Genetic differentiation in two cryptic species of Ostreidae, *Ostrea edulis* (Linnaeus, 1758) and *Ostreola stentina* (Payraudeau, 1826) in Mar Menor Lagoon, southwestern Mediterranean Sea

M. González-Wangüemert[1]
A. Pérez-Ruzafa
Departamento de Ecología e Hidrología
Facultad de Biología
Universidad de Murcia
30100 Murcia
SPAIN
mergonza@um.es

M. J. Rosique
A. Ortiz
Departamento de Biología Animal
Facultad de Veterinaria
Universidad de Murcia
30100 Murcia
SPAIN

ABSTRACT

Ostrea edulis is a target species for aquaculture but its hatchery has suffered as a result of the lack of morphological differentiation between individuals with a low growth and those that reach commercial size. Two sympatric species of oysters, *Ostrea edulis* and *Ostreola stentina,* have been reported at the Mar Menor Lagoon, Spain. A third nominal species, *Ostreola parenzani,* is now considered a synonym of *O. stentina.* The external morphology of *O. edulis* and *O. stentina* is very similar and this prevents their differentiation at the morphological level, except for maximum size. Oysters were collected from 3 locations along the Mar Menor Lagoon and examined for variation at the *PGI* locus. Principal component analysis of allozyme data revealed the existence of two groups, which confirms the presence of two species: *Ostrea edulis* and *Ostreola stentina.* The genetic variability of the glucose-6-phosphate isomerase (*PGI*) locus was also compared in *Ostrea edulis* and *Ostreola stentina* from the Mar Menor Lagoon. *Ostrea edulis* has high levels of homozygosis and shows an important deviation from the Hardy-Weinberg equilibrium. *Ostreola stentina* shows high heterozygosis and significant differentiation among coastal lagoon samples. The allele frequencies at the *PGI* locus can be used as a diagnostic character at the species level.

INTRODUCTION

Molecular techniques, including cytogenetics (Thiriot-Quiévreux, 1994) and flow cytometry (Partensky et al., 1997), provide a range of methods for quantifying the phylogenetic relationships between species and higher taxa, defining species limits, and identifying and quantifying cryptic species (Féral, 2002).

Biochemical methods helped demonstrate that many abundant and ecologically important "species" are, in fact, groups of species or species complexes (Avise, 1974).

Genetic studies have indicated a remarkably high incidence of cryptic speciation in marine invertebrates (Knowlton, 1993; Thorpe and Solé-Cava, 1994) including marine bivalves (Koehn, 1991; André et al., 1999; Daguin, 2000) and gastropods (Munksgaard, 1990; Palmer et al., 1990; Liu et al., 1991; Corte-Real et al., 1996a; 1996b) and sometimes even in comparatively well studied commercially important species (Yeatman and Benzie, 1994; Chan and Chu, 1996; Thorpe et al., 2000). These genetically differentiated groups often show minor differences in shell morphology that are not always consistent with genetic (allozyme) characters (Sarver et al., 1992). This has important implications for studies on the biology of the involved species. The overlooked presence of cryptic species may produce unexpected variation in physiological or ecological studies.

The demand for high-quality protein, especially from aquatic sources, is rising dramatically. Increased aquaculture production is clearly needed to meet this demand (Dunham et al., 2000). However, aquaculture productivity cannot be optimized if the biological potential of cultured species is not realized. Due to the abovementioned difficulties in differentiating some species on the basis of external morphological characters, the genetic identification and discrimination of aquaculture stocks and species is a fundamental requirement in any culture program (Ferguson, 1994).

Oysters have been exploited since the time of the Roman Empire (Magenis et al., 1983), but harvesting on a large scale began in France around 1850. Spat have been collected from natural beds and cultured with varying success due to epizootic diseases (Jaziri et al., 1987).

In the Mar Menor Lagoon, *Ostrea edulis* have undergone rapid expansion since the early 1980s after the

[1] Author for correspondence

artificial enlargement of one of the inlets that connect the lagoon with the Mediterranean. In 1992, 177 million individuals were tallied in a survey with a mean density of 2 oysters/m^2 reaching 22 oysters/m^2 in the most populated areas (Rosique and García-García, 1997). As a result of these high densities, several attempts at hatching were made and the spats of this species have been collected from natural beds and cultured with varying degrees of success due to epizootic diseases and the irregular growth of percentile individuals, which does not allow for profitable exploitation. Blanc et al. (1986) cited a similar finding in Nador Lagoon (Morocco). They studied two populations of oysters that belonged to the same cohort: 49 individuals of a normal growth population and 49 individuals of slow growth. They concluded that the fast-growing sample was *Ostrea edulis*. Of the slow-growing oysters, only 19% were considered to be *Ostrea edulis*, while 81% belonged to another species. Moreover this second species differed from *Ostrea edulis* by three loci and appeared to be a dwarf sibling species of *Ostrea edulis* with similar larvae and spat.

A second species (*Ostreola stentina*) inhabits the Mar Menor Lagoon (Murillo and Talavera, 1983; Olmo and Ros, 1984; Pérez-Ruzafa, 1989) and is undifferentiated from *Ostrea edulis* except for the maximum size reached by each species. A third species, *Ostreola paranzani*, has been reported at the lagoon (Murillo and Talavera, 1983) although it is considered a synonym for *Ostreola stentina* by some authors (Parenzan, 1974).

Ostrea edulis can reach 94 g and 95 mm in weight and size respectively after thirteen months in culture, *Ostreola stentina* does not exceed 20 g in weight and 45 mm in size (Rosique et al., 1995).

Ostrea edulis has a high commercial value and its populations have suffered a strong decline due to overexploitation (Yonge, 1960). It is a hermaphroditic, infralitoral species with a wide geographical distribution along the Atlantic coastline from Norway to Morocco, and all along the Mediterranean as well as the Black Sea (Yonge, 1960; Launey et al., 2002). It has also been introduced into many other parts of the world (e.g., United States, Canada and Japan) due to its aquaculture potential (Korringa, 1976; Launey et al., 2002). Its life history is characterized by fertilisation occurring inside the pallial cavity and the brooding of larvae (Yonge, 1960). As a result of a brooding period of 8 to 10 days, the length of the plankton larval phase is reduced compared to that of other oyster species (Buroker, 1985).

Ostreola stentina is small to medium in size and lives in shallow subtidal waters to a few meters depth, in tropical and temperate seas (Harry, 1985).

The systematic position of Ostreidae has been studied in several works (Pasteur-Humbert, 1962; Harry, 1985; Orton, 1928; Nelson, 1938; Montero, 1971; Stenzel, 1971; Parenzan, 1974) but most of them have not resolved all the taxonomic problems. Harry (1985) presented a good synopsis of the supraspecific classification of living oysters in which he considered not only the structure of the flesh and shells but also the environments, geographic range, and behavior of oysters. The author concluded that the intraspecific variation of oyster shells, which is probably greater than in any other group of living bivalves, precluded the preparation of a simple and satisfactory taxonomic key. The use of molecular genetic techniques in oyster systematics has increased over the past several years, largely due to the increased availability of techniques and increased awareness of the value of genetic data (Littlewood, 1994; Hare and Avise, 1998; Jozefowick and Ó Foighil, 1998; Lee et al., 2000).

Variation in enzyme coding genes has been studied in recent years in several species of marine bivalves, providing differentiation among similar species and information regarding genetic structure in populations of these organisms. Several studies of variations at enzyme loci in *Ostrea edulis* have been made (Wilkins and Mathers, 1973; Buroker, 1982; Maggenis et al., 1983; Johannesson et al., 1989; Le Pennec et al., 1986; Blanc et al., 1986; Saavedra et al., 1987; 1993; 1995; Álvarez et al., 1989). Electrophoretic studies have been mainly restricted to Atlantic populations, which have been very much affected by human harvesting activities (Yonge, 1960; Maggenis, et al., 1983). These studies indicated high genetic uniformity, covering restricted areas of the total range of the species' distribution (Le Pennec et al., 1986; Jaziri et al., 1987; Saavedra et al., 1987). Saavedra et al. (1995) showed that broad macrogeographical clines are a major feature of allozyme interpopulation variability in this species. The origin of these clines probably implied the contact of two Atlantic and Mediterranean oyster stocks that became differentiated in allopatry. Launey et al. (2002) studied the genetic differentiation in *Ostrea edulis* by means of variation at five microsatellite loci. The results showed a mild but significant isolation-by-distance profile, a noticeable between–sample variance in expected heterozygosity, and a tendency for Atlantic populations to be less variable than Mediterranean ones. Comparison with data on allozyme variation in relevant literature confirms this view.

MATERIALS AND METHODS

Study Area

The Mar Menor is a hypersaline coastal lagoon with a surface area of about 135Km^2. It is located in a semi-arid region of the southeast of Spain (37°44′ N, 0°47′ W) on the Mediterranean coast. The mean depth is 3.5 m with a maximum depth of 6 m (Pérez-Ruzafa, 1996). It has five open inlets, which permit the interchange of water with the Mediterranean Sea. In the 1970s, one of these channels (El Estacio) was dredged and widened, inducing important changes in the hydrodynamics and biological communities of the lagoon, including colonization by new species (Pérez-Ruzafa et al., 1987; 1991).

Sampling

In order to analyze the causes of the observed differential growth in oyster populations and to confirm the

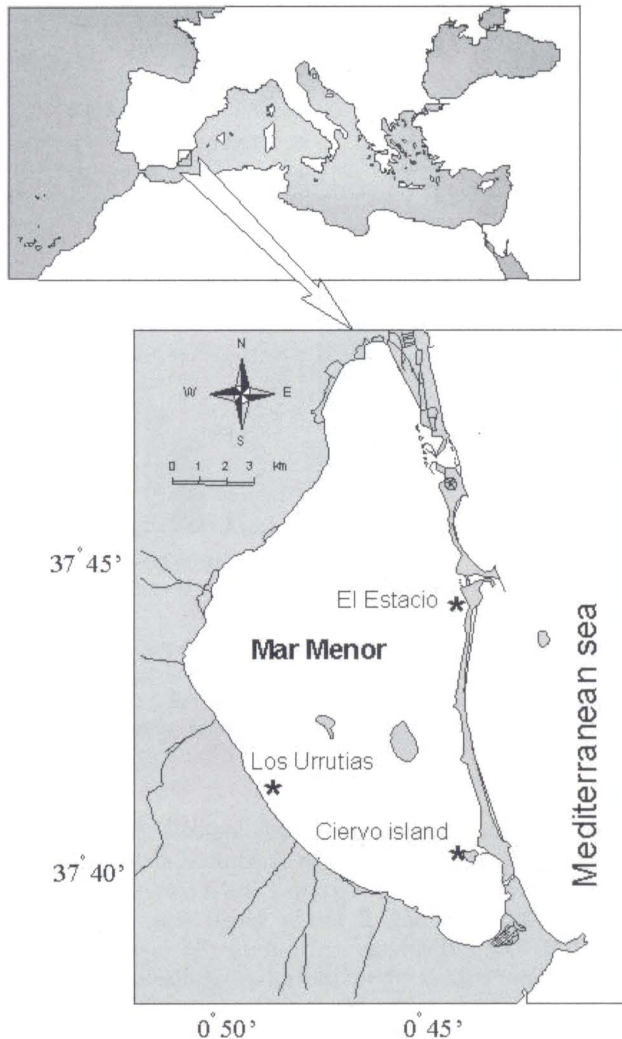

Figure 1. Sampling localities in Mar Menor Lagoon.

existence of the two reported species in the Mar Menor Lagoon, thus determining their importance in oyster hatchery, three localities were sampled at the lagoon in 1996 (Figure 1). Two samples were taken in natural oyster beds at Los Urrutias and Ciervo Island. The third sample was collected as spat at El Estacio in January and moved to aquaculture installations at Marbella (southern Spain) where after 8 months they were collected as adult oysters. This ensures that all individuals belong to the same cohort.

Electrophoresis

All oysters were transported live to the laboratory where they were dissected. Portions of adductor muscle were removed from each individual, homogenized in 1.5M Tris buffer (pH 9), and centrifuged at 4°C and 13500xg. They were stored at −40°C until electrophoresis.

Vertical polyacrylamide gel electrophoresis was carried out at a constant voltage (125 V) for 5 hours at 4°C. Gels were stained for *PGI* activity as described in Harris and Hopkinson (1976) with some modifications in the proportion of reagents (see González-Wangüemert, 1997).

Isozymes were numbered in decreasing order of mobility starting from the most anodal; allozymes were encoded according to the mobility of the most common allele (100).

Population Genetic Analysis: The existence of homogeneous genetic groups was explored performing a Principal Component Analysis (PCA) (ter Braak and Prentice, 1988) on the matrix of genotypes. The results of the ordination analysis are displayed in a biplot, scaling the axes, adjusting genotype scores to genotype variance: the resulting scores are correlations between genotypes and eigenvectors. All these calculations were done using the CANOCO v. 3.15 package (ter Braak, 1990).

The groups identified by the PCA were characterized morphologically comparing the maximum length of the shells (L1) using analyses of variance (ANOVA). According to the results, identified groups were assigned to the species *Ostrea edulis* and *Ostreola stentina*.

The genetic variability of the samples was recorded as expected and observed heterozygosity (H_e and H_o respectively) and the deviation coefficient were calculated. Differences in gene frequencies among three samples of *Ostreola stentina* were tested using χ^2 test (two degrees of freedom).

To analyze spatial differences in populations, a second PCA analysis was performed on the allelic frequency matrix for the two species and genetic variability descriptors at each locality.

F-statistics following Wright (1951) were calculated to detect non-random mating within populations (F_{IS}) and differentiation between populations (F_{ST}). Both statistics were calculated via the Weir and Cockerham method (1984). Probabilities of random departure from zero for F-values, according to the null hypothesis, were read directly from the distribution of 1000 randomized matrices computed via permutation of individuals among populations. This was performed using the "Genetix" F-testing procedure, thus providing a test of significance.

Genetic distance (Nei's D; Nei, 1978) was computed between pairwise samples. Probabilities of random departure from zero for Nei's D-values, according to the null hypothesis, were read directly from the distribution of 1000 randomized matrices computed by permutation.

Gene flow between samples was estimated as the number of migrants exchanged between populations *per* generation at equilibrium (N_e m). Values for N_e m were derived from one approach with F_{ST} values, following Wright's island model (1951).

The data was analyzed using the Genetix package (Bonhomme et al., 1993) (available at: www.univ-montp2.fr/genome-pop/genetix.htm).

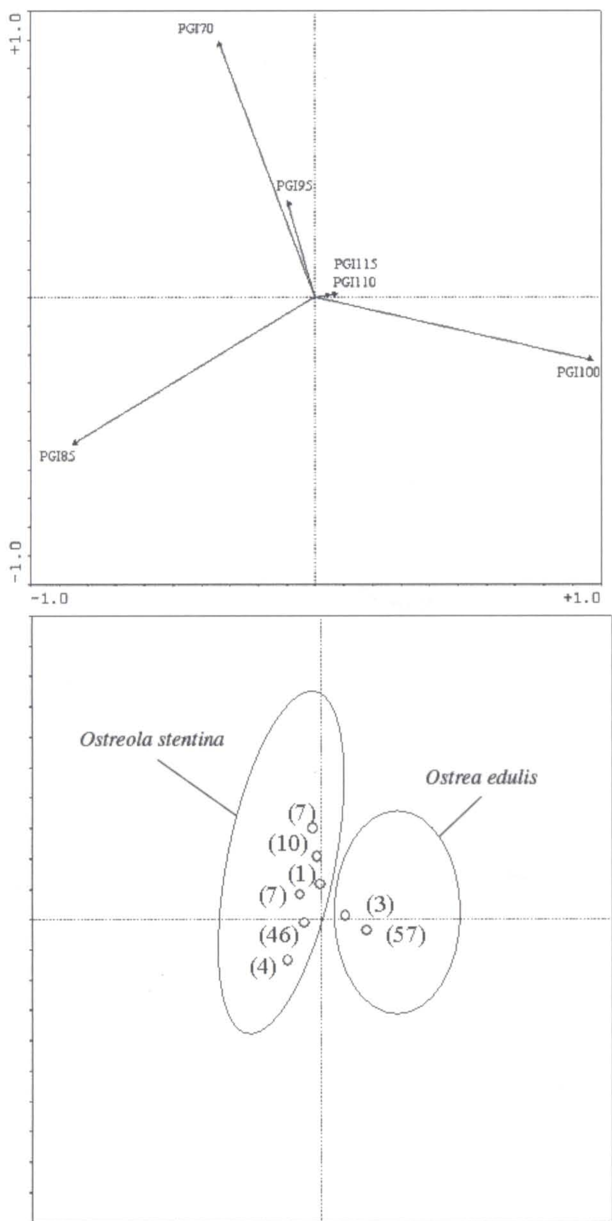

Figure 2. Ordination of first two axes of principal component analysis (PCA) of individual genotypes that jointly explained 91.7% of the variance in the global data set (numbers in brackets correspond to number of individual in that particular point).

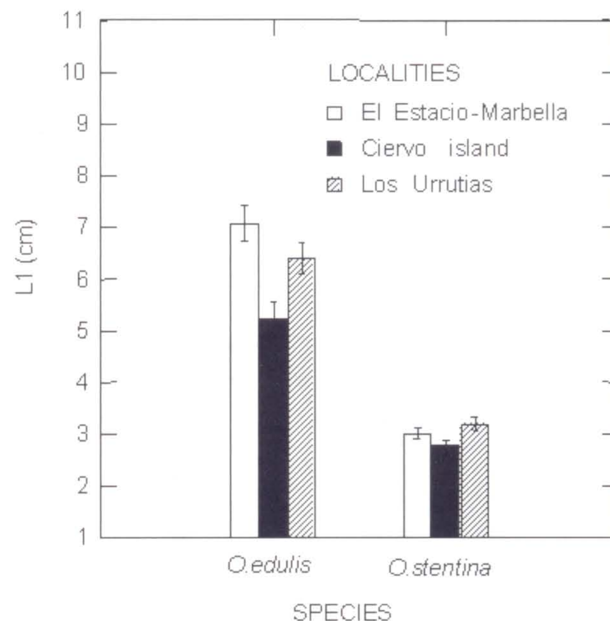

Figure 3. Significant differences in maximum diameter of the shell in the two species of oysters in the three sampling sites in Mar Menor. All individuals grouped as a species in this figure correspond to the individuals of same species in Figure 2 above.

RESULTS

A total of 168 individual oysters have been analyzed for the glucose-6-phosphate isomerase (*PGI*). The electrophoretic survey shows nine different genotypes. The first two axes of the PCA analysis (Figure 2) explain 91% of the total variance in data. The results shows two well-differentiated groups along the first ordination axis, which accounted for most of the variation, explaining 67.2% of the total variance.

The results of the ANOVA performed in order to characterize these groups morphologically show significant differences ($p<0.001$) in maximum size among genetic groups (Figure 3). Group 1 has a mean size of 6.61 cm ($\pm$ 0.21). Group 2 has a mean size of 3.16 cm ($\pm$0.04). The individuals cultivated in Malaga, belonging to the same cohort, show the same significant differences in size which are in line with genetic differentiation. So the two groups, which do not share any alleles, would correspond to the two species reported at the Mar Menor Lagoon, *Ostrea edulis* with only three alleles and three genotypes and *Ostreola stentina* with three alleles and six genotypes, respectively. The relative frequencies of all detected genotypes are shown in Table 1.

PGI was encoded by three alleles in *Ostrea edulis* (Table 2), though only three individuals exhibited the *PGI**115 and one individual showed the allele *PGI**110

Table 1. Relative frequencies of *PGI* genotypes in coastal lagoon oysters.

Genotypes	*Ostrea edulis*	*Ostrea stentina*
N	57	111
**100/100*	0.9452	0
**100/115*	0.0410	0
**100/110*	0.0136	0
**70/70*	0	0.0930
**70/85*	0	0.3813
**70/95*	0	0.1101
**85/85*	0	0.3644
**85/95*	0	0.0762
**95/95*	0	0.0084

Table 2. Allele frequencies at *PGI* locus of *Ostrea edulis* and *Ostreola stentina* (N: number of individuals; EE: El Estacio; IC: Ciervo Island; U: Los Urrutias).

Species	Samples	N	*PGI 70°*	*PGI 85°*	*PGI 95°*	*PGI 100°*	*PGI 110°*	*PGI 115°*
Ostrea edulis	EE	20	0	0	0	0.950	0.025	0.025
	IC	19	0	0	0	1	0	0
	U	18	0	0	0	0.970	0	0.030
	EE	76	0.263	0.645	0.092	0	0	0
	IC	22	0.386	0.568	0.046	0	0	0
Ostreola stentina	U	13	0.385	0.461	0.154	0	0	0

in heterozygous combination (Table 1). The locus can be regarded as essentially monomorphic in this species.

In *Ostreola stentina* three alleles of the phosphoglucose isomerase were expressed with frequencies higher than 0.10, as such the locus can be regarded as polymorphic.

Little difference was detected between the observed and expected heterozygosity. The highest deviation coefficient (D) was 0.0278 for *Ostrea edulis* and 0.111 for *Ostreola stentina* (Table 3). The observed heterozygosity in *Ostrea edulis* showed low values (ranging from 0.00 to 0.0952) due to *PGI*°100 being mainly combined as a homozygote and only four individuals being heterozygotes. *Ostreola stentina* has higher observed heterozygosity than *Ostrea edulis*, since 56% of the individuals analyzed were heterozygotes.

Allele frequencies at *PGI* differed significantly among the three sampled populations of *Ostreola stentina* (χ^2=5.99; P=0.035).

The PCA analyses performed on the allelic frequency matrix and genetic variability descriptors at each locality separate both species along the first axis which explains 97.8% of the total variance in data (Figure 4). *Ostrea edulis* samples groups in the positive part of the axis are characterized by a high homozygosity and a low heterozygote deficit. *Ostreola stentina* populations in the negative part are characterized by a higher expected and observed heterozygosity and a high heterozygote deficit. The second axis explains an additional 1.7% of the total variance and discriminates mainly among *Ostreola stentina* populations, with the El Estacio population, closer to the Mediterranean, in the positive part, with a dominance of *PGI* 85° allele, and that of Los Urrutias in the negative part with a dominance of *PGI* 95° allele and a higher heterozygote deficit.

Deviations from Hardy-Weinberg proportions within samples are shown by means of F_{IS} statistic. F_{IS} values indicated a significant heterozygote excess ranging from −0.006 to −0.101 in *Ostreola stentina* (Table 3). *Ostrea edulis* showed a deviation from Hardy-Weinberg expectations within the El Estacio sample, although it was not significant. (Table 3). Nei's genetic distances (Nei, 1978) were estimated using *PGI* locus (Table 4) in *Ostreola stentina.* Values ranged from −0.024 to 0.017. All distances were not significant at the 0.05 level. Estimates of genetic subdivision (F_{ST}) in the three samples are given in Table 4. The minimum positive F_{ST} value derived from allelic variation was found between the El Estacio and Ciervo Island samples (0.008), showing low divergence in gene frequencies between the two populations. In contrast, F_{ST} was considerably higher (0.026) between El Estacio and Los Urrutias samples, suggesting the possible occurrence of restricted gene flow between these populations. F_{ST} values between samples were significant at the 0.05 level.

Assuming equilibrium between genetic drift and migration, we calculated the number of migrants ($N_e m$) *per* generation (Table 5), based on F_{ST} values and according to the island model. Estimates of the number of migrants ranged from 9.16 (El Estacio-Los Urrutias) to infinite (Ciervo Island-Los Urrutias).

DISCUSSION

Allele frequencies at the *PGI* locus, used as a species-diagnosing character, allow the differentiation of the two sympatric oyster species studied. The coexistence of both species could explain the disastrous oyster hatchery attempt in the Mar Menor Lagoon (Rosique et al., 1995).

Table 3. Observed and expected heterozygosities (H_o and H_e), deviation coefficient (D) and F_{IS} (ns: non-significance; *: p<0.05; EE: El Estacio; IC: Ciervo Island; U: Los Urrutias).

Species	Samples	H_o	H_e	D	F_{IS}
Ostrea edulis	EE	0.0952	0.0963	−0.0106	0.013 ns
	IC	0	0	0	—
	U	0.0556	0.0540	0.0278	0 ns
	EE	0.5132	0.5066	0.0129	−0.006*
	IC	0.5909	0.5258	0.1101	−0.101*
Ostreola stentina	U	0.6923	0.6154	0.1111	−0.085*

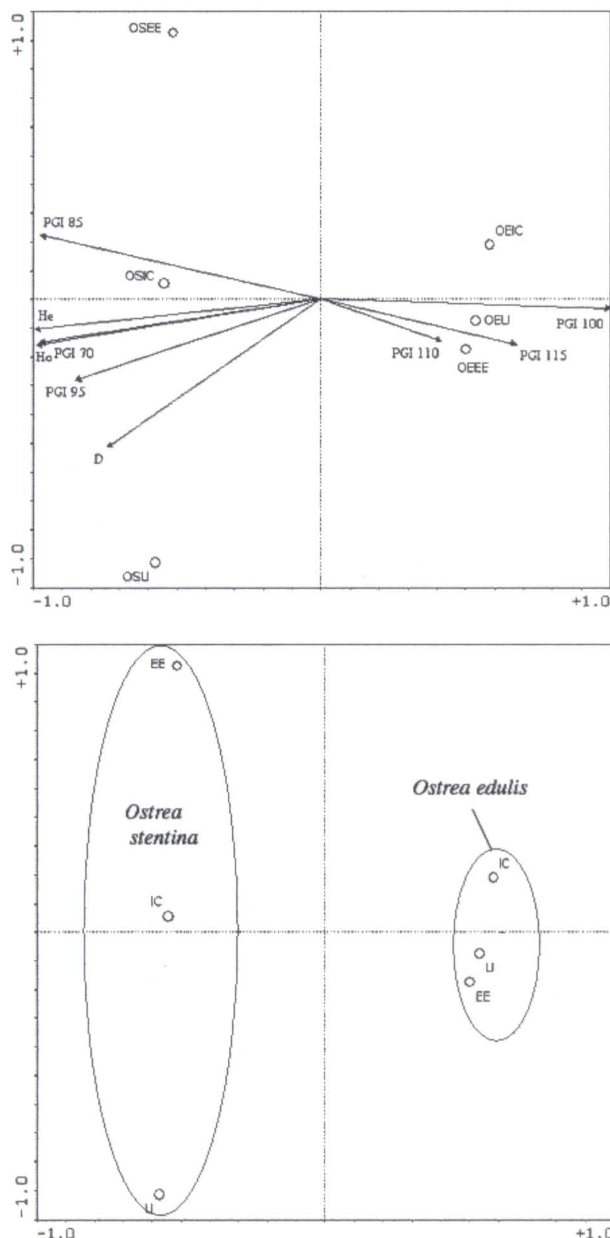

Figure 4. Ordination of first two axes of principal component analysis (PCA) of allele frequencies that jointly explained 99.5% of the variance in the global data set. (EE:, El Estacio; IC: Ciervo Island; U: Los Urrutias).

Genetic variation within and between populations has been demonstrated by the use of electrophoresis. We now have some information regarding the frequencies and distribution of alleles in wild populations of *Ostrea edulis* and *Ostreola stentina* in the Mar Menor Lagoon. Lower levels of genetic variation and heterozygote deficit were detected in the *Ostrea edulis* population.

All the electrophoresis studies on *Ostrea edulis* populations coincide in that this species displays lower levels of allozyme variation than other bivalves (Buroker, 1982; Saavedra et al., 1987) and the overall differentiation among its populations is usually slight (Johannesson et al., 1989). In fact Saavedra et al. (1993) showed, through an UPGMA dendrogram based on Nei's unbiased genetic distances, two main clusters, one formed by the eastern Mediterranean samples and the other by the remaining populations (western Mediterranean and Atlantic samples).

Two of the *Ostrea edulis* populations studied (El Estacio and Los Urrutias) have very low observed heterozygosity, though a significant deficit in heterozygotes is not observed. The Ciervo island population has 100% homozygotic individuals, so that this population shows an excessive heterozygote deficit and an important deviation from the Hardy-Weinberg equilibrium.

Some researchers have documented a deficit in heterozygotes for populations of *Ostrea edulis* from Atlantic oyster beds (Buroker, 1982; Maggenis et al., 1983; Johannesson, et al., 1989; Saavedra et al., 1995; Launey et al., 2002). The biological origin of these heterozygote genotype deficiencies may be related to fecundation. This takes place inside the pallial cavity of the female, which favors mating between nearest-neighbors. Also, larvae are brooded for a period of 8 or 10 days before the plankton phase, which limits dispersal. In addition, the extremely low levels of variability detected may to some extent be due to the recent history and exploitation of these populations (Saavedra et al., 1993).

Apart from chance alone, a number of factors may be responsible for causing deficiencies in heterozygotes against the H-W model in allozyme data. These include, null alleles, the Walhund effect, inbreeding and selection against heterozygotes or strong directional selection as a consequence of the geographic isolation of some populations (Zouros and Foltz, 1984; Mamuris et al., 1998; Rossi et al., 1998).

The low levels of observed allozyme variation in the

Table 4. Pairwise Nei's genetic distances (below the diagonal) and F_{ST} values (above the diagonal) in *Ostreola stentina*. F_{ST} and Nei's D considered to be significantly different from zero (°) if they fall within the 5% most extreme values in the permutation test. (ns: non-significance; ° = $p<0.05$; EE: El Estacio; IC: Cievo Island; U:Los Urrutias).

	EE	IC	U
EE	~	0.082°	0.0266°
IC	0.0080 ns	~	−0.0076°
U	0.017 ns	−0.024 ns	~

Table 5. *Ostreola stentina*. Estimates of N_em using F_{ST} values (Wright, 1951) (EE: El Estacio; IC: Ciervo Island; U: Los Urrutias).

	EE	IC	U
EE	~		
IC	30.28	~	
U	9.16		~

Ostrea edulis populations of the Mar Menor Lagoon, may be due to the recent history and exploitation of these populations. The current *Ostrea edulis* oyster bed in the Mar Menor Lagoon could come from oyster beds harvested for commercial purposes in NW Spain (Rosique per. com.). The transplantation of farmed stocks from Atlantic populations to Mediterranean populations has been a common occurrence (Launey et al., 2002). This hypothesis is reinforced due to the fact that the *Ostrea edulis* population from the Mar Menor Lagoon showed a lower heterozygosity than Mediterranean populations, and similar values to Atlantic populations (Arousa and Ares, NW Spain; Saavedra et al., 1993) and those of NW France (Jaziri et al., 1987). Allozyme and microsatellite studies have shown a lower genetic variability in Atlantic populations than in Mediterranean ones. This result could be explained by an overall smaller evolutionary effective size for Atlantic populations compared to Mediterranean populations and two main explanations have been put forward for such a difference: variance in effective sizes and oyster parasites (Launey et al., 2002).

High levels of variation were evident in populations of *Ostreola stentina*. This species shows six different genotypes for phosphoglucose isomerase and a high observed heterozygosity. This high variability could be due to long larval period (Harry, 1985) which could favor the dispersion of the gene pool. Some authors affirm that patterns of variability at the *PGI* locus in bivalves suggest that species inhabiting temporally variable or spatially heterogeneous environments exhibit higher levels of genetic variability than those from less variable or more monotonous environments (Valentine and Ayala, 1978). This agrees with the fact that the Mar Menor Lagoon shows a high degree of isolation with respect to the Mediterranean and highly variable environmental conditions (Pérez-Ruzafa, 1996) explaining the high genetic variability in *Ostreola stentina*.

F_{ST} values among *Ostreola stentina* populations are always lower than 0.1, and although significant, are indicative that there is little divergence among populations (Hartl, 2000). The fact that the Ciervo Island and Los Urrutias localities show infinite rates of interchange of individuals and negative F_{ST} and genetic distance values, suggests that both localities have the same *Ostreola stentina* population. Further genetic studies using several loci are required to confirm this hypothesis.

The results of this study confirm that there are two species (*Ostrea edulis* and *Ostreola stentina*) in the Mar Menor Lagoon stock and the alleles at the *PGI* locus can be used as a species-diagnosing character. As this situation can be a common state in the distribution area of both species, some works related to ecological and physiological adaptations or ecotoxicological responses of any of them should be reviewed. Some marine molluscs regulate their body tissue levels of particular trace metals to constant levels over a wide range of metal levels in their environment (Rainbow et al., 1990). The laboratory experiments have also provided evidence that this regulation is species-specific (Bryan et al., 1985; Rainbow et al., 1990; Dallinger and Rainbow, 1993) so that the existence of two cryptic species could change the conclusions of some toxicological works in *Ostrea edulis* (George et al., 1978; Auffret et al., 2002). Similar considerations could be applied to *Ostrea edulis* physiological studies (Beiras et al., 1995; Labarta et al., 1999; Culloty et al., 2001; Culloty et al., 2002) and works on the resistance of this species to the parasite *Bonamia ostreae* (Elston et al., 1987; Culloty and Mulcahy, 1996; Naciri-Graven et al., 1998; Naciri-Graven et al., 1999).

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