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The use of genetic markers in the study of marine natural populations

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ABSTRACT

Crassostrea angulata (Lamarck, 1819), known as the Portuguese oyster, is a species naturally located in the southwest Iberian Peninsula, mainly at river mouths and the heads of estuaries with suitable hard substrates. Genetic data on *C. angulata* are scarce, and Spanish natural populations (the main beds of the species) have not been studied previously. Electrophoresis is a good detector of variability, and isozyme polymorphisms have been widely used in genetic studies. The present paper reports on the electrophoretic examination of seven allozyme loci at 14 sites located between the mouth of the Guadiana River and the Straits of Gibraltar, which found a high level of genetic variability in terms of average number of alleles (min. of 2.71 and max. of 3.71), polymorphism ($P_{95} \geq 71.43$ %) and mean heterozygosities (H = 0.264). All populations showed a deficit of heterozygotes as well as the genes Lap, Mdh-1, Mdh-2, Me-2, Pgi and Xdh across populations. However, Pgm showed heterozygous excess. Null alleles appeared in Lap and Mdh loci, which could be responsible for this disequilibrium, at least in Mdh, because the increase in frequencies of null alleles was also related to the increase in heterozygote deficit. Values of genetic distance between populations have been used to cluster the populations, and the F-statistics showed a mean F_{ST} value across loci of 0.023 and Mdh-1 surveyed the highest value (0.046). At the same time, heterogeneity in allelic frequencies found in all genes indicated genetic differentiation between populations, in spite of the high dispersal of the species, probably due to local selective conditions.

Key words: *Crassostrea angulata,* allozymes, polymorphisms, oysters.

RESUMEN

Uso de marcadores genéticos en el estudio de poblaciones naturales marinas

Crassostrea angulata *(Lamarck, 1819), conocida como ostra portuguesa, es una especie que se encuentra localizada en el suroeste de la península Ibérica, principalmente en la desembocadura de los ríos y en la cabecera de estuarios con sustrato duro disponible. Los datos sobre la genética de* C. angulata *son escasos y las poblaciones naturales de España (la principal fuente de esta especie) no habían sido estudiadas. La electroforesis es un buen detector de la variabilidad, y los polimorfismos isoenzimáticos han sido ampliamente usados en estudios genéticos. Este trabajo está basado en el análisis electroforético de siete* loci *y catorce puntos de muestreo entre la desembocadura del río Guadiana y el estrecho de Gibraltar y se ha encontrado un alto nivel de variabilidad genética en términos de número medio de alelos (mínimo de 2,71 y máximo de 3,71), polimorfismos ($P_{95} \geq 71{,}43$ %) y heterocigosis media (H = 0,264). Todas las poblaciones mostraron un déficit de heterocigotos en los genes Lap, Mdh-1, Mdh-2, Me-2, Pgi y Xdh. En cambio, el sistema Pgm mostró un exceso de heterocigotos. Aparecieron alelos nulos en los* loci *Lap y Mdh que podrían ser los responsables del desequilibrio, al menos, en Mdh debido a que el incremento en la frecuencia de los alelos nulos estaba relacionado con un incremento en el déficit de heterocigotos. Las poblaciones se han agrupado según los valores de distancia genética obtenidos y también se han calculado los estadísticos F, obteniéndose un valor medio de FST en los* loci *de 0,023; Mdh mostró el valor mayor (0,046). Simultáneamente, la heterogeneidad en las frecuencias alélicas encontradas en todos los genes indicaban diferenciación genética entre las poblaciones, a pesar de la gran dispersión de la especie, probablemente debido a condiciones selectivas locales.*

Palabras clave: Crassostrea angulata, *aloenzimas, polimorfismo, ostras.*

INTRODUCTION

Crassostrea angulata (Lamarck, 1819), known as the Portuguese oyster is a species naturally located in the southwest Iberian Peninsula, mainly at river mouths and the heads of estuaries with suitable hard substrates (Marteil, 1957). Adult oysters are sessile, with a tendency toward protandrous hermaphroditism: first-time spawners are predominantly males, while older age-classes often change to females. Simultaneous production of eggs and sperm by an individual is rare, and hence self-fertilisation is minimal (Galtsoff, 1964). Spawning takes place several times during the summer months, with both sexes synchronously releasing gametes into the water column. Sperm and eggs remain viable up to 5 and 24 hours, respectively. Fertilisation is external, and zygote reach a free-swimming planktonic larval stage within 48 hours; then the veliger larvae may swim and drift for 2-3 weeks, and providing a potential for high dispersal (Korringa, 1952).

Published genetic data on *C. angulata* are scarce and incomplete, and Spanish populations (the main natural beds of the species) have not been studied previously. These populations were overexhausted in the 1940s, and nowadays they not only have an obvious taxonomic and ecological interest, but also an economic one, because they are the sources of progenitors taken to the hatcheries.

Geographic genetic variability is abundant in both morphology and gene frequency in most species, and its extent results from a balance of forces tending to produce local genetic differentiation and forces tending to produce genetic homogeneity. The main producers of variation are migration, selection depending on environmental heterogeneity, and genetic drift, although their effects on genetic markers are difficult to measure in marine organisms. Gene flow can inhibit genetic evolution by preventing natural selection, and genetic drift from establishing and maintaining local genetic differences. In this sense, if the geographic distribution of a species remains the same and if local populations persist for long time, then gene flow occurs primarily through the movement of gametes, individuals or groups of individuals from one place to another, which may prevent them from evolving into different species, with the amount of gene flow dependent on the breeding biology of the species.

The population genetics of marine molluscs present several characteristics: significant and persistent departures of genotypic frequencies from the Hardy-Weinberg expectations (Zouros, Singh and Miles, 1980; Zouros and Foltz, 1984); relationships between genetic structures and environmental changes appearing sometimes as gene frequency geographic clines, even maintained by a balance between gene flow and differential survival among genotypes (Koehn, Newell and Immermann, 1980; Rose, 1984; Nevo, Lavie and Noy, 1987); the relationship between heterozygosities and/or allozyme genotypes and quantitative traits, e.g. viability and growth rate (Koehn and Shumway, 1982; Koehn and Gaffeney, 1984; Foltz and Chatry, 1986; Zouros, Romero-Dorey and Mallet, 1988).

Most of the above data come from enzyme polymorphism studies, because electrophoresis technology is ideally suited to population studies and hence isozyme data constitute the largest existing data set for many organisms (Koehn, Milkman and Mitton, 1976; Buroker, Hershberger and Chew, 1979; Hedgecock and Okazaki, 1984; Sanjuan, Pérez-Losada and Guerra, 1996).

The aim of the present study was to examine the genetic variability of the Portuguese oyster *C. angulata*, using isozyme electrophoresis as genetic markers, in order to gain insight into the genetics and population structure of the species.

MATERIALS AND METHODS

Samples

Samples of *C. angulata* were taken from Spanish natural populations collected during 1994 to 1997 from nine estuarine and five nearshore marine sites along the southwestern Spanish coast, from the mouth of the Guadiana River to the Straits of Gibraltar, over a distance of more than 300 km (figure 1). The estuarine sites were Punta del Moral (Ayamonte) (PM); Punta Umbría (PU); Mazagón (MZ); Torre de la Higuera (Matalascañas) (TH); Sanlúcar de Barrameda (SBD), located at the mouth of the Guadalquivir River; and the nearby Punta de Montijo (SBPM), Chipiona Faro (CHF) and Chipiona Corral (CCP). Barbate (BR), at the mouth of the Barbate River, was also an estuarine population, but far away from the others. The rest of them were marine locations: La Caleta (CLC), Santa María del Mar (CSMM), San Fernando (SFT), Sancti Petri (CSP) and Vejer (VP).

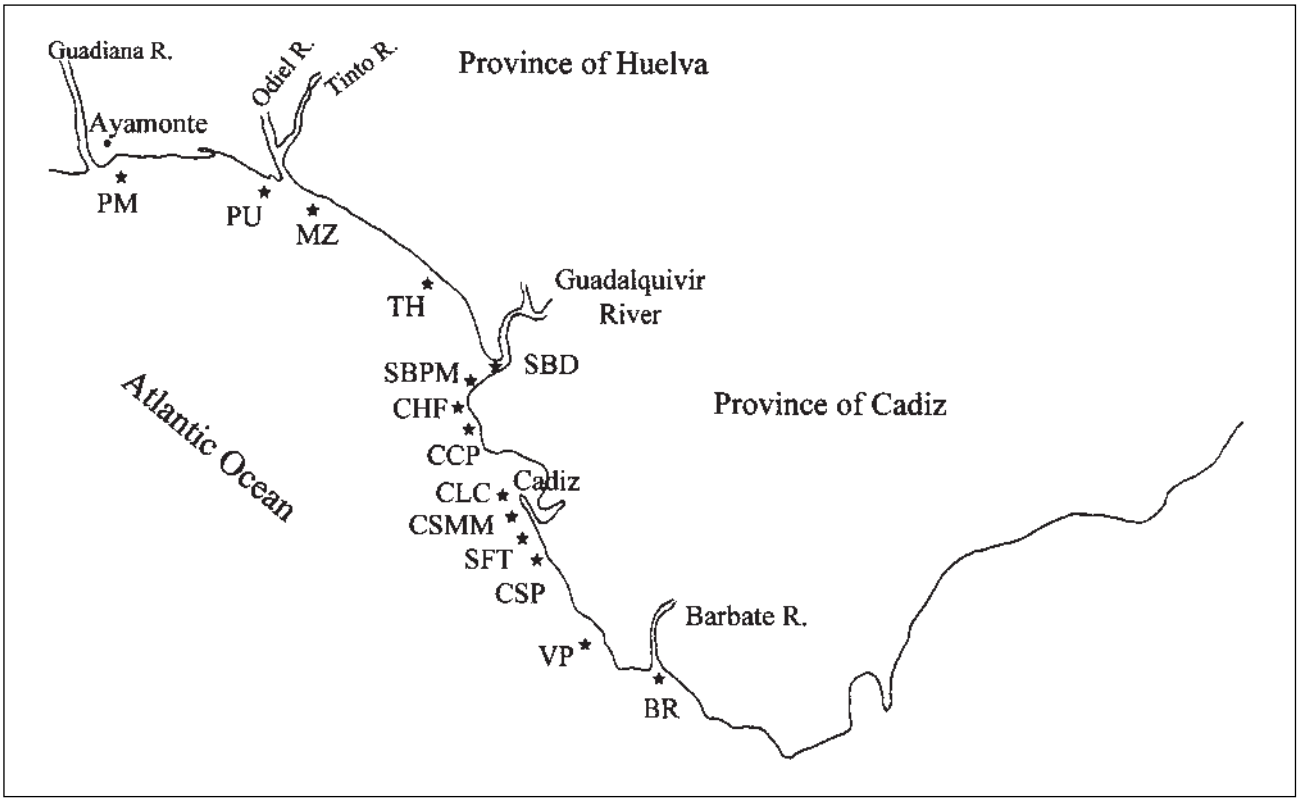

Figure 1. Map of localities where *C. angulata* was sampled. (PM): Punta del Moral; (PU): Punta Umbría; (MZ): Mazagón; (TH): Torre de la Higuera; (SBD): Sanlúcar de Barrameda; (SBPM): Punta de Montijo; (CHF): Chipiona Faro; (CCP): Chipiona Corral; (CLC): La Caleta; (CSMM): Santa María del Mar; (SFT): San Fernando; (CSP): Sancti Petri; (BR): Barbate; (VP): Vejer

Electrophoretic methods

Specimens were brought to the laboratory and, after being cleaned of fouling organisms, kept alive in a tank containing chlorated water and allowed to purge themselves for 24 hours. Starch gel electrophoresis was conducted using methods outlined by Selander *et al.* (1971). Enzyme extraction from digestive glands was made with potassium phosphate 0.2 M, pH 7.0 containing 0.01 % -mercaptoethanol.

Six enzymatic systems controlled by seven loci were studied: a) two locus-controlled Malic enzymes (E.C. 1.1.1.40), Me-1 and Me-2 (although only the Em-2 locus has been included in this study); b) Malate dehydrogenase (1.1.1.37) Mdh-1 and Mdh-2 respectively instead one gene encoded; c) Phosphoglucomutase (5.4.2.2); d) Pgm, Phosphoglucoisomerase (E.C. 5.3.1.9.); e) Pgi, Leucine aminopeptidase (E.C. 3.4.11.1); and f) Lap and Xanthine dehydrogenase (E.C. 1.1.1.204) Xdh.

Data analysis

Genetic variability was determined by three estimators: i) the unbiased estimate of heterozygosity for each locus (h) and the average heterozygosity over loci (H); ii) the percentage of polymorphic loci at a 95 % level (P_{95}), at 99 % (P_{99}) and under non-criterion (P_{nc}: every allele was considered without restriction of allelic frequencies); and iii) the average number of alleles per locus (n_a).

Departures from expected Hardy-Weinberg genotypic proportions were tested using χ^2 tests for goodness of fit, applying the Yates correction for continuity, and both with and without pooling of rare alleles, so that the expected number of genotypes in a given class was increased. The degree of heterozygote deficiency below Hardy-Weinberg expectations was recorded as the D Index through the relation D = = (Het. Observed – Het. expected)/Het. expected (Selander, 1970). Values of F-statistics involved FIT that indicated differentiation within the total population, FIS within populations, and FST among popula-

tions, and were calculated for each locus and over all loci, and statistical significance was tested by a contingency χ^2. Values of $p < 0.05$ indicated significant differentiation between areas that could not be explained by sampling error alone. To study the amount of gene flow between populations, the genetically effective number of migrants between populations, Nm, was estimated using Wright's (1943) island model of population structure. Under this model, genetic divergence is related to gene flow by the formula $Nm = (1/F_{ST} - 1)/4$, using the average FST across loci. The model assumes that migrants are randomly distributed among subpopulations, ignores any effects of natural selection, and considers that equilibrium has been reached between gene flow and genetic drift; therefore it provides only a rough estimate, but it helps to put the values of F_{ST} into a populational perspective.

Calculations of gene frequencies, conformance to Hardy-Weinberg equilibrium, F_{ST} determinations and unbiased genetic distance measures were performed with the BIOSYS program by Swofford and Selander (1981).

RESULTS

The seven studied genes were polymorphic, with allele frequencies shown in table I. Polymorphism

Table I. Allelic frequencies of 7 loci in 14 natural populations of *C. angulata*. (N): sample size

Locus	Allele	PM	PU	TH	MZ	SBD	SBPM	CHF	CCP	CLC	CSMM	SFT	CSP	VP	BR
Lap	(N)	(56)	(60)	(39)	(45)	(70)	(52)	(99)	(94)	(71)	(58)	(55)	(61)	(10)	(50)
	73	0.000	0.008	0.026	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000
	91	0.098	0.158	0.167	0.122	0.143	0.115	0.136	0.112	0.141	0.155	0.200	0.115	0.100	0.130
	100	0.866	0.758	0.731	0.733	0.779	0.808	0.697	0.766	0.775	0.784	0.755	0.738	0.650	0.750
	122	0.036	0.075	0.077	0.133	0.064	0.077	0.086	0.059	0.070	0.043	0.018	0.082	0.250	0.120
	00	0.000	0.000	0.000	0.000	0.014	0.000	0.081	0.064	0.014	0.017	0.018	0.066	0.000	0.000
Mdh-1	(N)	(72)	(72)	(54)	(67)	(60)	(52)	(99)	(99)	(71)	(58)	(55)	(61)	(53)	(50)
	85	0.000	0.007	0.009	0.000	0.000	0.000	0.020	0.020	0.021	0.009	0.018	0.041	0.019	0.010
	100	1.000	0.993	0.954	0.970	0.983	0.981	0.949	0.929	0.979	0.836	0.982	0.959	0.959	0.990
	00	0.000	0.000	0.037	0.030	0.017	0.019	0.030	0.051	0.000	0.155	0.000	0.000	0.000	0.000
Mdh-2	(N)	(72)	(72)	(54)	(67)	(70)	(52)	(99)	(99)	(71)	(58)	(55)	(61)	(53)	(50)
	100	1.000	0.984	1.000	0.978	1.000	1.000	0.995	0.980	1.000	1.000	1.000	1.000	1.000	1.000
	118	0.000	0.014	0.000	0.022	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Me-2	(N)	(70)	(72)	(58)	(57)	(55)	(52)	(95)	(81)	(71)	(58)	(55)	(61)	(53)	(50)
	90	0.150	0.229	0.155	0.096	0.309	0.202	0.200	0.148	0.225	0.121	0.255	0.230	0.189	0.060
	100	0.650	0.514	0.552	0.658	0.482	0.654	0.589	0.648	0.556	0.707	0.545	0.541	0.613	0.720
	110	0.200	0.257	0.293	0.246	0.209	0.144	0.211	0.204	0.218	0.172	0.200	0.230	0.198	0.220
Pgi	(N)	(71)	(68)	(53)	(58)	(70)	(52)	(77)	(80)	(71)	(58)	(55)	(61)	(53)	(50)
	54	0.007	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	77	0.085	0.096	0.094	0.060	0.114	0.067	0.084	0.063	0.099	0.086	0.073	0.074	0.075	0.050
	100	0.887	0.882	0.858	0.897	0.886	0.933	0.903	0.938	0.901	0.914	0.927	0.926	0.925	0.950
	127	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	157	0.021	0.022	0.028	0.043	0.000	0.000	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	167	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pgm	(N)	(62)	(71)	(50)	(54)	(60)	(52)	(98)	(99)	(71)	(58)	(55)	(61)	(53)	(50)
	80	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
	90	0.008	0.049	0.030	0.028	0.025	0.000	0.000	0.020	0.056	0.026	0.009	0.000	0.038	0.020
	94	0.032	0.056	0.040	0.083	0.117	0.038	0.061	0.040	0.028	0.026	0.073	0.074	0.000	0.040
	96	0.097	0.134	0.120	0.130	0.058	0.038	0.046	0.086	0.014	0.043	0.055	0.090	0.094	0.010
	100	0.524	0.465	0.340	0.343	0.258	0.375	0.332	0.338	0.324	0.388	0.345	0.303	0.292	0.340
	114	0.226	0.176	0.290	0.324	0.275	0.221	0.250	0.202	0.218	0.233	0.145	0.246	0.179	0.220
	132	0.097	0.106	0.150	0.093	0.267	0.308	0.286	0.308	0.317	0.284	0.345	0.287	0.396	0.360
	138	0.016	0.014	0.030	0.000	0.000	0.010	0.026	0.005	0.042	0.000	0.027	0.000	0.000	0.000
Xdh	(N)	(64)	(67)	(53)	(50)	(70)	(52)	(94)	(94)	(71)	(58)	(55)	(45)	(53)	(50)
	99	0.133	0.299	0.189	0.110	0.221	0.183	0.170	0.170	0.331	0.216	0.282	0.389	0.189	0.470
	100	0.484	0.522	0.557	0.500	0.543	0.625	0.638	0.638	0.444	0.474	0.500	0.522	0.623	0.450
	101	0.383	0.179	0.255	0.390	0.236	0.192	0.191	0.191	0.225	0.310	0.218	0.089	0.189	0.080

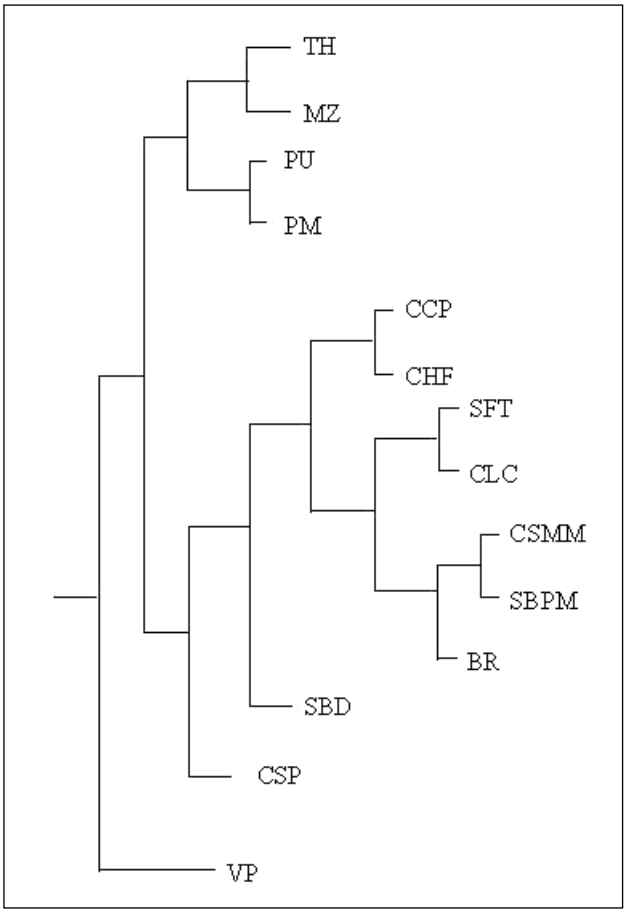

Figure 2. UPGMA dendogram showing the relationships between populations of *C. angulata* based on genetic distances

ranged between 71.43 % and 85.71 % at P95 level; however, it increased to 85.71 % in nine populations, and reached 100 % in another two at P_{99}. The average heterozygosity in populations ranged from a maximum value of 0.334 ± 0.114 at CLC, with a mean allele number of 3.14 ± 0.74, to a minimal value of 0.181 ± ± 0.068 at MZ, with an average allele number of 3.29 ± 0.52. VP and CSP showed the lowest allele number of 2.71 ± 0.47 and 2.86 ± 0.51, with an H of 0.268 ± 0.268 and 0.290 ± 0.096, respectively (table II).

Pgi, Pgm, and Mdh-2 genes appeared in equilibrium in most populations. However, Mdh-1, Em-2, Xdh and Lap reported significant χ^2 values of fit to Hardy-Weinberg conditions (table III). When pooling of alleles was carried out, non-significant values of χ^2 were obtained for most populations on the Lap gene, indicating a conformance to Hardy-Weinberg proportion expectations. However, Me-2 at SBD and Mdh-1 at both CHF and CLC increased their χ^2 values, and none changed for the Xdh gene.

Table II. Estimators of genetic variability in natural populations of *C. angulata*. P95 and P99 mean the percentage of polymorphic loci at 95 and 99 %, respectively. H is the average heterozygosity and na the average number of alleles per locus. (SE): standard error

Population	P_{95}	P_{99}	H ± SE	na ± SE
PM	71.43	71.43	0.209 ± 0.070	3.14 ± 0.77
PU	71.43	85.71	0.183 ± 0.060	3.43 ± 0.65
TH	71.43	85.71	0.292 ± 0.090	3.71 ±0.71
MZ	71.43	100	0.181 ± 0.068	3.29 ± 0.52
SBD	71.43	85.71	0.300 ± 0.113	3.00 ± 0.62
SBPM	71.43	85.71	0.266 ± 0.124	3.00 ± 0.72
CHF	85.71	85.71	0.279 ± 0.106	3.43 ± 0.48
CCP	85.71	100	0.248 ± 0.101	3.43 ± 0.65
CLC	71.43	85.71	0.334 ± 0.114	3.14 ± 0.74
CSMM	85.71	85.71	0.264 ± 0.104	3.14 ± 0.59
SFT	71.43	85.71	0.322 ± 0.129	3.29 ± 0.78
CSP	71.43	85.71	0.290 ± 0.096	2.86 ± 0.51
VP	71.43	85.71	0.268 ± 0.268	2.71 ± 0.47
BR	71.43	85.71	0.269 ± 0.121	3.00 ± 0.72

According to the results of D index, genes can be included in two different categories: i) negative values, indicating high heterozygote deficiencies for Xdh (–0.520), Mdh-1 (–0.453), Me-2 (–0.421) and Lap (–0.296); ii) close to zero or positive values on Pgi (–0.016), Pgm (–0.068) and Mdh-2 (–0.190). Mean D across populations reported negative values for all of them. Coincident results were shown by F-statistics, so that FIT showed positive values at most loci, indicating a general deficiency of heterozygotes. Pgm, however, produced a negative value (–0.052) and Pgi (0.038) was close to zero. FIS contributed most to the FIT in all values, indicating deviations from Hardy-Weinberg proportions within populations (table IV). The greatest differentiation in allelic frequencies were shown in Mdh-1 (F_{ST} = 0.046) and Xdh (F_{ST} = 0.036). Values of χ^2 of contingency indicated that Lap, Mdh-1, Mdh-2, Me-2, Pgm and Xdh were statistically significant at a level of 0.001, and Pgi was homogenous. The average Nm was 15.36, ranging from 41.41 in Pgi to 5.18 in Mdh-1. Population divergence was also estimated in terms of genetic distances, finding a maximum unbiased Nei's genetic distance (1978) of 0.034 between BR and PM, and a minimum value of 0.000 between CHF, SBPM and CCP (table V).

DISCUSSION

Isozymes are functionally similar but separable forms of enzymes. Isozyme electrophoresis has

Table III. Chi-square values of fit to genotype Hardy-Weinberg conditions in 14 populations of *C. angulata*. (*): $p < 0.05$; (**): $p < 0.01$; (***): $p < 0.001$; (ns): not significant; (mn): monomorphic; (D): Het. obs. – Het. exp./Het. exp

Pop.	Lap	Mdh-1	Mdh-2	Me-2	Pgi	Pgm	Xdh	D ± SE
PM	ns	mn	mn	***	ns	ns	***	
	0.046			–0.639	0.030	–0.162	–0.558	–0.256 ± 0.144
PU	ns	ns	ns	***	***	*	***	
	–0.366	0.007	0.014	–0.820	–0.236	–0.333	–0.606	–0.334 ± 0.115
TH	ns	***	mn	***	ns	ns	***	
	–0.109	–0.792		–0.293	–0.180	–0.254	–0.727	0.392 ± 0.119
MZ	***	***	ns	***	ns	ns	***	
	–0.586	–1.000	0.023	–0.612	–0.277	–0.254	–0.727	0.490 ± 0.129
SBD	***	***	mn	*	ns	*	***	
	–0.419	–1.000		–0.306	0.129	0.127	–0.405	0.392 ± 0.171
SBPM	ns	***	ns	***	ns	ns	***	
	–0.122	–0.584	0.005	–0.435	0.072	0.349	–0.643	0.194 ± 0.139
CHF	***	***	ns	***	ns	ns	***	
	–0.413	–0.584	0.005	–0.555	–0.052	0.145	–0.334	0.255 ± 0.109
CCP	***	***	***	***	ns	ns	***	
	–0.351	–0.697	–1.000	–0.618	0.067	0.078	–0.394	0.416 ± 0.149
CLC	***	ns	mn	ns	ns	ns	**	
	–0.211	0.022		–0.144	0.109	0.178	–0.343	0.065 ± 0.082
CSMM	**	***	mn	**	ns	ns	***	
	–0.182	–0.938		–0.395	0.094	0.164	–0.591	0.308 ± 0.171
SFT	***	ns	mn	*	ns	ns	***	
	–0.347	0.019		–0.300	0.078	0.368	–0.358	0.090 ± 0.119
CSP	***	ns	mn	**	ns	ns	***	
	–0.316	–0.374		–0.374	0.080	–0.019	–0.257	0.210 ± 0.079
VP	ns	ns	mn	**	ns	**	***	
	–0.604	0.019		–0.244	–0.189	0.267	–0.616	0.227 ± 0.141
BR	**	ns	mn	ns	ns	ns	***	
	–0.163	0.010		–0.162	0.053	0.306	–0.754	0.118 ± 0.145
D	–0.296	–0.453	–0.190	–0.421	–0.016	0.068	–0.522	
±SE	±0.049	±0.117	±0.202	±0.053	±0.038	±0.063	±0.045	

Table IV. F-statistics (Nei, 1977) indicate deviations from Hardy-Weinberg proportions within the total population (FIT), within populations (FIS) and among populations (FST). χ^2_C: Chi-square value of contingency tests of allelic frequences. (Nm): gene flow value

	Lap	Mdh-1	Mdh-2	Me-2	Pgi	Pgm	Xdh
FIT	0.328	0.683	0.326	0.433	0.038	–0.052	0.518
FIS	0.316	0.668	0.316	0.422	0.032	–0.079	0.500
FST	0.017	0.046	0.015	0.018	0.006	0.025	0.036
χ^2_C	115.21***	127.56***	58.92***	57.65***	69.14	227.26***	132.16***
Nm	14.45	5.18	16.41	13.63	41.41	9.75	6.69

been widely used over the past 20 years for the determination of genetic variability in populations, because this technique is ideally suited to population studies: it is relatively inexpensive and requires little in the way of specialised equipment; it is a fairly rapid procedure to perform on a large scale; and a large number of loci can be screened simultaneously. Protein electrophoresis has proved to be useful for defining genetic markers in aquatic organisms. In spite of some limitations of the technique (some changes in a gene can be underdetected at the protein level, and genes that produce proteins

Table V. Unbiased genetic distances (below diagonal) and genetic identities (above diagonal) (Nei, 1978) based on 7 loci for 14 populations of *C. angulata*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. PM	–	0.991	0.996	0.994	0.982	0.989	0.985	0.984	0.984	0.990	0.983	0.974	0.974	0.967
2. PU	0.009	–	0.986	0.998	0.992	0.991	0.988	0.990	0.993	0.984	0.994	0.994	0.984	0.981
3. MZ	0.004	0.014	–	0.999	0.985	0.988	0.988	0.988	0.982	0.990	0.980	0.976	0.983	0.969
4. TH	0.006	0.002	0.001	–	0.997	0.996	0.996	0.998	0.994	0.992	0.993	0.992	0.991	0.980
5. SBD	0.018	0.008	0.015	0.003	–	0.996	0.993	0.997	0.998	0.986	0.999	0.996	0.992	0.978
6. SBPM	0.011	0.009	0.012	0.004	0.004	–	1.000	1.000	0.996	0.995	0.998	0.994	0.999	0.987
7. CCP	0.015	0.012	0.013	0.004	0.007	0.000	–	1.000	0.992	0.995	0.995	0.993	0.998	0.985
8. CHF	0.016	0.010	0.012	0.002	0.003	0.000	0.000	–	0.994	0.991	0.996	0.995	0.999	0.983
9. CLC	0.016	0.007	0.018	0.006	0.002	0.004	0.008	0.006	–	0.992	1.000	0.999	0.993	0.993
10. CSMM	0.010	0.017	0.010	0.008	0.014	0.005	0.005	0.009	0.008	–	0.991	0.984	0.986	0.985
11. SFT	0.017	0.006	0.020	0.007	0.001	0.002	0.005	0.004	0.000	0.009	–	0.998	0.994	0.989
12. CSP	0.027	0.006	0.025	0.008	0.004	0.006	0.007	0.005	0.001	0.016	0.002	–	0.993	0.995
13. VP	0.026	0.016	0.018	0.009	0.008	0.001	0.002	0.001	0.007	0.014	0.006	0.007	–	0.987
14. BR	0.034	0.020	0.032	0.020	0.022	0.013	0.015	0.017	0.007	0.015	0.011	0.005	0.013	–

detectable with a histochemical stain are only a small percentage of total genomes), it is generally a better estimator of genetic variability than the others available (Carvalho and Pitcher, 1994).

After applying allozyme electrophoresis, the two features that stand out from our data analysis are the high level of genetic variability and the slight genetic differentiation among local Spanish natural populations of *C. angulata*. Studied enzymes were randomly chosen and non due to their variabilility; in fact two of the systems controlled by three loci (Mdh-1, Mdh-2, and Me-2) had been described as monomorphic in *C. virginica* (Foltz, 1986b). Moreover, Got-1 (glutamate oxaloacetate transaminase), Cat (catalase) and Sod (superoxide dismutase) also appeared to be variable (data not shown).

A high number of alleles in low frequencies has been described in bivalves (Sarver, Katoh and Foltz, 1992), and should be considered in relation to the mean allele number (na), because the restricted distribution of alleles occurring at frequencies of less than 0.03-0.04 can be attributed to sampling. Mean heterozygosities (0.181-0.334) are greater than those described for *C. virginica* (0.183-0.254) by Buroker (1983) and *C. gigas* (0.214-0.238) by Buroker, Hershberger and Chew (1979), although these authors analysed different numbers of populations and genes as well as monomorphic loci; similar levels of genetic variability have been reported in natural populations of *C. virginica* (Foltz and Chatry, 1986), studying a similar number of variable loci.

D index showed a deficiency of heterozygotes in all populations, with the extent of homozygote excess varying greatly among loci, as has been repeatedly observed in marine molluscs (Holborn, Johnson and Black, 1994; Singh and Green, 1986) and some species of fishes (Johnson, Hebbert and Morán, 1993). One of the most widely accepted theories for explaining this phenomenon is the presence of null alleles in populations, because active/null heterozygotes can be scored as active homozygotes. Null alleles have been reported in leucine aminopeptidase and mannosephosphate isomerase in *C. virginica* (Foltz, 1986a) and leucine aminopeptidase in *Mytilus edulis* (Mallet *et al.*, 1985); an inactive variant was also reported for an alkaline phosphatase system in *Macoma balthica* (Singh and Green, 1986). Experimental evidence supporting null alleles as a possible cause of heterozygote deficiency in *C. virginica* has been reported by Foltz (1986a). We found evidences of null alleles in two of the studied loci, which showed high negative D values for Lap (D = –0.296) with null allele frequencies (Lap^{00}) ranging between 0.014 at both CLC and SBD, and 0.081 at CHF, and Mdh-1 (D = –0.403) with frequencies ($Mdh\text{-}1^{00}$) ranging between 0.017 at SBD and 0.155 at CSMM.

The Lap gene showed a negative D-index value in all populations, except at PM (D = 0.046), which presented both null alleles missing and χ^2 of fit to Hardy-Weinberg conditions not significant. PU, SBPM, TH and VP populations exhibited a negative D value, but their genotypic proportions were statistically not significant.

Populations CLC, CSMM, CSP and BR had a statistically significant χ^2, which became not signifi-

cant when pooling of alleles was carried out. However, CHF and CLC, where null alleles appeared at frequencies of 0.129, yielded an χ^2 of fit significant at a level of 5 %, even after pooling.

Related to the Mdh-1 locus, a relationship appeared between departures from Hardy-Weinberg conditions and the presence of null alleles, so that populations without null alleles were in Hardy-Weinberg equilibrium (PU, CLC, SFT, CSP, VP, BR), whereas populations showing null alleles gave an χ^2 of fit significant at a level of 0.001, and negative D values. In the MZ population only a reduced number of individuals was studied compared with the remaining populations, hence a large sample should be analysed before reaching definite conclusions. CCP and CSMM, with the highest null allele frequencies (0.051 and 0.155, respectively), also showed high negative D values; however, the disequilibrium proportions could also be due to the presence of alleles at low frequencies. In this sense, Siegismund (1985) reported that frequencies greater than 0.05 for null alleles could have significant impact on the genetic structure of populations, including apparent deficiencies in the number of heterozygous individuals. However, except for species with duplicate genomes or loci (in which null alleles have been interpreted as being part of the process of gene silencing), the biological significance of allozyme null alleles is poorly understood (Rebordinos and Pérez de la Vega, 1987).

The remaining loci deviations could only be attributed to null alleles if it is assumed that null allele homozygotes are lethal, since no non-staining individuals were observed, which would imply that the silent alleles occur in the heterozygote form only.

Another explanation for genotypic frequency departures could be the occurrence of selective forces causing post-settlement microgeographic differentiation, because if functional differences exist among genotypes and if certain heterozygotes represent disadvantageous levels of enzyme activity, it could be assumed that natural selection will act against those heterozygous genotypes and thus account for the observed heterozygote deficiency. Although in most cases, the action of selection at gene loci is difficult to demonstrate experimentally, evidence for the correlation of changes in allele frequencies with heterogeneous environmental parameters has been used to infer selection in natural populations of different species (Beaumont, 1982; Buroker, 1983; Rose, 1984). In this sense, higher frequencies of homozygotes have been found in juveniles than in adults, possibly as a result of selection against homozygosity (Koehn, Turano and Mitton, 1973). A case of heterozygosity-dependent growth has also been established in the American oyster (Singh and Zouros, 1978; Zouros, Singh and Miles, 1980), and studies in other bivalve species point to selection as a major factor causing heterozygote deficiency (Beaumont, Beveridge and Budd, 1983; Hilbish and Koehn, 1985).

Two other theories, inbreeding and Wahlund effect, are probably irrelevant to our study, since external fecundation and a long pelagic larval stage, in addition to tidal movements and currents, should permit a good mixing of the species's progeny. The Wahlund effect is not so clear when populations have very similar allele or genotype frequencies at each locus.

Restricted gene flow between populations has been claimed to be responsible for departure from equilibrium proportions. Nm indicates the relative strengths of gene flow, and Nm estimates from all loci (table IV) indicate that gene flow is sufficiently strong to prevent genetic drift from causing local genetic differentiation. The locus Mdh-1 differs sufficiently from the others, and either it or a closely linked locus could be subject to strong natural selection favouring different alleles in different populations.

The association between gene exchange and dispersal capabilities at early life-cycle stages has been investigated for marine animals (Waples, 1987; Palumbi, 1992) and generally species with long-lived planktonic larvae have high dispersal capabilities and are less likely to show high genetic variation. Planktonic dispersal also creates a potential for variation, and relatively small changes in the rate of mortality can have major effects on the numbers of offspring available to recruit into the adult habitat after the dispersive stage (Underwood and Faireweather, 1989).

Mdh-2 showed a mean D value of –0.190, but was in Hardy-Weinberg equilibrium for all populations except at CCP ($p < 0.01$). Pgi and Pgm produced D values close to zero, coincident with the goodness-of-fit of proportions in almost all populations. Instead, Me-2 and Xdh yielded high negative values (0.421 and 0.520, respectively), and did not show null alleles, and the frequencies of all electromorphs were > 0.05. In spite of this, Me-2 showed

departures from equilibrium in all populations, and CLC and BR populations were also the only ones adjusted to equilibrium for the Xdh gene. Selection and/or hidden variability could be a likely explanation for disequilibria in these genes. The second possibility relies on the finding that many electromorphs are known to harbour hidden variation that can be uncovered by varying the assay conditions (Rebordinos and Pérez de la Vega, 1989, 1990), because some bands visualised as unique can actually be the result of several overlapping bands, and their separation could increase the number of heterozygotes as opposed to homozygotes.

The amount of allele frequency variance as measured by FST was fairly small at most loci (0.006-0.046), and the overall differentiation between populations was slight, according to a mean F_{ST} of 0.023. This value was similar to the 0.034 reported by Buroker (1983) for eight populations in the Gulf of Mexico, and on the order of magnitude of that reported for other local populations of bivalves (Buroker, Hershberger and Chew, 1979; Hedgecock and Okazaki, 1984). On the other hand, the F_{ST} value is in the range described for other sedentary marine invertebrates with widespread dispersal of planktonic larvae (Nash, Goddard and Lucas, 1988; Johnson and Black, 1984; Holborn, Johnson and Black, 1994); however, notably higher values have been reported for species with no planktonic development (Johnson and Black, 1991).

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