Volume 37 Issue 4, October 2009 ISSN 0305-1978

ELSEVIER

biochemical
systematics
and ecology

Biochemical Systematics and Ecology 37 (2009) 325–333

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Phosphoglucose isomerase variability of *Cerastoderma glaucum* as a model for testing the influence of environmental conditions and dispersal patterns through quantitative ecology approaches

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ARTICLE INFO

Article history:
Received 8 October 2008
Accepted 24 May 2009

Keywords:
Isozymes
Principal component analysis
Generalised additive models
Synthesis maps
Seascape genetics

ABSTRACT

Extreme conditions of coastal lagoons could directly modify the genetic patterns of species. The aim of this work was to investigate the influence of environmental conditions and small scale dispersal patterns on the phosphoglucose isomerase (*PGI**) genetic variability of *Cerastoderma glaucum* from the Mar Menor coastal lagoon. For this purpose, 284 cockles were collected around the perimeter of the lagoon. Vertical polyacrylamide gel electrophoresis was used to scan for *PGI** polymorphisms, giving a total of seven alleles. The spatial genetic distribution of the *PGI** variability, which seems to be marked by the main circulation in the lagoon, discriminates four hydrological basins. In the central basin, a gradient of allelic composition reflects the circulation forced by the dominant winds and the main channel communicated to the open sea. This result is well supported by the salinity GAM model that defines this gradient.

The other three basins are defined by the distribution of fine sand in a more complex model that tries to explain the isolation of the three sites localized inside these basins. The southern, western and northern basins show the lowest degree of interconnection and are considered the most confined areas of the Mar Menor lagoon. This situation agrees with the confinement theory for benthic assemblages in the lagoon. The greater degree of differentiation seen in the Isla del Ciervo population is probably due to recent human intervention on the nearby Marchamalo channel, which has been drained in recent years thus altering the influence of the Mediterranean Sea on the southern basin.

1. Introduction

Coastal lagoons are naturally stressed systems which suffer frequent environmental disturbance and fluctuations (Pérez-Ruzafa et al., 2005). Brackish-water ecosystems are often exposed to wide variations in environmental parameters, including temperature and salinity, which may cause strong selective pressures on organisms. These extreme conditions directly

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0305-1978/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bse.2009.05.013

modify the genetic patterns of species (Cognetti and Maltagliati, 2000; Maltagliati, 2002; González-Wangüemert et al., 2006) and can play an important role in separating species into different populations, associated with geographical discontinuity (Trabelsia et al., 2004).

Studies of enzyme polymorphism have shown that certain ecological events may result in the replacement of particular genotypes or may maintain some observed variability. For example, the 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 environments (Valentine, 1976; Rand et al., 2002; Véliz et al., 2004). Several studies point to patterns of allelic distribution associated with clinal variations on environmental factors, such as temperature and salinity (reviewed in Mitton, 1997; Watt, 2000). In some cases, coastal lagoon populations, regardless of their geographic distance, have shown a high degree of genetic homogeneity, probably driven by the manifold array of selective factors, characterizing lagoon environments (Allegrucci et al., 1997).

The cockle *Cerastoderma glaucum* (Poiret, 1789) typically inhabits closed brackish-water lagoons and estuaries in the Atlantic and the Mediterranean (Nikula and Vaïnölä, 2003). Its distribution along coastal waters is limited by intolerance to air exposure and wave action, which prevent this species from colonising open, tidal, coastal areas (Mariani et al., 2002). Most benthic marine species, especially bivalves, achieve high rates of dispersal by means of planktonic larval stages. *C. glaucum* can be considered gonocoric species with a long pelagic larval phase (Ducrotoy et al., 1987; Nikula and Vaïnölä, 2003). The larvae settling time of the species is about 11–30 days. Gametogenesis occurs in early spring and spawning takes place from May to July. Individuals live for about five years. Both its ecological and biological features make *C. glaucum* an interesting species, among marine bivalves, for studying patterns of geographic genetic structuring.

C. glaucum adopts several adaptive strategies in lagoons. For example, it seems to show an ability to regulate its metabolism at higher temperatures, reducing metabolic energy expenditure and representing an advantage in the saving of energy (Wilson and Elkaim, 1997). Since lagoon habitats are liable to long periods of high temperature during the summer, the energy conservation achieved by *C. glaucum* would confer on it a distinct advantage. Other strategies adopted in brackish lagoons include juvenile/adult distribution partitioning through migration and a shortened larval life span (Wilson and Elkaim, 1997). Larval stages of cockle can be transported live by currents for more than one week across hundreds of kilometres. On a smaller scale, lagoon-specific hydrodynamics and morphology are likely to account for the distribution of cockle larvae more than geographical distances and to determine the consequent recruitment within the lagoons (Mariani et al., 2002).

The aim of this work was to investigate the influence of environmental conditions and small scale dispersal patterns (inferred from a circulation model of the lagoon) on the phosphoglucose isomerase (*PGI**) variability of *C. glaucum* from the Mar Menor coastal lagoon.

2. Material and methods

2.1. Study area

The Mar Menor is a hypersaline coastal lagoon, with a surface area of 135 km^2 located on the Southwestern Mediterranean coast (Fig. 1) with a mean depth of 3.6 m and maximum of about 6 m. It is separated from the Mediterranean Sea by a 22 km long sandbar. Five channels connect the lagoon with the open sea. Some temporal watercourses flow into the lagoon, collecting run-off water from the watershed which is mainly used for intensive agriculture.

The salinity of the lagoon waters range from 43 to 46 and maximal and minimal salinities are registered in summer and spring, respectively. The temperature varies from 10 °C in winter to 32 °C in summer. The bed sediment grain composition is predominantly muddy and sandy; with some areas of natural rocky bottoms around islands and a few calcareous and volcanic outcrops. Muddy bottoms cover the whole central area of the lagoon and those shallow bottoms showing a lower hydrodynamism, at the same time being covered by a dense meadow of the algae *Caulerpa prolifera* or patches of the sea grass *Cymodocea nodosa*.

2.2. Genetic data as dependent variables

Cockles (284) were collected at 10 sampling sites distributed around the perimeter of the Mar Menor (Fig. 1). Samples were transported live to the laboratory where they were dissected and frozen.

Vertical polyacrylamide gel electrophoresis was used to scan for phosphoglucose isomerase (*PGI**) patterns in enzymatic extracts from abductor muscle. Gels were run at 125 V for 5 h with a current intensity in the 40–60 mA range. The *PGI** staining followed the methodology of Harris and Hopkinson (1976) with some modifications.

2.3. Hydrographical data as independent variables

Environmental variables expected to influence genetic structuring were represented by a set of estimators. We used these data to describe every 100 × 100 m unit grid of the study area.

Salinity was determined with a Beckman RS 7B salinometer and *in situ* determinations were performed using a WTW Multiline F/Set3 multiple probe. Descriptors of the substrate composition were grouped into five grain size classes: fine and coarse sands, fine and coarse silts and clays.

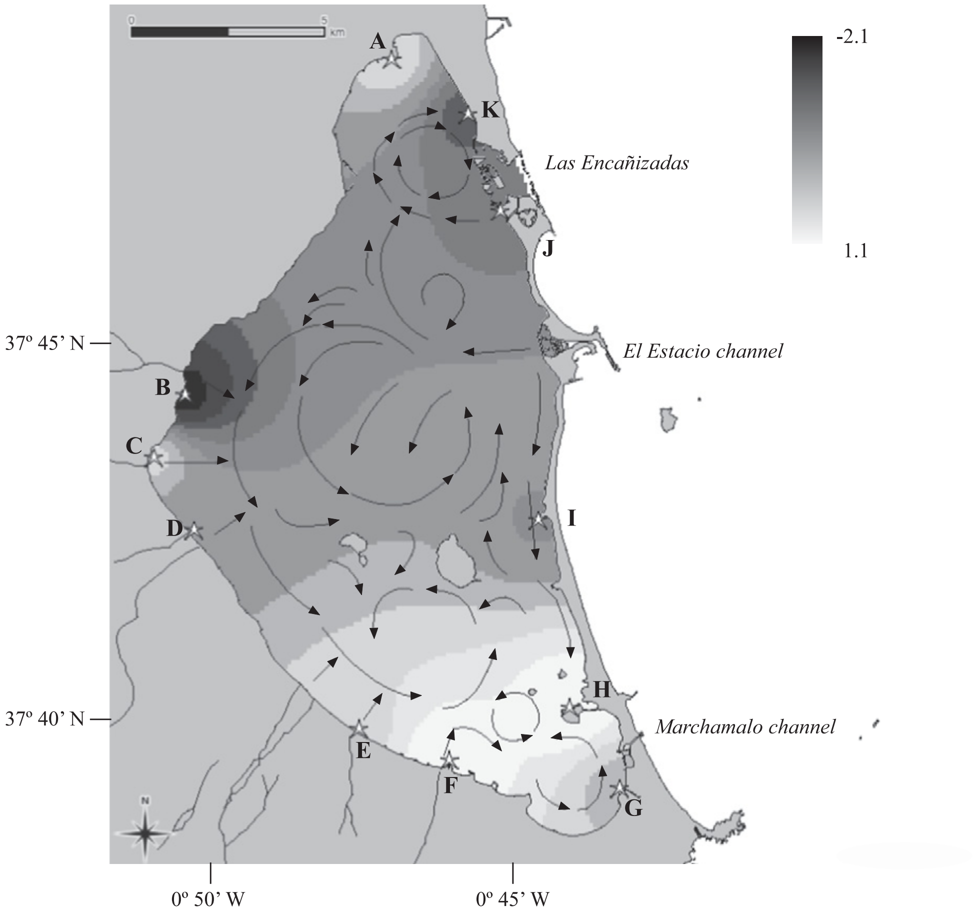

Fig. 1. Synthetic map illustrating the geographical variation of the first component which accounts for 53.12% of the total *PGI** allelic variation in *C. glaucum*. The components show a complex pattern that peaks (lightest shading) in the Isla del Ciervo, the most genetically isolated locality, related to the current pattern. Arrows represent the current pattern.

Sediment samples were stored and transported in darkness and cold in polyurethane bags to the laboratory. They were dried at room temperature, stirring at the same time to break up lumps. They were then sieved through a 2 mm mesh and the gravels (>2 mm fraction) were weighed. Grain size distribution in the <2 mm fraction was determined by the Boyoucos hydrometer method (Day, 1965; Soil Conservation Service, 1973) after dispersion of clusters by mechanical agitation in a sodium-hexametaphosphate and Na_2CO_3 solution. Prior to the analysis, salts were removed by washing and centrifugation and the organic matter by hydrogen peroxide treatment. Grain size classification followed the International Association for Soil Science (Duchaufour, 1975).

Hydrodynamism (as represented by wave exposure) was estimated according to Keddy (1983).

The values of wave exposure obtained at each site were corrected for measured depth at the same point and the wave length for each wind condition was calculated since the effect of waves on the bottom depends on these parameters (Bretschneider, 1964; Pond and Pickard, 1978).

Detailed information on the Mar Menor's hydrographic conditions, temperature and salinity spatial–temporal variations, wave exposure and current pattern were previously described in Pérez-Ruzafa et al. (2004, 2005).

The correlations between predictors were calculated and any correlated predictors were rejected to avoid the trouble involved in estimating responses. Lehmann et al. (2002) consider that a correlation of 0.79 is not high enough to justify the removal of any variable. The highest absolute values of correlation in our dataset were 1 and 0.92 among clays and silt which was considered sufficiently high to remove any of these variables from the model.

2.4. Statistical analysis

Genetic variability was recorded as: observed (H_o) and expected (H_e) heterozygosity and number of alleles per population (Ap). Data were analysed using the GENEPOP software (Raymond and Rousset, 1995).

Genetic distance (Cavalli-Sforza and Edwards, 1967) was computed between pairwise samples. Probabilities of random departure from zero for distance values, according to the null hypothesis, were read directly from the distribution of 1000 randomized matrices computed by permutation (Belkhi et al., 2004).

Rogers (1991) compared several genetics distances and concluded that Euclidean distances verify the properties of a genetic distance. A dendrogram of genetic distances was constructed using the complete linkage method (farthest neighbour) of cluster analysis.

Populations were spatially clustered using principal component analysis (PCA), which utilized the allele frequencies of populations as the variables. PCA summarizes all the variations in the study area and accommodates each population as a study unit (Manel et al., 2003). Then, the inverse distance weighted (IDW) interpolation of the major principal components leads to a synthesis map, which gives insight into the spatial pattern concerned (Hanotte et al., 2002). However, this approach does not test for the presence of a spatial pattern (Piertney et al., 1998).

Formulation of an oceanographic-based model to explain the spatial variation may fail to provide satisfactory results because the response could depend on the range and distribution of the observations. The experimental design and the biological response together could lead us to unreal trends of the data. In such a case, the model predictions could be improved by applying a particular category of statistical algorithm. Generalised additive models (GAM) are non-parametric extension of generalised linear models (GLM) that fit a wide variety of forms of stochastic variation in the response. GAMs represent the relationship between the response variable and the predictors by smooth functions, which can take virtually any form (Hastie and Tibshirani, 1990). The GAM model was developed for the first principal component of the PCA, using a procedure with an α set at 0.05. The explanatory variable was considered for inclusion in the model as a smoothed function, initially with 4 degrees of freedom. The GAM model was evaluated by examining the proportion of explained deviance and minimizing the generalised cross validation (GCV; Wood, 2000; Wood and Augustin, 2002) and the Akaike information criterion (AIC; Venables and Ripley, 2004) scores. The adjusted D (D^2_{adjusted}) is an ideal measure to compare models that include different combinations of variables and interaction terms, and is equivalent to R^2 in linear models (Guisan and Zimmermann, 2000). The standard errors of the smooth term were computed as the 95% bootstrap confidence interval for the smooth function based on 500 replications (Hastie and Tibshirani, 1990).

PCA analyses and GAM models were performed using “ade4” (Chessel, 1992) and “mgcv” (Wood, 2006) packages from R statistical software (R Development Core Team, 2007).

3. Results

Electrophoresis performed on *PGI** locus gave a total of seven alleles. Allele richness in *C. glaucum* localities varied from 3 alleles in Las Salinas to 7 alleles in Los Urrutias. The mean observed heterozygosity (H_o) ranged from 0.290 in Los Alcázares to 0.830 in La Carrasquilla (Table 1). The sampled localities showed some exclusive alleles, such as *PGI*104* in Los Urrutias or *PGI*88* in Lo Pagán and Los Urrutias.

The dendrogram constructed using genetic distances (Cavalli-Sforza and Edwards, 1967) is given in Fig. 2. Five groups were differentiated in this cluster. The Isla del Ciervo, Las Salinas and Lo Pagán populations (H, G and A, respectively) were isolated in independent groups. Los Urrutias and El Pedruchico formed the fourth group. The rest of the populations constituted the other group and included the localities that receive the direct influence of Mediterranean waters.

The surface plot of the first-axis principle component scores (which account for 53.12% of the variance) derived from the actual sampling points and interpolated over the whole lagoon is given in Fig. 1. The change in the first component scores was greater than would be expected if the difference between populations was due to an “isolation by distance” effect alone, and suggested barriers to gene flow. The surface could be viewed as three levels: i) the north, west and south areas which are isolated from the general spatial pattern (A, C and G), ii) localities from the centre of the lagoon (B, D, I, J and K), and iii) all other southern localities (E, F and H). These two levels of grouping followed a gradient that peaks (lightest shading) in the Isla del Ciervo population (H), the most genetically differentiated locality. The second component of the analysis revealed the genetic differentiation of this last locality (account until 77.44% of the variance together with the first component).

The relationship between the smooth terms in the GAM and the first-axis principle component scores was plotted in Fig. 3. The vertical axes represent the variable contribution of mean salinity and fine sand to the component (expressed on the scale

Table 1
Genetic diversity in analysed populations of *C. glaucum*. Number of alleles per population (Ap), observed (H_o) and expected heterozygosities and standard deviations ($H_e \pm \sigma$).

Localidad	Abr.	n	H_o	$H_e \pm \sigma$	Ap
Lo Pagán	A	40	0.450	0.558 ± 0.193	6
Los Alcázares	B	21	0.290	0.257 ± 0.110	4
El Albujón	C	27	0.560	0.508 ± 0.093	5
Los Urrutias	D	52	0.400	0.473 ± 0.147	7
Los Nietos	E	20	0.420	0.534 ± 0.205	4
La Carrasquilla	F	18	0.830	0.600 ± 0.388	5
Las Salinas	G	25	0.480	0.470 ± 0.022	4
Isla del Ciervo	H	21	0.480	0.618 ± 0.229	5
El Pedruchico	I	20	0.400	0.455 ± 0.121	5
Las Encañizadas	J	20	0.400	0.374 ± 0.070	4
Molino de la Calcetera	K	20	0.400	0.346 ± 0.155	4

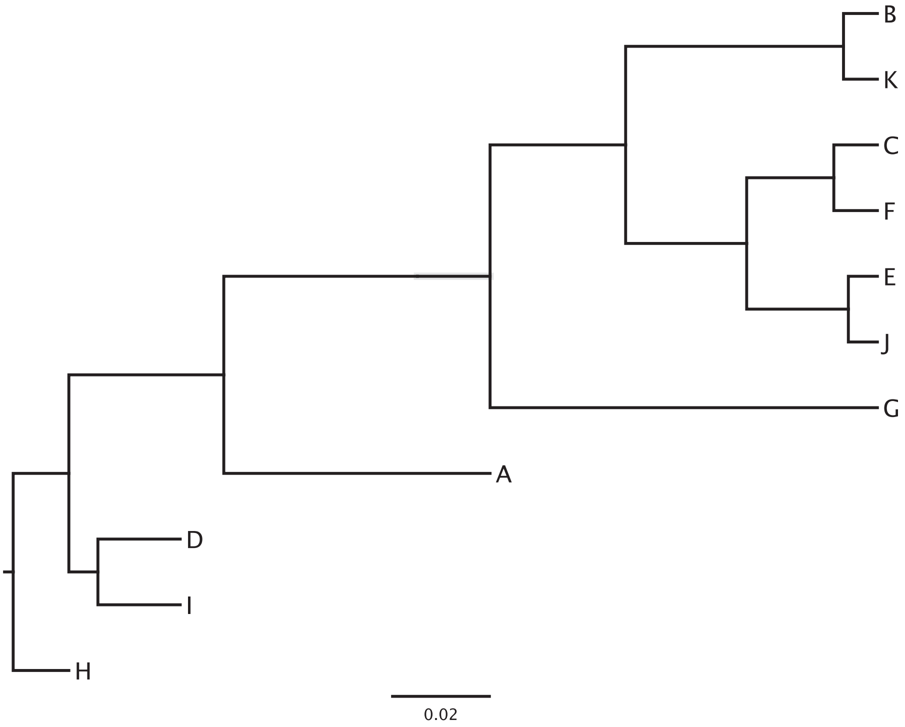

Fig. 2. Complete linkage method of cluster analysis from Cavalli-Sforza and Edwards (1967) genetic distances among populations of *Cerastoderma glaucum* in the Mar Menor. Abbreviations correspond to Table 1.

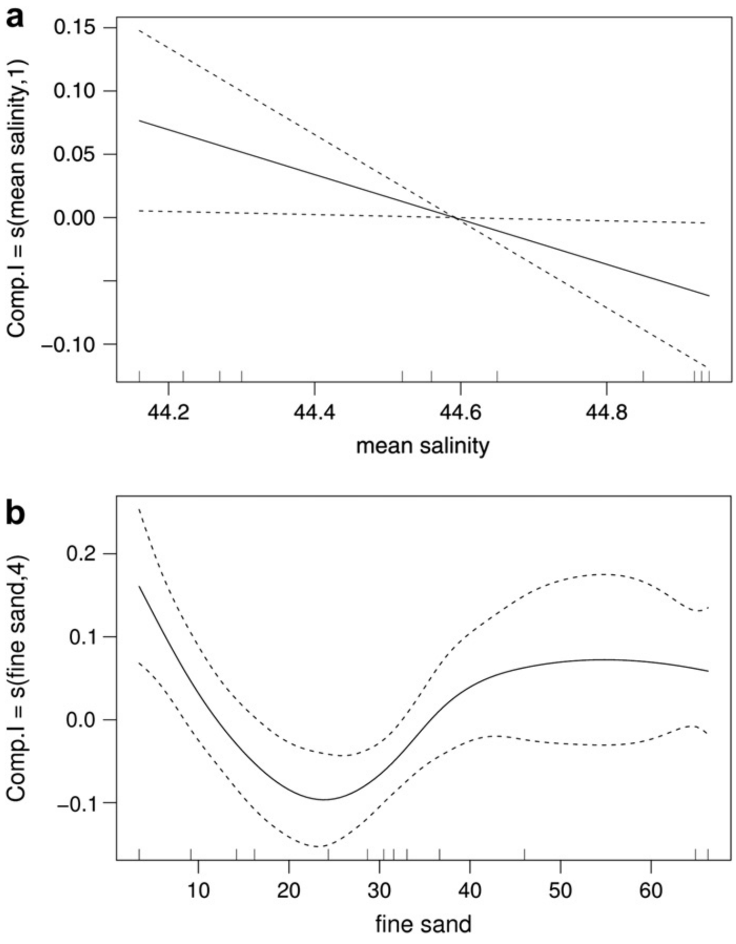

Fig. 3. Fitted smooth terms (indicated as *s* (*name of the predictor*; *number degrees of freedom*)), for the first component of PCA (solid lines) and ±95% bootstrapped confidence intervals (dashed lines) (a: mean salinity; b: fine sand).

of the linear predictor). A linear response was observed to mean salinity and a non-linear response with 4 degrees of freedom to the fine sand. The deviance explained by the GAMs was significantly high, as assessed by the approximate chi-square test and the D^2_{adjusted} (Table 2). The GCV and AIC scores were significantly low in both cases. All the components of the substrate composition showed significant fitted terms but less adjust and proportion of explained deviance than fine sand. The rest of the hydrographical variables did not fit significant by the terms according to GAM.

The smooth function fitted by mean salinity could be a GLM and presented an inverse relationship with the allelic composition gradient described by the first component of the PCA (Fig. 3a). This model described the gradient of the general pattern between the two margins of the spatial genetic distribution (Isla del Ciervo population – H – on the south side, and Los Alcázares and Molino de la Calcetera populations – B and K, respectively – on the centre and north side of the lagoon). Mean salinity described a similar gradient between the northern, central and southern localities (Fig. 4a). The percentage of deviance explained by this model was low because the three populations did not follow this general gradient.

Two parts could be appreciated in the second smooth function fitted by fine sand (Fig. 3b): an exponentially decreasing part (lower than 25% fine sand composition) and an increasing part of a gaussian curve (up to 25%). This independent variable (fine sand) predicted the behaviour of the three populations in the first component of the PCA as shown by the non-significant model values made without these three populations in the analyses ($\chi^2 = 0.757$, GCV = 0.005, AIC = 29.75). The variable represented a gradient between the areas exposed to the circulation model of the lagoon (Fig. 4b). The localities of Lo Pagán, Albujón (together with Los Alcázares and Los Narejos) and Las Salinas were the areas outside the influence of the current pattern. The GAM model used this particularity in the distribution of the variable to explain the spatial discontinuities of the component that represents the genetically differentiated population resulting from the current pattern (hydrographical effect). For this reason, the bivariate GAM model increased the significance and the cumulative deviance that explained the first component of the genetic ordination.

4. Discussion

*PGI** locus is an appropriate marker for revealing the spatial distribution of the *C. glaucum* genetic variation and for establishing the relationships between allelic composition and the environmental variables that are expected to influence it. In spite of using a single locus, genetic differences between *C. glaucum* populations within the lagoon are similar to those reported among populations of other molluscs (*Mytilus edulis*, *Mytilus trossulus*, *Mytilaster minimus*, *Littorina* spp., *Ostrea edulis* and *Ostreola stentina*) on a geographical gradient extending from a few metres to several km (Cognetti and Maltagliati, 2000; Riginos et al., 2002; Riginos and Cunningham, 2004; González-Wangüemert et al., 2004).

The differences among populations demonstrate the spatial consequences of the variation in environmental conditions due to the hydrographical characteristics and management of the Mar Menor coastal lagoon. If we assume that the alternating environmental conditions affecting a population promote the selection of the most genetically adapted individuals, this would imply the differentiation of populations with greater heterozygosity and genetic diversity (Wright, 1977). The populations isolated from the general current pattern show such characteristics. Therefore, the spatial genetic distribution of the *PGI** variability seems to be driven by the main circulation in the lagoon.

The principal component analysis based on allelic composition and the dendrogram based on genetic distances discriminated among four hydrological basins. The first basin is related with the main channel (El Estacio), which is responsible for most of the interchange between lagoon water and the Mediterranean Sea. The isocline surface of allelic composition reflects the circulation driven by the dominant winds and the El Estacio channel. This result is well supported by the GAM model of salinity that defines a gradient between these two localities in the central basin as was defined by Pérez-Ruzafa et al. (2004) and it is represented in Fig. 4a.

In the case of the other three basins that are defined by Albujón, Las Salinas and Lo Pagán populations, the GAM model fits a more complex function that tries to explain the isolation of these three localities. The selected variable is the distribution of percentage of the fine sand and according to this distribution, these three localities show a decrease in gene flow and are isolated from the general circulation model of the lagoon. In this sense, the southern, western and northern basins are the least connected and are considered the most confined areas on the Mar Menor Lagoon (Pérez-Ruzafa et al., 2004). This situation agrees with the gradients observed in species richness, diversity and abundance, and with those predicted by the confinement theory for benthic assemblages in the Mar Menor (Pérez-Ruzafa et al., 2004). The gradient is expressed as levels of isolation using the genetic data in *C. glaucum*: the more confined areas show greater isolation and greater differentiation in their populations. These areas show a substrate composition related with non-exposure zones with higher percentage of fine sand (Fig. 4b).

Table 2

Stages of the stepwise regression procedure in fitting the GAM model for the first component of the PCA for *C. glaucum GPI** allelic variation (D^2_{adjusted}: analogous to R^2 in linear regression; GCV: Generalised Cross Validation score; AIC: Akaike Information Criterion; Significance of χ^2: $0 < {}^{***} < 0.001 < {}^{**} < 0.01 < {}^{*} < 0.05$).

Variable	Cum. Dev.	D^2	GCV	AIC	χ^2
s (mean salinity)	29.6	0.77	0.008	32.70	*
s (mean salinity) + *s* (fine sand)	84.4	0.83	0.005	29.47	**

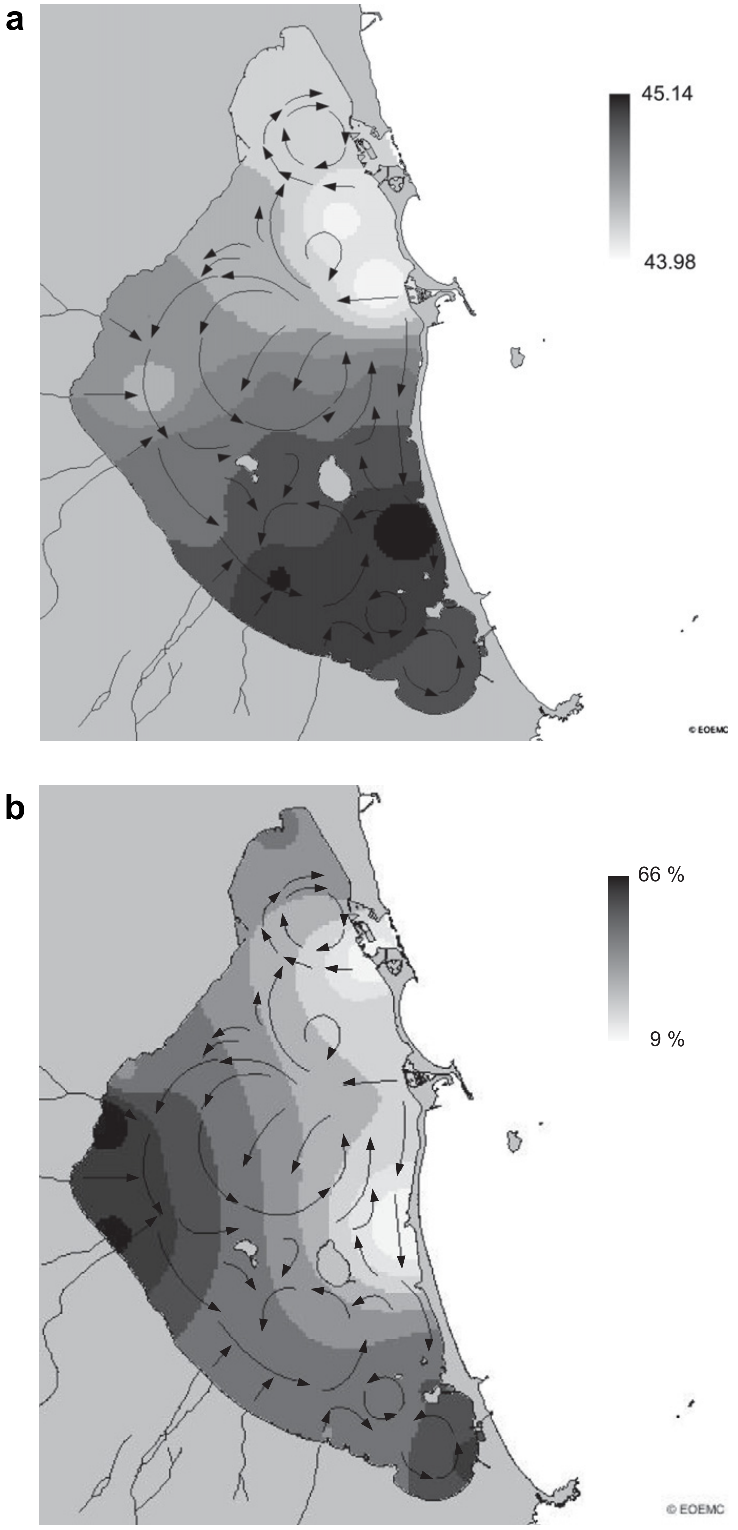

Fig. 4. Interpolated surface of mean salinity (a) and percentage of fine sand in the substrate (b). Arrows represent the current pattern.

The Isla del Ciervo population shows the highest degree of differentiation and a slight deviation from the general distribution pattern in all the analyses. The highest heterozygosities ($H_o = 0.480$, $H_e = 0.618 \pm 0.229$) and greatest genetic distances result in a high degree of genetic differentiation of this population. The part of the genetic gradient that peaks in Isla del Ciervo and the nearest populations grouped in the PCA (La Carrasquilla and Los Nietos), is probably due to recent human intervention in the Marchamalo channel. This channel was drained several years ago changing the way in which the Mediterranean Sea influences the southern basin, and showing a gradient from Isla del Ciervo to the nearest populations which were restricted by the general circulation pattern of the lagoon (Fig. 1).

5. Conclusions

The spatial variability of a single locus at a fine scale suggests the existence of population differentiation processes due to the wide variations in hydrographical parameters such as salinity, together with the current patterns of the Mar Menor. The spatial genetic distribution of brackish-water species together with the analysis of the relationships with environmental conditions could help understanding allopatric speciation and the evolutionary roles of coastal lagoons.

Acknowledgements

We are very grateful to Dr. Machado, Dr. Costa and Dr. Brock for helping with the taxonomy and to the many volunteers who assisted in the field and laboratory. This work received partial financial support from the SENECA Program Murcia University (03000/PI/05). M.G.W. was supported by Ministerio de Educación y Ciencia (M.E.C) and University of Murcia postdoctoral grants.

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