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Temporal genetic variability in the Mediterranean common sea urchin *Paracentrotus lividus*

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ABSTRACT: In sedentary benthic invertebrates with pelagic larval stages, the genetic composition of individuals that recruit at a given site is determined by the interaction between factors such as non-random mating, differential reproductive success, dispersal ability, selective pressures, and environmental factors. We studied the temporal variation of genetic structure of several cohorts of the sea urchin *Paracentrotus lividus* (Lamarck) collected over 2 consecutive years (2006, 2007) from a locality in the NW Mediterranean. Sea urchins bigger than ca. 1 cm in diameter were collected and aged using growth bands visible in the tests. Small recruits of the year were collected by scraping off algal substrates. A fragment of the mitochondrial gene of the cytochrome c oxidase subunit I (COI) and a fragment of the nuclear gene of the gamete recognition protein bindin were sequenced for 374 and 316 urchins, respectively. Differentiation between cohorts was smaller than between spatially separated populations for both markers. Likewise, our results do not show a reduction of diversity within cohorts over time or a reduced diversity of the juveniles relative to adult populations. No significant changes in allelic frequencies were detected between cohorts for COI, indicating that sweepstake episodes—in which few individuals dominate reproductive events, resulting in high levels of genetic variance—are not common in this species. In contrast, a stronger signal of differentiation was found for bindin, with many pairwise comparisons among cohorts being significant. This differentiation was mainly due to positively selected codons in this gamete recognition protein, which suggests some degree of non-random mating in *Paracentrotus lividus*. This can be due to spatial and temporal heterogeneity in the pool of gametes resulting in inter-cohort differences in the composition of bindin alleles that maximized fertilization. Our results indicate that processes occurring previous to fertilization are important in shaping the genetic structure of populations. This information is relevant if management plans are to be designed for this commercially interesting species.

KEY WORDS: Temporal genetic structure · Cohorts · Bindin · COI · *Paracentrotus lividus* · Sea urchins

INTRODUCTION

Many marine invertebrates present sedentary adult stages with a planktonic larval phase that ensures dispersal. In such species, the genetic composition of recruits determines the genetic makeup of populations (Watts et al. 1990). Many studies attribute the genetic patchiness frequently detected at small geographic and temporal scales in marine invertebrates to the annual recruitment of genetically variable cohorts within a site (Johnson & Black 1982, Hedgecock 1994, Li & Hedgecock 1998, reviewed in Planes & Lenfant 2002). In this context, the 'sweepstake' hypothesis suggests that only a random subset of adult individuals succeeds at each reproductive event (Hedgecock 1994), potentially causing stochastic variations in the composition of recruits (e.g. Li & Hedgecock 1998, Planes & Lenfant 2002, Pujolar et al. 2006, Hedgecock et al. 2007). Organisms with high fecundity and high larval mortality are prone to show large variance in the

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number of offspring contributed by adults to subsequent generations. Mechanisms generating variance in reproductive success can be linked to biological factors such as adult densities, sperm availability, and gamete traits (Levitan & Ferrell 2006, Levitan 2008), and to environmental conditions such as current patterns, temperatures, or upwellings, modulating survival and gene flow of larvae from different spawning populations (Kordos & Burton 1993, Ruzzante et al. 1996, Banks et al. 2007). These and other factors vary in time, potentially generating an underlying cohort structure that is often neglected when populations are sampled for genetic analyses lumping together different cohorts. As a consequence, an important source of genetic structure is frequently overlooked, and only recently has this temporal structure been taken into account in studies of population genetics.

Several hypotheses have been proposed to explain temporal patterns of genetic variation for many marine invertebrates, suggesting that genetic exchange may be limited over ecological time scales and that populations may be less demographically 'open' than previously thought (e.g. Hellberg et al. 2002). For instance, cohesion of groups of genetically similar planktonic larvae can result in within-cohort spatial genetic patchiness (Avise & Shapiro 1986, Johnson et al. 1993, Li & Hedgecock 1998). Likewise, settlement and recruitment are crucial processes in population dynamics, especially in invertebrates with a planktonic larval phase. These 2 processes are determined among other factors by larval availability, substrate selection, effect of presence of adults, and mortality during metamorphosis and juvenile stages, which are key factors that are difficult to evaluate (Cameron & Schroeter 1980, Harrold et al. 1991, Tomas et al. 2004). If these processes vary in space and time, fine-scale patchy genetic structure may arise even in the absence of restricted dispersal (Johnson et al. 1993, Waples 1998, Banks et al. 2007). This demographic heterogeneity of settlers and recruits can also lead to differentiation among cohorts at a given site, especially in benthic organisms with adult sedentary lifestyles.

Alternatively, temporal variation among recruits may be the consequence of selection acting at different levels, from pre-zygotic stages to larval and post-recruitment periods (Koehn et al. 1980, Johnson & Black 1982, Watts et al. 1990, Vacquier 1998, Zigler et al. 2005). In broadcast spawners with external fertilization, male and female interaction occurs between gametes, with no intervention of adult individuals other than their spawning behaviour. In effect, mate recognition occurs primarily at the level of cell-cell interactions between gametes (Palumbi 1992, Lennarz 1994). In this perspective, mechanisms determining gamete compatibility in broadcast spawners (Lee et al. 1995, Metz & Palumbi 1996, Palumbi 1999) may be a key factor regulating genetic diversity of progeny, thereby influencing temporal genetic structure of populations.

Sea urchins show high dispersal abilities and generally feature large variations in settlement and recruitment from one year to the next (Ebert 1983, Sala et al. 1998, Hereu et al. 2004, Tomas et al. 2004). Differences in genetic composition between years and cohorts have already been detected for several species of sea urchins (Palumbi & Wilson 1990, Edmands et al. 1996, Moberg & Burton 2000, but see Flowers et al. 2002). The common sea urchin *Paracentrotus lividus* has an Atlanto-Mediterranean distribution (Boudouresque & Verlaque 2001), and previous studies suggest that panmixia occurs within both the Atlantic and Mediterranean basins (Duran et al. 2004, Calderón et al. 2008). Thus, it can be assumed that larvae are theoretically able to disperse over long distances and are well homogenized during the 20 to 40 d they remain in the plankton (Pedrotti 1993). However, recent studies are revealing significant excess of homozygotes (ANT gene, Calderón et al. 2008; bindin gene, Calderón et al. 2009a), suggesting that reproduction is not completely random in *P. lividus*.

In the present study we analyze temporal variability of cohorts of *Paracentrotus lividus* in a locality of the NW Mediterranean. This species features 2 reproductive events in the area studied, with a main recruitment episode occurring in spring, and a smaller recruitment event taking place in autumn (López et al. 1998, Tomas et al. 2004). A previous study based on microsatellites (Calderón et al. 2009b) analyzed variability of the cohorts that arrived during the main spawning events in spring of 2004, 2005 and 2006. These authors found only a mild differentiation between cohorts, as well as between cohorts and the adult population (comprising a mixture of individuals of different ages) of the same locality. The aim of the present research was to perform a broader study of temporal variability in Tossa de Mar, including 9 different cohorts and comparing the information gleaned from a mitochondrial marker and a nuclear marker directly implicated in reproduction (gamete recognition). The temporal variability was compared with the spatial variability detected with the same markers in a stretch of coast of ca. 1000 km. Our goal was to obtain a picture of the temporal patterns of differentiation and of the importance of natural selection at pre-zygotic stages upon the establishment of genetic structure in this edible species that plays a major role in structuring benthic Mediterranean ecosystems (Palacin et al. 1998, Boudouresque & Verlaque 2001).

MATERIAL AND METHODS

Sampling and aging methods. In June 2006 and June 2007, samples of *Paracentrotus lividus* were collected by SCUBA at the locality of Tossa de Mar (41° 43.160′ N, 2° 56.241′ E; Fig. 1). Details on the sampling site are described in Calderón et al. (2009b). The undersides of boulders were scrutinized, and sea urchins between 10 and 40 mm in diameter were collected and kept in 96% ethanol at –20°C until processed. Of these, 319 individuals could be unambiguously aged using growth rings in the test, following the method detailed in Calderón et al. (2009b), and were used for genetic analyses. 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 in winter–spring (see Turon et al. 1995) and appears as opaque bands in the test plates, while looser stereom corresponds to periods of slow growth in summer–autumn, visible as more translucent bands once embedded in xylene. The alternance of opaque and translucent bands is interpreted as yearly growth rings. The pattern of translucent/opaque bands was then transformed into age of individuals. The annual formation of the growth bands in this species was noted by Crapp & Willis (1975) and Turon et al. (1995) and was further validated by a tagging experiment using tetracycline, performed in the study locality between 2005 and 2007 (see details in Calderón et al. 2009b).

It is crucial for our study to distinguish the 2 settlement periods, in late spring and late autumn, occurring in Tossa de Mar (López et al. 1998, Tomas et al. 2004). For this purpose, we examined the nucleus or central part of the plates, which is the first zone to be formed. Individuals recruited in late spring experience an initial period of low growth in summer and present a translucent nucleus in the oldest plates, whereas individuals recruited in autumn grow actively during the following winter and show a central opaque nucleus. A whole oral–aboral series of interambulacral plates was examined to discern true growth bands from smaller, supernumerary bands that may occur in some individuals due, for instance, to periods of stress.

Fig. 1. Sampling sites of *Paracentrotus lividus* along the south and east Iberian coast. Populations were separated by a maximum of ca. 1000 km

Additionally, recruits arriving on the same year of sampling (i.e. corresponding to the spring recruitment) that were too small for direct underwater observation with our sampling method were collected by delimiting a 20 × 20 cm square using an aluminum frame and scraping off all organisms present within the frame. Samples were carefully cleaned under the stereomicroscope, and recruits of *Paracentrotus lividus* were separated and kept in absolute ethanol until analyzed.

Our dataset consisted of individuals for whom recruitment season could be assessed with the aging method and of small recruits from the scraped samples arrived on the same season of collection (spring 2006 and spring 2007). Sea urchins arriving at Tossa de Mar within a single recruitment period (spring or autumn of a given year) were considered to be a cohort. The data were organized by age classes for some analyses (from 0 to 4 yr, pooling individuals of a given age in 2006 with those that had the same age in 2007).

Analysis of mitochondrial and nuclear markers. DNA was extracted using REALPURE extraction kit (Durviz) from gonads or, when individuals were too small to have gonads, from Aristotle's lanterns. In the case of recruits, the whole individual was used for DNA extraction with DNeasy Tissue Kit (Qiagen).

Samples were analyzed for one mitochondrial and one nuclear gene. A fragment of COI (cytochrome c oxidase subunit I) was amplified using primers from Arndt et al. (1996) with PCR conditions described in Duran et al. (2004). A fragment of the second coding region of the nuclear protein bindin was also analyzed. This fragment was amplified using specific primers designed for *Paracentrotus lividus* (Calderón et al. 2009a): Bindin2F2 (5′-GCC ACC AAG ATT GAC CTA CCA-3′) and EndCodeR (5′-CCC TTC CCC TA(AT) ACA ATT CA -3′) with the following PCR protocol: 94°C for 3 min, 35 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 1.5 min, and a final elongation step of 8 min at 72°C. Go*Taq*® polymerase (Promega) was used in all PCR reactions. PCR products were verified on a 1.5% agarose gel. PCR amplicons were vacuum-cleaned and labeled using BigDye® Terminator v.3.1 (Applied Biosystems). Sequences were obtained on an ABI 377 automated sequencer (Applied Biosystems, belonging to the Serveis Científico-Técnics of the Uni-

versity of Barcelona). COI fragments were sequenced with the reverse primers and bindin sequences with both primers.

Bindin is a single copy nuclear gene presenting several repeated motifs that play a determinant role in gamete recognition during fertilization (Zigler & Lessios 2003, Zigler 2008). Heterozygotes were identified by the presence of insertion/deletions of repeated motifs or of double peaks in the sequences. The allelic phase of heterozygote individuals was assessed by cloning of a subset of 30 individuals and using the program PHASE v. 2.1 (Stephens et al. 2001, Stephens & Scheet 2005). This Bayesian-based program can provide a more accurate inference of allelic phases than cloning, which can create false alleles by PCR recombination and cloning errors (Harrigan et al. 2008). Sequences from the homozygote individuals and alleles obtained from clones were used as 'known' sequences for the remaining inferences. PHASE was run with option –MS, as no recombination was detected for our sequences using several algorithms implemented in the Recombination Detection Program (Martin et al. 2005). We performed 5 runs of PHASE with default values for iterations, thinning, and burn-in, and compared the haplotype frequency estimates and the goodness-of-fit measures in the output. Differences between runs were minimal, and we selected the one with the highest average value for the goodness-of-fit. PHASE allows controlling for the uncertainty of results by assigning a probability to each haplotype pair suggested for each individual. Only haplotype pairs with probabilities >90% were considered. For the best pair inferred, a probability is also assigned to each ambiguous position; we also examined in the output the number of phased positions in the best haplotype guess for each individual whose certainty was <90%.

Cloning was performed with pGEM-Easy Vector cloning kit (Promega), and 5 clones per individual were sequenced using colonies directly as template for PCR amplification with vector primers M13 F and M13 R. Sequencing of both strands was performed on an ABI 377 automated sequencer (Applied Biosystems). Mutations that appeared only once in the whole data set of cloned sequences were considered to be PCR and cloning artefacts and were replaced so as to match the consensus sequence (Villablanca et al. 1998, Calderón et al. 2009a).

In order to compare temporal with geographic differentiation, we used as a reference the study of Duran et al. (2004), where the genetic structure of several populations of the Atlanto-Mediterranean zone was assessed based on COI. No reference was available for bindin, so adult samples (49 individuals larger than 40 mm in diameter) were collected and sequenced from Tossa de Mar and from 4 additional populations, spanning ca. 1000 km along the Iberian coast (Fig. 1). As before, a subset of 12 heterozygote individuals was cloned, and the program PHASE was used to determine allelic phase.

Sequence and statistical analyses. Sequences were aligned using BioEdit v. 7.5.0.2 (Hall 1999). Arlequin v. 3.1 (Excoffier et al. 2005) was used to calculate haplotype and gene diversity for COI and bindin, respectively, as well as nucleotide diversity. Levels of cohort differentiation were calculated as F_{ST} measures based on allele frequencies (using the estimator described in Weir & Cockerham 1984), and their significance was assessed with 10 000 permutations with Arlequin v. 3.1. The same software was used to perform analysis of molecular variance based on allele frequencies (AMOVA; Excoffier et al. 1992). For bindin, observed and expected heterozygosities were estimated with Arlequin based on the individuals' genotypic composition. Departures from Hardy-Weinberg equilibrium and inbreeding coefficients F_{IS} were computed and tested using Genetix (Belkhir et al. 2004). Previous studies with PAML (Yang 2007) confirmed a scattered action of positive selection along the bindin gene in *Paracentrotus lividus* (Calderón et al. 2009a). Of the 12 codons identified by Calderón et al. (2009a) as being under positive selection, 3 are located within the analyzed region. To assess the role of selection upon the genetic structure of cohorts, measures of differentiation were also computed only for neutrally evolving sites.

In all cases, statistical significance was determined after correcting for multiple comparisons using the false discovery rate method in Benjamini & Yekutieli (2001; hereafter B-Y correction), which is adequate for non-independent tests (Narum 2006). The critical value of this test is determined by:

$$\alpha \Big/ \sum_{i=1}^{k} (1/i) \qquad (1)$$

where k is the number of hypothesis tests performed (Narum 2006).

We compared diversity and F_{ST} values using standard parametric tests (*t*-tests and paired-sample *t*-tests) after checking the normality and homoscedasticity of data. The software SigmaStat 3.1 (Systat Software) was used for these analyses.

RESULTS

Using the information from the band patterns and the nature of the nuclei observed in the test plates, 9 cohorts were identified in our samples (Table 1). Nevertheless, due to the small number of individuals

sampled, the cohort Autumn 2002 was excluded from subsequent analyses. Combining data per age classes (pooling classes of 3.5 and 4 yr, due to the small number of individuals sampled), our results showed a good correlation between size and estimated age (Fig. 2).

Results based on mitochondrial COI

A fragment of 631 bp of COI was sequenced for a total of 374 individuals (187 individuals per sampling year). Of these, 319 corresponded to sea urchins aged with growth rings, and 55 represented recruits sampled on the same year of recruitment (28 collected in 2006 and 27 collected in 2007). A total of 93 variable sites were observed, of which 7 presented non-synonymous substitutions. A total of 133 different haplotypes were identified in our sample, of which 95 (71.4%) were singletons. Table 1 summarizes sample sizes, haplotype numbers, and diversity values. Of the 133 haplotypes detected in our study, 31 had already been identified by Duran et al. (2004). In each of the 2 years studied, 5 haplotypes accounted for almost half of the individuals (average 49% for 2006 and 44% for 2007). These haplotypes were also the most frequently represented in the dataset analyzed by Duran et al. (2004), corresponding to 33% of their samples. All the remaining haplotypes were present in <2.5% of our dataset (<10 urchins).

High haplotype diversity and low nucleotide diversity were observed for each cohort as well as for the whole sample set (Table 1). These measures do not globally differ from those observed when samples from 2006 and from 2007 were analyzed independently, suggesting that, in both cases, we obtained a good representation of the existing variability with the number of individuals collected. When haplotype diversity from our cohorts was compared with that found in 12 adult populations (urchins >40 mm diameter) analyzed by Duran et al. (2004; overall $H = 0.961 \pm 0.009$; mean ± SE), no significant differences were found (*t*-test: $t = 1.431$, df = 18, p = 0.169). No reduction in diversity was observed between recruits and older individuals (Table 2; Age classes 3.5 and 4 were excluded due to small sample size), as would have been expected if large mortalities were acting upon the first year of benthic life.

When data from the two sampling years were available for a given cohort (i.e. cohorts Spring 2003, Autumn 2003, Spring 2004, Autumn 2004, Spring 2005,

Table 1. *Paracentrotus lividus*. Cohorts identified in the samples (sampling years for each cohort in brackets. Number of individuals (n) sequenced for COI (cytochrome c oxidase subunit I) with the number of haplotypes in brackets, haplotype diversity (mean ± SD) and nucleotide diversity for mitochondrial COI. The cohort Spring 2006 includes recruits (28, sampled in 2006) and 1 yr old (22, sampled in 2007) individuals. The cohort Spring 2007 comprises only recruits sampled in 2007

Cohort (sampling year)	n (haplotypes)	Haplotype diversity	Nucleotide diversity
Autumn 2002 (2006)	5 (5)	1 (±0.126)	8.56×10^{-3}
Spring 2003 (2006–2007)	23 (13)	0.889 (±0.051)	5.27×10^{-3}
Autumn 2003 (2006–2007)	31 (21)	0.944 (±0.028)	6.49×10^{-3}
Spring 2004 (2006–2007)	85 (43)	0.946 (±0.016)	6.81×10^{-3}
Autumn 2004 (2006–2007)	42 (24)	0.907 (±0.096)	5.58×10^{-3}
Spring 2005 (2006–2007)	85 (41)	0.929 (±0.020)	6.72×10^{-3}
Autumn 2005 (2007)	26 (19)	0.932 (±0.042)	6.33×10^{-3}
Spring 2006 (2006–2007)	50 (27)	0.925 (±0.026)	5.55×10^{-3}
Spring 2007 (2007)	27 (20)	0.952 (±0.032)	6.52×10^{-3}
Spring total	270 (105)	0.931 (±0.011)	6.45×10^{-3}
Autumn total	104 (51)	0.928 (±0.019)	6.11×10^{-3}
Total	374 (133)	0.930 (±0.0095)	6.36×10^{-3}

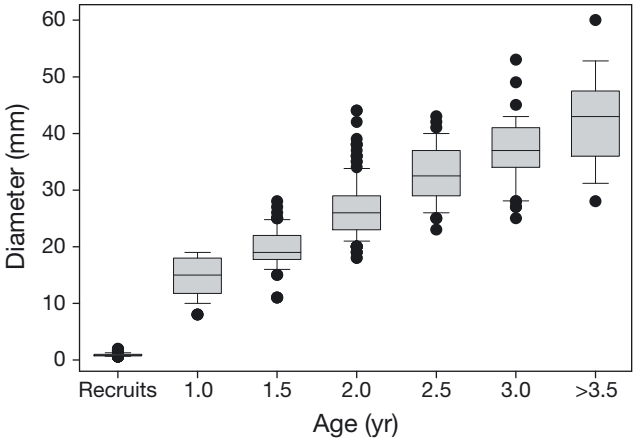

Fig. 2. *Paracentrotus lividus*. Estimated age in relation to diameter from samples collected in 2006 and 2007. Urchins aged 3.5 and 4 yr were pooled due to small sample sizes. Horizontal lines in boxes: medians; lower and upper edges: quartiles; whisker caps: 10th and 90th percentiles; dots: outliers

Table 2. *Paracentrotus lividus*. Haplotype diversity for mitochondrial COI and gene diversity for nuclear binding for each age class (mean ± SD). Age classes 3.5 and 4 yr excluded due to small sample size

Age class (yr)	Haplotype diversity for COI	Gene diversity for binding
Recruits	0.944 ± 0.024	0.929 ± 0.016
1	0.925 ± 0.036	0.909 ± 0.024
1.5	0.913 ± 0.032	0.941 ± 0.012
2	0.926 ± 0.017	0.929 ± 0.009
2.5	0.937 ± 0.026	0.945 ± 0.012
3	0.942 ± 0.023	0.942 ± 0.017

and Spring 2006; Table 1), intra-cohort comparisons were performed by calculating F_{ST} between the 2 sampling years. This comparison permitted us to assess temporal differentiation within cohorts, which could be related to either selection acting between the 2 sampling years or to sampling stochastic errors. Our results showed no significant differentiation in any of the 6 intra-cohort comparisons (data not shown), so further analyses were performed pooling together data from the 2 sampling years for each cohort.

F_{ST} analyses between cohorts for COI showed no significant differentiation based on haplotype frequencies (Table 3). The average F_{ST} was −0.004 (not significantly different from zero), which is much lower than the average differentiation detected in the spatial study conducted by Duran et al. (2004; F_{ST} = 0.010, based on allelic frequencies). In that study, samples were collected from the whole distribution area of the species. For comparative purposes, we also computed population differentiation based on a subset of samples from Duran et al. (2004), spanning a geographical zone comparable to the one covered by populations analyzed for bindin in the present study (zones 1–7 in Duran et al. 2004). Differentiation between our cohorts was still lower than differentiation detected between these populations (F_{ST} = 0.0005, based on allelic frequencies), separated by up to 1000 km.

Finally, AMOVA did not detect a significant structure when comparing cohorts arriving in spring with cohorts arriving in autumn (p = 0.779), with most of the variation located within cohorts. Similarly, no significant structure was detected when comparing cohorts made up of recruits of the year with cohorts of older individuals (p = 0.396), with almost all the variability located within cohorts.

Results based on nuclear bindin

With regard to bindin, we sequenced a total of 316 individuals (Table 4): 167 in 2006 (of which 19 were recruits arrived on that same spring) and 149 in 2007 (17 recruits). A fragment of 359 bp was amplified for these samples, representing a total of 119 amino acids.

Table 3. *Paracentrotus lividus*. F_{ST} comparisons based on allelic frequencies between cohorts with mtCOI (mitochondrial cytochrome oxidase I) (lower diagonal). Upper diagonal represents p values. No significant comparisons were detected

Cohort	Spring 2003	Autumn 2003	Spring 2004	Autumn 2004	Spring 2005	Autumn 2005	Spring 2006	Spring 2007
Spring 2003	–	0.706	0.138	0.679	0.654	0.817	0.591	0.554
Autumn 2003	−0.0089	–	0.256	0.360	0.502	0.786	0.529	0.464
Spring 2004	0.0084	0.0029	–	0.303	0.426	0.570	0.537	0.612
Autumn 2004	−0.0079	0.0009	0.0015	–	0.332	0.879	0.722	0.544
Spring 2005	−0.0042	−0.0021	−0.0010	−0.0042	–	0.829	0.856	0.837
Autumn 2005	−0.0125	−0.0083	−0.0027	−0.0102	−0.0056	–	0.738	0.940
Spring 2006	−0.0064	−0.0023	−0.0003	0.0012	−0.0049	−0.0084	–	0.567
Spring 2007	−0.0048	−0.0017	−0.0032	−0.0034	−0.0029	−0.0126	−0.0082	–

Table 4. *Paracentrotus lividus*. Summary of results for bindin. Cohort codes as in Table 1. Number of individuals (n) with the number of haplotypes in brackets, gene diversity (mean ± SD), nucleotide diversity, observed and expected heterozygosities (H_o/H_e) and inbreeding coefficient F_{IS}. $^{**}p < 0.01$ $^{***}p < 0.001$. The cohort Spring 2006 includes recruits (19, sampled in 2006) and 1 yr old (18, sampled in 2007) individuals. The cohort Spring 2007 comprises only recruits sampled in 2007

Cohort	n (haplotypes)	Gene diversity	Nucleotide diversity	H_o/H_e	F_{IS}
Autumn 2002 (2006)	5 (7)	0.911 (±0.077)	0	0.800/0.911	0.135
Spring 2003 (2006–2007)	21 (20)	0.941 (±0.017)	1.858×10^{-3}	0.762/0.941	0.196
Autumn 2003 (2006–2007)	29 (24)	0.937 (±0.015)	2.178×10^{-3}	0.724/0.937	0.230**
Spring 2004 (2006–2007)	77 (54)	0.931 (±0.012)	2.241×10^{-3}	0.610/0.931	0.347***
Autumn 2004 (2006–2007)	36 (32)	0.948 (±0.012)	0.939×10^{-3}	0.750/0.948	0.215***
Spring 2005 (2006–2007)	69 (55)	0.918 (±0.015)	1.714×10^{-3}	0.710/0.918	0.228***
Autumn 2005 (2007)	25 (27)	0.931 (±0.024)	1.959×10^{-3}	0.800/0.931	0.143
Spring 2006 (2006–2007)	37 (33)	0.923 (±0.020)	2.221×10^{-3}	0.759/0.923	0.182
Spring 2007 (2007)	17 (16)	0.905 (±0.029)	0.713×10^{-3}	0.765/0.905	0.160
Spring total	221 (126)	0.933 (±0.006)	1.890×10^{-3}	0.688/0.933	0.198***
Autumn total	95 (64)	0.943 (±0.086)	1.589×10^{-3}	0.756/0.943	0.259***
Total	316 (165)	0.937 (±0.005)	1.868×10^{-3}	0.707/0.937	0.240***

This fragment coded from amino acid 24 in the conserved core (which corresponds to amino acid 144 in the mature bindin sequence; Calderón et al. 2009a) down to the stop codon. A total of 56 variable positions (excluding presence/absence of indels) were observed, of which 11 were located within the core region and corresponded to synonymous substitutions. The remaining 45 variable positions, located 3′ from the core, represented 24 synonymous substitutions and 21 amino acid changes. In the region located downstream from the conserved core, variations were due to a high extent to the presence of indels. No nucleotide variation was observed in any of the insertions. Interestingly, when samples from 2007 were analyzed, 11 individuals from different cohorts presented an insertion that had not been detected in 2006. This corresponded to the duplication of a poly-glycine fragment QGPGGGGM, which had been previously observed in samples from Cyprus (Calderón et al. 2009a).

A total of 90 urchins (28.5%) were homozygotes. These known sequences, together with the allelic phases of the 30 cloned heterozygotes, were used to infer allelic phases of the remaining 196 individuals. A total of 86 haplotypes were detected in 2006 and 103 in 2007, with a total of 165 haplotypes inferred in the whole data set of 316 individuals (632 gene copies); 4 haplotypes accounted for 46.9% of the sequences, while 107 haplotypes appeared only once. Homozygotic sequences provided a total of 28 haplotypes, while clones represented 23 haplotypes. For the remaining individuals, the allelic phase of 84 urchins was assigned with a probability of 100% by PHASE; 24 individuals were assigned with >99% confidence, and the remaining urchins were assigned with a probability between 90 and 99%. Over the ambiguous positions over the whole data set, only 0.8% were phased below the 90% threshold.

Every cohort presented fewer heterozygotes than would be expected under Hardy-Weinberg (HW) equilibrium, with inbreeding coefficients being significant for several of the cohorts analyzed, as well as overall for the cohorts of spring and autumn (Table 4). The same results were found when considering amino acid sequences instead of nucleotides. As already observed for COI, no reduction in diversity was observed in recruits relative to older cohorts (Table 2).

The study of 49 urchins from 5 adult populations (Fig. 1) detected a total of 31 different haplotypes (Table 5), using the same stringent conditions for PHASE as before. Of these haplotypes, 14 had not previously been observed in the cohorts studied. The overall gene diversity of the cohorts of Tossa de Mar (0.937 ± 0.0051) was higher than that of the adult samples of 5 geographically separated populations (0.877 ± 0.0223), and this difference was significant (*t*-test between cohorts and localities: $t = 2.303$, df = 11, $p = 0.042$).

Intra-cohort differentiation (as measured by F_{ST}) between sampling years within cohorts (i.e. for cohorts for which we had samples from 2006 and from 2007) was not significant (not shown), so these data were pooled by cohort for subsequent analyses. Several cohorts appeared significantly differentiated from others, according to F_{ST} values: 13 out of 18 pairwise comparisons were significant in the permutation test, although only 7 of these comparisons were still significant after B-Y (2001) correction (Table 6). Overall, the cohort from Spring 2007 had the highest F_{ST} values when compared to the other cohorts.

Table 5. *Paracentrotus lividus*. No. of haplotypes and gene diversity (mean ± SD) for bindin of 5 adult populations (including Tossa de Mar) along Iberian coasts

Population	n (haplotypes)	Gene diversity (±SD)	Nucleotide diversity
Cadaqués	12 (14)	0.934 (±0.031)	13.4×10^{-3}
Tossa	13 (14)	0.908 (±0.035)	12.9×10^{-3}
Gata	6 (3)	0.621 (±0.087)	12.7×10^{-3}
Tarifa	7 (5)	0.813 (±0.066)	5.10×10^{-3}
Cádiz	11 (9)	0.870 (±0.044)	11.6×10^{-3}
Total	49 (31)	0.877 (±0.022)	13.7×10^{-3}

Table 6. *Paracentrotus lividus*. F_{ST} for bindin based on allelic frequencies (lower diagonal). Upper diagonal represents p values. *Significant after Benjamini & Yekutieli (2001; B-Y) correction ($\alpha < 0.0127$)

Cohort	Spring 2003	Autumn 2003	Spring 2004	Autumn 2004	Spring 2005	Autumn 2005	Spring 2006	Spring 2007
Spring 2003	–	0.062	0.527	0.508	0.018	0.604	0.202	0.032
Autumn 2003	0.0114	–	0.107	0.313	0.051	0.109	0.013	0.004*
Spring 2004	−0.0013	0.0049	–	0.143	0.002*	0.617	0.384	0.002*
Autumn 2004	−0.0013	0.0015	0.0034	–	0.133	0.105	0.025	0.051
Spring 2005	0.0153	0.0081	0.0116	0.0040	–	0.003*	0.008*	0.025
Autumn 2005	−0.0031	0.0076	−0.0021	0.0071	0.0201	–	0.196	0.0028*
Spring 2006	0.0043	0.0159	0.0001	0.0113	0.0138	0.0038	–	0.001*
Spring 2007	0.0220	0.0317	0.028	0.0136	0.0175	0.0352	0.0383	–

Of 119 codons, 3 were subject to positive selection in the fragment of bindin analyzed (Calderón et al. 2009a), and 6 variable positions were scored in the corresponding 9 base pairs. Surprisingly, these 3 codons represented 24 different haplotypes, suggesting that these positions are responsible for a large degree of the variability detected. In fact, when these positions were removed from the sequences, the number of haplotypes present decreased from 165 to 110. In contrast, when 3 codons including the same total number of variable positions were removed at random from our sequence, the number of haplotypes was not appreciably reduced (data not shown). To confirm the role of selected positions in the variability observed, the 9 bp were excluded and the F_{ST} analysis repeated. Most of the differentiation signal was lost, with only 1 pairwise comparison remaining significant (Spring 2003 with Spring 2005). The mean F_{ST} values dropped from 0.011 ± 0.002 to 0.004 ± 0.001, and the difference between these 2 values was highly significant (paired sample *t*-test: $t = 4.509$, df = 27, $p < 0.001$). No equivalent reduction in differentiation was detected when 3 variable codons were removed at random (data not shown). F_{ST} measures are taken here simply as an estimate of genetic differentiation when the signal given by a particular subset of the sequence was removed. Clearly, sequences are inherited as a whole and no interpretation of these measures in terms of gene flow is intended.

Concerning the spatial study, average F_{ST} detected for populations separated from 80 to >1000 km was low but larger than values detected between cohorts. The average F_{ST} value detected between spatially separated populations was $F_{ST} = 0.107$, which is almost 8 times higher than the average value detected for F_{ST} differentiation between cohorts ($F_{ST} = 0.011$).

Finally, when hierarchical analyses of molecular variance were computed comparing cohorts recruited in spring with cohorts recruited in autumn, no significant differentiation was found ($p = 0.463$), and most of the variability was found within cohorts, with a small but significant percentage associated to differentiation among cohorts within groups (0.99%; $p < 0.001$). Similar results were obtained when cohorts of older individuals were compared against cohorts made up of recruits of the year, with no significant differentiation observed among groups ($p = 0.101$), and most of the variability occurring within cohorts.

DISCUSSION

The study of band patterns confirmed the existence of 2 recruitment periods (spring and autumn) in the study area. The level of differentiation between cohorts of spring and autumn of the same year (compared for years for which we had samples of both cohorts: 2003, 2004, and 2005) was of the same magnitude than the differentiation between cohorts of different years. This fact, together with the lack of overall differentiation between cohorts recruited in spring and autumn found in AMOVA analyses, supports the idea that the 2 recruitment events occurring each year belong to independent reproductive episodes.

Mitochondrial DNA has been frequently analyzed for population studies (Avise et al. 1987, reviewed in Avise 2000). In contrast, to date only Debenham et al. (2000) have performed population studies based on bindin, using a neutrally evolving fragment. The large dataset analyzed in this study (374 individuals for COI and 316 for bindin) allowed us to assess patterns of genetic diversity in Tossa de Mar for several cohorts of *Paracentrotus lividus*. We did not find genetic differences between sea urchins belonging to the same cohort sampled over the 2 consecutive years, indicating that selective forces at these stages were not strong enough to leave a signature in the form of changes in haplotype frequencies. Mortality in *P. lividus*, as well as in other marine invertebrates, mainly affects young stages and much less adult individuals (Turon et al. 1995, Verling et al. 2005). In our case, however, no reduction in diversity was observed between recruits and subsequent age classes. Although we are unable to assess mortality in larval stages, it seems that juvenile mortality, even if demographically important, does not have a major effect on genetic composition of cohorts at least once individuals have reached the size that can be observed with our sampling method (ca. 0.8 mm).

The 2 main signatures of sweepstake events characterizing species with high variance in reproductive success are that (1) cohorts have reduced genetic diversity compared to adult samples representing a mix of several generations and (2) there is genetic differentiation among cohorts due to stochastic effects (Hedgecock 1994). Results obtained in our study showed no reduction of diversity in our cohorts relative to adult populations (data from Duran et al. 2004) based on COI. For bindin, in fact, the opposite pattern was detected: genetic diversity of recruits was significantly higher than that of adults from 5 different populations (including Tossa de Mar). This result, however, should be taken with caution due to low sample sizes in the spatial study.

Concerning temporal genetic differentiation between cohorts of *Paracentrotus lividus*, none of the F_{ST} values observed for COI evidenced a significant differentiation between cohorts, based on haplotype frequencies. This is in agreement with results found for microsatellite markers on a more restricted set of samples (Calderón et al. 2009b). Levels of temporal variation, on the other hand, were much smaller than spatial

differentiation detected for this species (Duran et al. 2004). Taken together, results based on mitochondrial COI indicate that there is no evidence of sweepstake events acting upon reproduction in this species, a result coincident with that found for other species of sea urchins (Flowers et al. 2002).

A different picture was obtained for the nuclear bindin. This marker showed a higher degree of variation among cohorts than COI. With similar number of alleles and genetic diversity, mean F_{ST} values were significantly higher (paired sample *t*-test: $p < 0.001$) for bindin (0.011 ± 0.002) than for COI (–0.004 ± 0.001). This indicates that a marker implied in gamete recognition and subject to positive selection (Calderón et al. 2009a) captures a different signal than largely neutrally evolving markers such as COI and microsatellites (Calderón et al. 2009b). This signal was due, to a high extent, to changes in positively selected positions. Indeed, significantly lower genetic differentiation was observed when selected positions were excluded from the analyses than when the whole data set was used. Although only a fragment of the second exon of bindin was analyzed, we assume that positively selected sites may account for functional properties of this protein.

A deficit of heterozygotes for bindin was found in all cohorts, with significant departures from HW equilibrium in many of them (Table 3). While the role of other factors cannot be ruled out, differential fertilization efficiency within species related to bindin type has been reported in other sea urchins (Palumbi 1999). Therefore, incompatibilities in gamete recognition proteins may explain the observed departures from HW equilibrium.

The discrepancy of the patterns observed with COI and bindin is likely to be the consequence of the role played by gamete recognition proteins in pre-zygotic selection, effectively leading to assortative mating and resulting in a temporal signal of genetic differentiation. Neutral markers, which are required for the estimation of demographic parameters (e.g. effective population sizes, gene flow), may not be suitable to reflect adaptive changes in the short term. Thus, the study of markers under selection directly implicated in reproductive success can provide a more accurate view of ongoing differentiation processes. A similar result was obtained recently for *Paracentrotus gaimardi* (Calderón et al. 2010), for which comparisons among the different color morphs using different markers revealed contrasting patterns: a nuclear intron revealed no significant differentiation, a mitochondrial gene showed divergence only for a color morph, and bindin provided a strong evidence for differentiation among all morphs.

The patterns of evolution of bindin differ greatly among sea urchin species (reviewed in Zigler 2008). Among the processes originating these patterns, the interplay between adult densities, sperm availability, and frequency of bindin genotype seems to play an important role (Levitan & Ferrell 2006, Levitan 2008). Rare alleles would be favored at high sperm densities to avoid polyspermy, whereas common bindin alleles would be selected at low sperm densities (Levitan & Ferrell 2006). Echinoids undergo large changes in population size as well as large temporal and spatial variations in settlement and recruitment (Ebert 1983, Hereu et al. 2004, Uthicke et al. 2009). This also applies to the studied population. Heterogeneity in the gamete patches and variance of sperm density is probably high, and, as a result of these and other stochastic processes, different bindin alleles may be selected at different spawning events, resulting in the observed differences. It would be interesting to expand the temporal frame of our studies, as well as to analyze the whole bindin molecule, including the hotspot region located 5′ of the conserved core. Besides, studies looking at fertilization success related to bindin variability are called for to clarify the effect of this variation on reproductive success.

Populations present at Tossa de Mar at a given time are a mosaic of cohorts arriving at different settlement events, which show a lack of differentiation based on mitochondrial haplotypic frequencies and higher variation based on nuclear bindin, mainly related to positively selected sites. Besides, our study showed no evidence of reduced diversity in newly arrived recruits. In addition, the genetic variability detected in *Paracentrotus lividus* was high, and the high demographic heterogeneity characterizing this species (Hereu et al. 2004, Tomas et al. 2004) did not leave a detectable signature in genetic structure of populations over the time frame studied. Therefore, we conclude that extreme sweepstake events are not common in reproduction of *P. lividus*. Our results also point to the existence of non-random mating in this species, suggesting that pre-zygotic events are determinant in shaping the genetic structure found when using a gene implicated in gamete recognition. *P. lividus* is a commercially interesting species, and over-harvesting has led to population depletion in many areas along its distribution range. If management plans are to be designed for this species, information about its genetic patrimony, as well as precise details about spatial and temporal population structure and reproductive success are essential to ensure the species′ sustainability.

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