Characterization of thirty two microsatellite loci for three Atlanto-Mediterranean echinoderm species.

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Abstract. Thirty two microsatellites were optimized from 454 pyrosequencing libraries for three Atlanto-Mediterranean echinoderms: *Coscinasterias tenuispina*, *Echinaster sepositus* and *Arbacia lixula*. We observed different frequency of microsatellite types (di-, tri-, tetra- and pentanucleotide) throughout the genome of the species, but no significant differences were observed in allele richness among different microsatellite repeats. No loci showed linkage disequilibrium. Heterozygosity deficit and departure from Hardy–Weinberg equilibrium were observed for some loci, in two species, probably due to high levels of inbreeding. Heterozygosity excess observed in *C. tenuispina* could be explained by selection against homozygotes and/or outcrossing.

Keywords. Pyrosequencing, inbreeding, clonality, conservation, starfish, sea urchin.

During last century, Mediterranean Sea has suffered an extensive loss of biodiversity due to high anthropogenic pressures and environmental perturbations (Coll et al. 2010). Introduction of non-native species, increase in water temperature and extensive gaps in the distribution of natural populations due to urbanization, are among the most important environmental pressures (Thibaut et al. 2005, Lejeusne et al. 2009).

In this study we described new microsatellite loci for three of the most common Atlanto-Mediterranean echinoderms with important implications for conservation; the starfishes *Echinaster sepositus* and *Coscinasterias tenuispina*, and the sea urchin *Arbacia lixula*. *E. sepositus* is an emblematic species along the Atlanto-Mediterranean area but some populations at the North-Western Mediterranean have suffered a severe decline (Villamor 2010, and authors' pers. obs.). This species is

now scarce in areas with high anthropogenic pressure and affluence of divers, and larger populations are only observed within marine protected areas. Due to the short-distance dispersal of its lecithotrophic larva, studies about populations' connectivity, inbreeding and genetic structure are crucial to design future management strategies for restoring their populations (Jones et al. 2007).

On the other hand, mitochondrial data suggested a recent colonization of the Mediterranean from the Atlantic Ocean by the thermophilous species *A. lixula* and *C. tenuispina* (Wangensteen et al. 2012 and authors' unpublished data), and whose densities may increase dramatically in the foreseeable future. Global warming might facilitate population blooms and thus turn these species into an ecological problem. Both species can modify sublittoral habitats because of their voracity generating barren grounds when populations reach high densities (Guidetti et al. 2003; Bonaviri et al. 2011). Populations' monitoring, including recruitment and connectivity studies between Atlantic sources and Mediterranean stocks based on microsatellites, is highly recommendable to evaluate the potential threat of these species for Mediterranean ecosystems.

We used 454 pyrosequencing to isolate novel microsatellite loci in *C. tenuispina*, *E. sepositus* and *A. lixula*. Genomic DNA was extracted using QIAamp® DNA Mini Kit (QIAGEN) to a final DNA concentration of 5 ng/μl and distributed in three physically separated lanes of a plate. Pyrosequencing was performed on a Roche Life Science 454 GS-FLX System at the Scientific-Technical Services of University of Barcelona. Sequences were trimmed to remove regions with a greater than 0.5 % chance of error per base using GENEIOUS version 5.5 (Drummond et al. 2010). Total number of sequences which passed quality filtering, number of microsatellites detected, and reads mode length were variable between species, and all details are summarized in Online Resource 1. Sequences were searched for perfect microsatellites (di-, tri-, tetra- and pentanucleotides) with at least eight repeats and enough priming regions with QDD1 v. 1.3 (Meglécz et al. 2010). Primers were designed with the software PRIMER 3 (Rozen and Skaletsky 1999).

Amplification success and polymorphism were tested in two populations per species: Costa Brava (42°29'N, 3°10'E) and Tenerife (28°25'N, 16°19'W) in *C. tenuispina*, Costa Brava (41°46'N, 3°05'E) and Marseille (43°16'N, 49°34'E) for *E. sepositus*, and Costa del Sol (36°34'N, 4°34'W) and Costa Brava (42°24'N, 3°07'E) in *A. lixula*. Total DNA was extracted from feet tube and amplified using the REDExtract-N-Amp Tissue PCR Kit (Sigma Aldrich). Forward primers were labelled with a fluorescent dye as shown in Table 1. PCR amplifications were performed as described in Valero-Jimenez et al. 2012. Allele length was estimated relative to the internal size standard 70-500 ROX (Bioventures) using the software Peak-Scanner (Applied Biosystems).

Dinucleotides were the most frequent microsatellites followed by tri, tetra and pentanucleotides throughout the genome of the species (see Online Resource 2). A total of thirteen, nine and ten polymorphic microsatellite were optimized for *C. tenuispina*, *E. sepositus* and *A. lixula*, respectively, including a selection of different microsatellite types (see Table 1). Linkage disequilibrium, observed and expected heterozygosity, and deviation from Hardy–Weinberg equilibrium were calculated with ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010). Bonferroni corrections of the p-values for multiple tests were run.

No evidence of linkage disequilibrium was detected across all pairwise comparisons. Failed amplifications due to presence of null alleles were not detected for any loci. Nineteen markers showed Hardy–Weinberg disequilibrium after Bonferroni corrections. Heterozygosity deficit observed in two species may be explained by high levels of inbreeding, as demonstrated in other marine invertebrates (Pérez-Portela et al. 2008; Calderón et al. 2009). The heterozygosity excess observed in *C.*

tenuispina may be explained by clonal reproduction, selection against homozygotes and/or outcrossing (Blanquer and Uriz 2010). After confirming normality and homoscedasticity of the dependent variable, we used a two-way ANOVA to test for differences in genetic diversity (measured as allelic richness) of different microsatellite types and species. Genetic diversity values were adjusted to population size with a rarefaction index calculated in CONTRIB V1.2 (Petit et al. 1998). Our results did not show differences in genetic diversity among di, tri, tetra and pentanucleotide repeats ($F=0.233$; $p=0.872$) but diversity was significantly different among species ($F=35.69$; $p<0.0001$) (see Online Resource 3). This result suggests that different microsatellite types are equally valid in terms of genetic diversity to assess population genetics in echinoderm species.

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Specie	Locus (dye), GenBank accession number	F and R primer sequence	Repeat motif	T_a (ºC)	Size range (bp)	Population 1				Population 2			
						N	N_A	H_O/H_E	H-W	N	N_A	H_O/H_E	H-W
C. tenuispina	m.ten1 (6'FAM)	F: TCAAGGCTGTGTAGTACTCT R: TCAATCAAACTGTGTACCTT	(ATT)*12	51 °C	171-174	22	2	0.045/0.045	1.0	16	2	0.812/0.498	0.014
	m.ten6 (NED)	F: CATGAGAGCTTACAGAAAAG R: CTTAGGTGTAATGAAGTGCT	(TAA)*7	51 °C	160-163	21	2	0.952/0.511	0.001*	16	2	0.812/0.498	0.014
	m.ten13 (6'FAM)	F: GACAGAGTGCTTTCTTAATG R: AGTTCTGGAATAAACTACCC	(ATAC)*12	51°C	360-364	19	1	0/0	-	15	2	0/0.331	0.001*
	m.ten14 (HEX)	F: CACTCTGAGCCTATAAGAGA R: GTTAATTTCTCCCTACCT	(TAA)*7	51 °C	137-138	22	2	1.0/0.512	0.001*	11	1	0/0	-
	m.ten19 (HEX)	F: CTGCTGGCTCCAGCTGCTAT R: TCAACCAGGTCGTTGATCTTTGT	(GATT)*8	51 °C	133-150	22	1	0/0	-	12	2	0.583/0.4311	0.487
	m.ten25 (HEX)	F: TAACTGTTGAATCCATCCT R: CCTGTCATGATTATGTTTGT	(GTA)*10	51 °C	295-298	22	1	0/0	-	16	2	1.0/0.217	0.001*
	m.ten24 (HEX)	F: CTCATAAGGGTGCTGTTT R: ATGAATCATACGTGTGTGG	(GT)*11	51 °C	365-367	22	1	0/0	-	16	2	0.437/0.353	0.543
	m.ten27 (6'FAM)	F: CTTCATAAGAGGTTAGTTGG R: TCCAAGTCATGGAATAACTA	(AT)*9	53 °C	293-295	13	1	0/0	-	10	2	0.6/0.442	0.480
	m.ten30 (NED)	F: GGTACCAGTCGTCATAAATA R: AGGTCCACACACTACAGAT	(AGTC)*17	51 °C	397-409	22	3	1.0/0.638	0.001*	16	2	0.812/0.497	0.014
	m.ten31 (6' FAM)	F: GTGAGTGAAGCCAGAAACTT R: ACATTTGGAATGTTTCCATC	(TGTT)*9	51 °C	298-302	18	1	0/0	-	16	2	1.0/0.516	0.001*
	m.ten32 (6'FAM)	F: ATGAGAGTGGATGACTGACA R: CCATAAGCTTAGCACTACAGG	(TAGA)*8	51 °C	245-249	19	2	0.947/0.512	0.002*	14	2	0.571/0.423	0.505
	m.ten33 (HEX)	F: CTGTTGAATCCATCCTTGTT R: GCCCTGTCATGATTATGTTT	(GTA)*10	51 °C	290-296	19	2	0.789/0.490	0.012	16	4	1.0/0.647	0.001*
	m.ten40 (6'FAM)	F: CCAGCTTGTTTCCATCCAAGGC R: TCTGCACCTCGGGCGCATAGA	(AG)*11	51 °C	151-154	19	1	0/0	-	16	4	0.312/0.635	0.001*
E. sepositus	mES 2 (JOE)	F: CGTATTTTATGTGCAGTTG R: ATCATCCCATTAGAGGTTTA	(TTA)*9	51 °C	232-254	25	7	0.520/0.619	0.012	11	8	0.636/0.740	0.272
	mES 4 (6'FAM)	F: GCCAAAGATGCCATAAAT R: CTGTAGGCTAGCTGAGTTT	(CAA)*6	51 °C	115-148	26	9	0.692/0.788	0.087	16	8	0.688/0.823	0.295
	mES 11 (FAM)	F: GTTGTAGTGATTTCCTGATG R: CCGTGTTGAGAATATGTAA	(TTA)*8	51 °C	128-256	21	3	0.143/0.138	1.000	8	3	0.250/0.242	1.000

	mES 23 (6'FAM)	F: ATCATTGTTCTTCAGTTTCC	(TG)*10	51 °C	85-91	19	5	0.611/0.607	0.771	1	2	1.00/1.00	1.000
		R: TTGTTAAATAGTCCCCAACT											
	mES 24 (HEX)	F: AGAGATCATTAACCCATTCA	(TTCA)*12	51 °C	87-195	26	10	0.115/0.838	0.000*	15	7	0.333/0.860	0.000*
		R: ACTAGTATGTATCCGTTGGC											
	mES 25 (HEX)	F: TAATTGATCCCATTCCTGTA	(TAAA)*10	51 °C	154-199	25	11	0.680/0.873	0.118	14	16	1.00/0.955	1.000
		R: TCACTGTATCCAGATTTCCT											
	mES 29 (6'FAM)	F: ACTAGAATGTGGAGTGACAG	(AC)*12	51 °C	203-288	26	13	0.833/0.891	0.465	16	12	0.938/0.885	0.876
		R: GTCGCTTAGGAAACATCT											
	mES 30 (HEX)	F: AAAGGTCTCTTTGAAGGTGTT	(CTG)*8	51 °C	262-290	26	8	0.269/0.767	0.000*	14	6	0.286/0.745	0.001*
		R: TTCAGGTAGTTGAAGAATTGC											
	mES 38 (HEX)	F: CCAGTTGACCCATCATAAAT	(GCA)*9	51 °C	256-317	25	9	0.320/0.796	0.000*	16	7	0.688/0.784	0.656
		R: GTGATTATGTCCAAAGTGC											
A. lixula	ALM 2	F: TGCTAAACGGCAACAATGAA	(AATC)*12	56 °C	283-355	23	12	0,739/0,756	0.5071	18	17	0,889/0,881	0.667
	(6-FAM)	R: TGGTCGCTAATGGAGGTTTC											
	ALM 4	F: TGAGACAACGGGAAAGTCAA	(AATC)*14	56 °C	239-308	23	17	0,435/0,912	0.000*	18	18	0,778/0,910	0.000*
	(6-FAM)	R: CGATGGTCCTAGAGGTGACA											
	ALM 5	F: GTGGAATGGTGATGGAAAGG	(AGAT)*14	57 °C	120-228	23	16	0,696/0,903	0.000*	18	14	0.722/0.866	0.008
	(6-FAM)	R: TCACGCCTGTTGAAATATCC											
	ALM 7	F: CATGGTTCATTTCTGCCTCA	(AATC)*11	56 °C	228-352	23	6	0,826/0,708	0.0835	18	13	0.500/0.866	0.000*
	(HEX)	R: GAATGGTTGACTTATTGGACGTT											
	ALM 8	F: CCATCCATTCATTCACTACTTCA	(AGGT)*11	57 °C	78-173	23	16	0,478/0,881	0.0906	18	14	0.444/0.886	0.000*
	(6-FAM)	R: ACAGATGGGTGGGTGGAG											
	ALM 9	F: TGTACGTACGTTGGCTGACGA	(AACT)*10	58 °C	221-275	23	11	0,261/0,857	0.000*	18	8	0.278/0.816	0.000*
	(HEX)	R: GCTCACATACAGCTCCCATGTT											
	ALM 11	F: CAGCTGAATCCGATGGTGTA	(AAATC)*9	57 °C	350-469	23	9	0,261/0,871	0.000*	18	8	0.222/0.841	0.000*
	(HEX)	R: TCACGTGCGAGATGTTCTTC											
	ALM 14	F: GCCTTATCATTAGGTGCAGGT	(AGT)*16	57 °C	181-259	23	18	0,609/0,911	0.000*	17	18	0,471/0,903	0.016
	(NED)	R: CCGTCTAAGTGGAGAGCTATGG											
	ALM 15	F: GAGGGCTTCATCCAACAATG	(ACT)*15	58 °C	75-125	23	14	0,478/0,797	0.000*	16	12	0,667/0,833	0.005
	(HEX)	R: TAATTGGCCGGCGTATATTG											
	ALM 17	F: GGATCCTACCATGAATTGTTACAT	(AC)*16	51 °C	177-356	23	13	0,799/0,911	0.259	18	11	0,625/0,865	0.007
	(NED)	R: AATCAACCTGCTCCGTGAAT											

Table 1. Characteristics of 32 microsatellite markers for three echinoderm species. T_a annealing temperature, *N* number of individuals, N_A number of alleles, H_o observed heterozygosity, H_E expected heterozygosity and *H-W* p-value of the Hardy-Weinberg equilibrium test (*) significant after Bonferroni corrections.

Class	Order	Species	Number of reads	Plate %	Average read length	% GC	Total SSR Loci with more than 8 repeats	% of reads containing microsatellites
Asteroidea	Forcipulatida	*Coscinasterias tenuispina*	220654	12.5	339.84	34.15	1974	0.89
Asteroidea	Spinulosida	*Echinaster sepositus*	101340	16.67	238.5	41.3	261	0.18
Echinoidea	Arbacioida	*Arbacia lixula*	315499	16.67	273.2	39.4	14041	3.38

Online resource 1. Reads sequenced with 454 from the three species, the different used plate percentages, average of read length (bp), percentage of GC, the number of SSR with 8 repeats or more found in all sequences and percentage of reads containing at least one microsatellite.