampled them, mortality occurring during the critical early benthic phase did not affect our genetic parameters.

As in previous studies on this species, we observe a deficit of heterozygotes in all our cohorts and all our loci relative to what would be expected for populations at HW equilibrium (Table 1). The lack of homozygotes for null alleles in our sample and the coincident results with other nuclear markers (Calderón *et al*. 2008, 2009; authors' unpublished research) suggest that null alleles are not the cause of this outcome. Deficits of homozygotes are usually explained by selection against heterozygotes, Wahlund effect, inbreeding or a combination of these. Although none of these possibilities can be completely ruled out, our results on the gamete recognition protein bindin suggest that positive selection acting upon this protein may be responsible for some degree of assortative mating in *Paracentrotus lividus* (Calderón *et al*. unpublished results) that can contribute to an excess of homozygotes in our samples.

For marine invertebrates with sedentary adults, the genetic composition of populations is mainly influenced by the genetic composition of recruits, and not of migrating adults (Watts *et al*. 1990). Temporal variability in recruitment of successive cohorts at a given locality is closely related to the effective population size, the smaller the *N*e the larger the effect of genetic drift, leading to cohort differentiation. Our estimates of effective population sizes should be taken with caution, considering that only three cohorts have been analyzed and that sample sizes considered were relatively small. However, the two independent methods used provided similar estimates of effective sizes of about one hundred progenitors for the recruits arriving at our restricted sampling spot. Although this figure may seem small at first sight, it should be noted that the meaning of *Ne* in sedentary species is necessarily linked to the sampling area covered. There is a marked spatial and temporal variability in recruitment of *P. lividus* at fine scales (Hereu *et al*. 2004; Tomás *et al*. 2004) which, coupled with the short movement range of this species (and particularly of juveniles that hide in cryptic habitats to avoid predation: Sala & Zabala 1996; Palacín *et al*. 1997), likely results in a high demographic heterogeneity of the recruits over small spatial scales, as found for other invertebrates (e.g., Johnson and Black 1982, 1984). Although this patchiness remains to be confirmed by genetic studies, our finding that ca. 100 individuals are involved in the fathering

of recruits from a spot of just several tens of square meters suggests that large *Ne* figures will be found when considering bigger spatial scales such as coastal stretches several km across.

Two main signatures are left by strong sweepstake events: reduction of genetic diversity within cohorts relative to adult populations (which are a mixture of several cohorts) and higher differentiation between different cohorts arrived at a given location relative to differentiation found among spatially distant populations. None of these features characterize the samples of *Paracentrotus lividus* analyzed, so we can infer a relatively high reproductive success of adults in this species, with no significant sweepstake effect that could reduce the genetic diversity of newly arrived cohorts or markedly alter allelic frequencies between them.

Pre- and post-settlement mortality are certainly taking place in this broadcast spawner but our results imply that the larval pool that is able to successfully recruit and survive at this given location is sufficiently large to maintain high degrees of genetic diversity within populations. Further studies on small-scale genetic heterogeneity of recruits and studies using markers under selective pressures along a longer time frame may shed more light on the processes explaining temporal genetic structure in *Paracentrotus lividus*.

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**Acknowledgements**

We are grateful to Manuel Ballesteros and Albert Corbacho for their help during the tagging with tetracycline. All the experiments comply with current Spanish and European laws. This research was funded by projects 119/2003 of the Spanish Network of National Parks and CTM2007-66635 of the Spanish Ministry of Science and Innovation.

## Figure legends

Fig. 1. Sampling sites for *Paracentrotus lividus*.

Fig. 2. Size-frequency histograms of individuals aged on the basis of growth rings. The asterisks mark the average size.

Fig. 3. Patterns of expected heterozygosity (*He*, in white) and allelic richness (corrected per sample size, in black) in the three age-classes contained in our samples. Bars represent standard deviations (across loci).

Fig. 4. Estimates of *Fs* corresponding to comparisons between cohorts of the same size of those studied obtained simulating an increasing number of progenitors (*N*). Solid lines indicate the *N* corresponding to the observed value of *Fs* between the cohort of 2004 and 2005, and dashed lines indicate *N* of the comparison between the cohort of 2005 and 2006. Error bars are standard deviations of 1000 replicates. Note log-log scale.

642

Table 1. Genetic characteristics of cohorts-by-year and adult population from Tossa de Mar.

| Cohort | Parameter | Spring04-06 (N= 31) | Spring04-07 (N= 24) | Spring05-06 (N= 15) | Spring05-07 (N= 29) | Spring06-07 (N= 22) | Adult population Tossa de Mar (N= 27) |
|---|---|---|---|---|---|---|---|
| Pl-B (6' FAM) | Nb. of alleles | 15 | 13 | 10 | 16 | 12 | 15 |
| | Allelic richness | 12.558 | 11.154 | 10 | 12.522 | 11.135 | 14.185 |
| | *Ho* | 0.3548 | 0.4667 | 0.4583 | 0.5862 | 0.5455 | 0.2593 |
| | *He* | 0.9191 | 0.8851 | 0.9007 | 0.9117 | 0.9080 | 0.9287 |
| | $F_{IS}$ | 0.618*** | 0.481*** | 0.497*** | 0.361*** | 0.405*** | 0.719*** |
| Pl-C (NED) | Nb. of alleles | 18 | 11 | 12 | 18 | 16 | 15 |
| | Allelic richness | 13.326 | 13.419 | 12 | 13.546 | 9.27 | 13.973 |
| | *Ho* | 0.8065 | 0.5427 | 0.9333 | 0.7586 | 0.8636 | 0.7037 |
| | *He* | 0.9239 | 0.8041 | 0.9034 | 0.9274 | 0.8890 | 0.8987 |
| | $F_{IS}$ | 0.129* | 0.331** | -0.034 | 0.185*** | 0.029 | 0.218*** |
| Pl-T (HEX) | Nb. of alleles | 19 | 15 | 15 | 16 | 13 | 14 |
| | Allelic richness | 13.808 | 13.42 | 15 | 11.748 | 12.622 | 13.524 |
| | *Ho* | 0.9032 | 0.75 | 0.9333 | 0.8966 | 0.6818 | 0.6296 |
| | *He* | 0.9164 | 0.9149 | 0.9310 | 0.9338 | 0.9059 | 0.9280 |
| | $F_{IS}$ | 0.015 | 0.183*** | -0.003 | 0.04 | 0.252*** | 0.318*** |
| Pl-Hist (HEX) | Nb. of alleles | 23 | 19 | 16 | 21 | 16 | 25 |
| | Allelic richness | 16.308 | 14.846 | 16 | 13.548 | 15.761 | 22.350 |
| | *Ho* | 0.5806 | 0.6250 | 0.4000 | 0.5862 | 0.4545 | 0.5556 |
| | *He* | 0.9476 | 0.9442 | 0.9562 | 0.9341 | 0.9049 | 0.9651 |
| | $F_{IS}$ | 0.391*** | 0.343*** | 0.590*** | 0.377*** | 0.504*** | 0.464*** |
| Pl-28F (NED) | Nb. of alleles | 22 | 21 | 16 | 19 | 19 | 22 |
| | Allelic richness | 16.459 | 15.492 | 16 | 16.214 | 16.599 | 20.183 |
| | *Ho* | 0.4516 | 0.75 | 0.7333 | 0.6552 | 0.5455 | 0.5185 |
| | *He* | 0.9508 | 0.9486 | 0.9609 | 0.9468 | 0.9471 | 0.9706 |
| | $F_{IS}$ | 0.529*** | 0.213*** | 0.243*** | 0.312*** | 0.43*** | 0.421*** |
| All | Nb. of alleles | 19.4 | 15.8 | 13.8 | 18 | 15.2 | 15 |
| | Allelic richness | 14.4918 | 13.0812 | 13.8 | 13.9182 | 13.2382 | 14.185 (±4.12) |
| | *Ho* | 0.6258 | 0.625 | 0.6933 | 0.6966 | 0.6182 | 0.5333 |
| | (±SD) | (±0.231) | (±0.1284) | (±0.2521) | (±0.1322) | (±0.1594) | (±0.1689) |
| | *He* | 0.9312 | 0.9025 | 0.9274 | 0.9302 | 0.911 | 0.9103 |
| | (±SD) | (±0.0164) | (±0.0585) | (±0.0329) | (±0.0126) | (±0.0216) | (±0.0265) |
| | $F_{IS}$ | 0.332*** | 0.312*** | 0.259*** | 0.255*** | 0.327*** | 0.719*** |

Table 1. Number of alleles and allelic richness for each locus and each cohort-by-year as calculated by FSTAT. $H_o$ and $H_e$: observed and expected heterozygosity respectively; $F_{IS}$: inbreeding coefficients (GENETIX). *: $P<0.05$; **: $P<0.005$; ***: $P<0.001$.

Table 2. Population differentiation among cohorts-by-year.

| | $\chi^2$ (df) | $F_{ST}$ |
|---|---|---|
| Spring04-06 vs Spring05-06 | 150.136 (107)* | 0.003 |
| Spring04-06 vs Spring04-07 | 152.548 (108)* | 0.008* |
| Spring04-06 vs Spring05-07 | 134.797 (111) | -0.002 |
| Spring04-06 vs Spring06-07 | 148.007 (108) | 0.006 |
| Spring05-06 vs Spring04-07 | 138.301 (94)* | 0.007 |
| Spring05-06 vs Spring05-07 | 120.915 (100) | -0.002 |
| Spring05-06 vs Spring06-07 | 128.323 (92) | 0.004 |
| Spring04-07 vs Spring05-07 | 134.908 (99) | 0.008* |
| Spring04-07 vs Spring06-07 | 148.946 (96)* | 0.01* |
| Spring05-07 vs Spring06-07 | 125.986 (102) | 0.001 |

Table 2. Measures of population differentiation among the five cohorts-by-year obtained from contingency table analysis. Degrees of freedom (df) are given in parentheses. $F_{ST}$ values are also indicated. Asterisks denote significant values at $P<0.005$ after Bonferroni correction.

11 Table 3. AMOVA of cohorts-by-year.

| Source | df | MS | % variance |
|---|---|---|---|
| **Among cohorts-by-year** | 4 | 3.485 | 0.422* |
| **Among individuals within cohorts-by-year** | 116 | 3.016 | 29.992** |
| **Within individuals** | 121 | 1.620 | 69.586** |

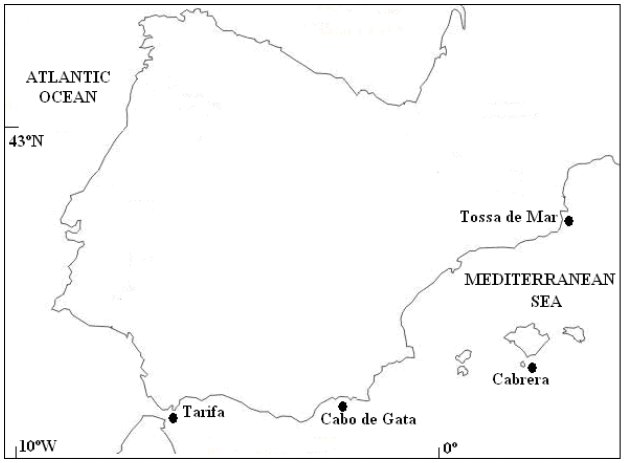
12

13 Table 3. AMOVA analysis of the cohort-by-year dataset (*: $P$<0.05; **: $P$<0.001). df: degrees of freedom; MS:
14 Mean square.

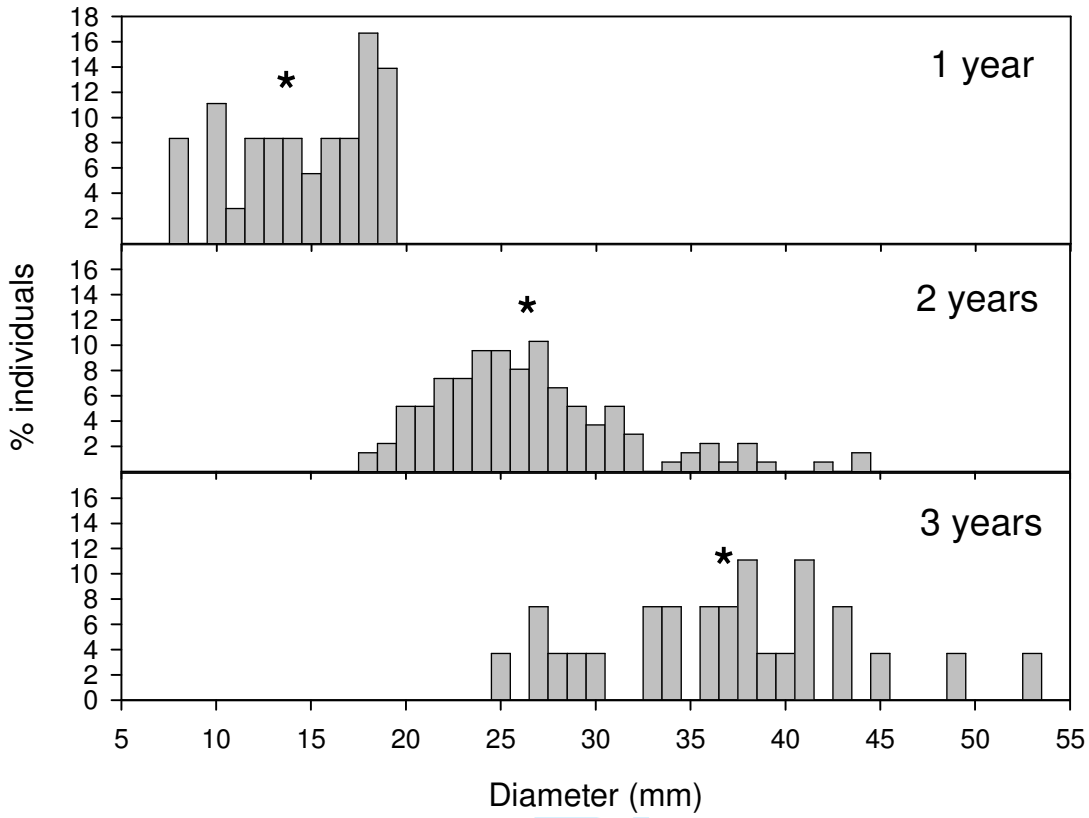
Table 4. Estimates of $F_s$ and $F'_s$ and corresponding $N_e$ estimates.

| | 2004 vs 2005 | 2005 vs 2006 |
|---|---|---|
| Temporal method | | |
| Mean $F_s'$= | 0.0097 | 0.0157 |
| *Ne*= | 132 | 81 |
| 95 % CI= | 66-13800 | 44-410 |
| | | |
| Simulation method | | |
| Mean $F_s$= | 0.0303 | 0.0501 |
| *Ne*= | 117 | 66 |
| 95 % CI= | 53-n.a. | 37-360 |

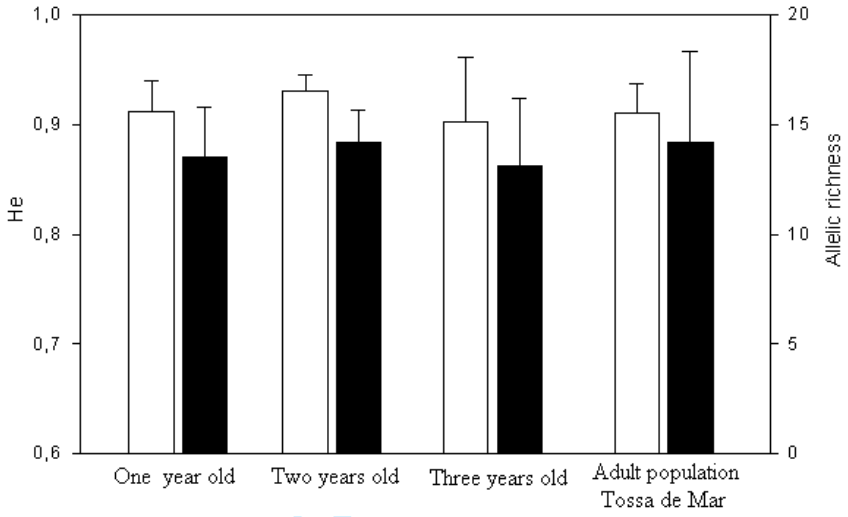
Table 4. Average temporal allele frequency shifts between consecutive cohorts expressed as $F_s'$ and $F_s$ (means over loci) and associated *Ne* estimates for the two methods used. When possible, 95% confidence intervals are provided (n.a.: not applicable, the lower bound for the mean $F_s$ is below the asymptotic part of the curve).




1




2

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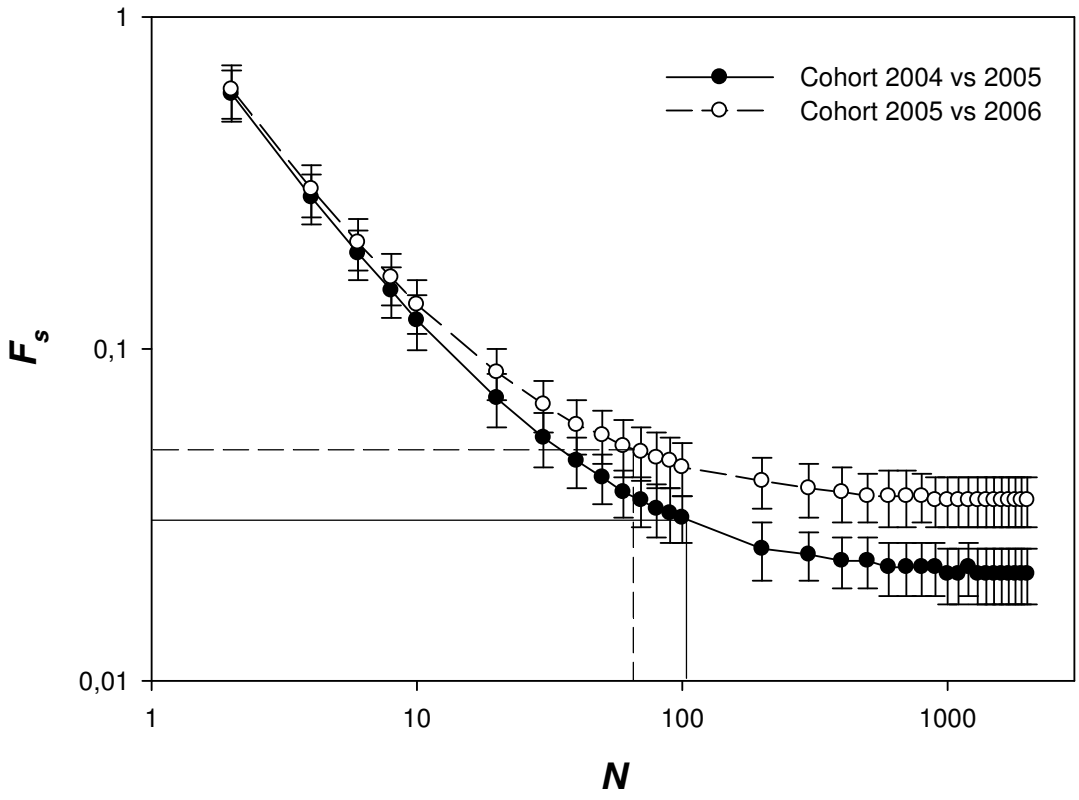
1
0,1
0,01
$F_s$
1
10
100
1000
N
Cohort 2004 vs 2005
Cohort 2005 vs 2006

4