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Genetic variation in the European hake, *Merluccius merluccius*. Description of protein loci and genetic divergence between Atlantic and Mediterranean populations*

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Abstract

We examined the genetic population structure of the european hake *(Merluccius merluccius)* using electrophoretically detectable population markers in 35 protein loci. Samples were collected from 7 locations in the Atlantic Ocean and Mediterranean Sea. Six loci were polymorphic using the 0.05 criterion of polymorphism. Sample heterozigosities ranged from 0.052 to 0.072 and averaged 0.0625. In this study, significant allele frequency differences were detected between Atlantic and Mediterranean populations in three polymorphic loci: *GAPDH-1*, GPI-2** and *SOD-1**. Two major genetic groups were considered: a North-Atlantic stock and the Mediterranean stock. The Nei genetic distance, D, (based on 33 loci) between samples from these two groups ranged from 0.002 to 0.006. Genetic differenciation between these areas appears to reflect the barrier effect of Strait of Gibraltar.
On average over loci, 96.92 % of the total gene diversity was contained within samples, 0.23 % expressed differences among locations within areas, and 2.64 % differences between regions. A review of morphological variation together with the genetic data presented here suggest that the populations of hake from these areas are subdivided into two different stocks: the North-Atlantic stock and the Mediterranean stock. The most conservative approach to the management of these stocks is to consider the Atlantic and Mediterranean stocks independently from one another.

KEYWORDS: *Merluccius merluccius*, Atlantic Ocean, Mediterranean Sea, electrophoresis, polymorphism, fish stocks, population markers, fisheries management.

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Resum

Anàlisi de la variabilitat genètica en el lluç europeu, *Merluccius merluccius*. Descripció dels loci electroforètics i divergència genètica entre les poblacions atlàntica i mediterrània

L'anàlisi electroforètica de mostres pertanyents a Port de la Selva, Palamós, Blanes, Sant Carles de la Ràpita, Gandia, Huelva i Donostia ha permès de detectar 35 loci electroforètics dels quals 16 presenten variabilitat genètica i 6 són polimòrfics: *GAPDH-1*, GPI-1*, GPI-2*, G3PDH* SOD-1* i CAT**.
El grau de diferenciació genètica trobat per aquests loci a les diverses localitats mostrejades indica que existeixen diferències significatives entre les poblacions mediterrànies i atlàntiques de lluç, per la qual cosa podem afirmar que aquestes poblacions formen part de dos estocs diferents. Aquesta diferenciació creiem que és deguda a la barrera ecològica que representa l'estret de Gibraltar, que redueix considerablement el flux de gens entre ambdues zones.
La variabilitat genètica, mesurada quant a nivell d'heterozigositat, indica que cap de les dues poblacions de lluç, mediterrani i atlàntic, han sofert recents «colls d'ampolla», és a dir, no han patit canvis dràstics quant a grandària i estructura de la població, tot i la sobreexplotació a la qual estan sotmeses.
Dels loci polimòrfics, *GAPDH-1*, GPI-2* i SOD-1**, presenten variabilitat genètica partint de factors geogràfics i poden ésser utilitzats com a marcadors genètics, ja que el seu comportament diferencial permet d'identificar i separar captures pertanyents a l'Atlàntic i a la Mediterrània. Quant al locus *GPI-2** també presenta una variant al·lèlica lenta, *GPI-2** (*90) només en les poblacions atlàntiques, per la qual cosa podem considerar també aquest al·lel com un possible marcador genètic que permetria de separar captures d'ambdues zones.
El valor de distància genètica trobat entre les localitats atlàntiques i les mediterrànies no permet de parlar d'espècies diferents de lluç. L'anàlisi de la diversitat genètica mostra que el percentatge més elevat de variació genètica es troba en els individus dins les poblacions, i entre les àrees mediterrània i atlàntica, per la qual cual deduïm que dins d'aquestes àrees existeix un flux de gens que redueix considerablement la diferenciació genètica entre les diverses localitats mostrejades. La menor diferenciació genètica existent entre les localitats atlàntiques mostrejades en comparació amb les mediterrànies pot ésser explicada a partir de majors grandàries poblacionals i/o majors taxes de migració en la població atlàntica de lluç. Aquest resultat és de gran interès per a la futura gestió i conservació d'aquest recurs biològic a les zones estudiades.

MOTS CLAU: *Merluccius merluccius*, oceà Atlàntic, mar Mediterrània, electroforesi, polimorfisme, estocs de peixos, marcadors genètics, gestió pesquera.

Resumen

Análisis de la variabilidad genética en la merluza europea, *Merluccius merluccius*. Descripción de los loci electroforéticos y divergencia genética entre las poblaciones atlántica y mediterránea

El análisis electroforético de muestras procedentes de Port de la Selva, Palamós, Blanes, Sant Carles de la Ràpita, Gandia, Huelva y Donostia ha permitido detectar 35 loci electroforéticos de los cuales 16 presentan variabilidad genética y 6 son polimórficos: *GAPDH-1*, GPI-1*, GPI-2*, G3PDH* SOD-1* i CAT**.
El grado de diferenciación genética encontrado para estos loci en las distintas localidades muestreadas indica que existen diferencias significativas entre las poblaciones mediterráneas y atlánticas de merluza, por lo cual podemos afirmar que estas poblaciones forman parte de dos estocs diferentes. Esta diferenciación creemos que es debida a la barrera ecológica que supone el estrecho de Gibraltar, el cual reduce considerablemente el flujo de genes entre las dos zonas.
La variabilidad genética, medida cuanto a nivel de heterozigosidad, indica que ninguna de las dos poblaciones de merluza, atlántica y mediterránea, han sufrido recientes «cuellos de botella», es decir, no han sufrido cambios drásticos en cuanto a tamaño y estructura de la población, a pesar de la explotación a que estan sometidas.
De los loci polimórficos, *GAPDH-1*, GPI-2* i SOD-1** presentan variabilidad genética en base a factores geográficos y pueden ser utilizados como marcadores genéticos, ya que su comportamiento diferencial permite identificar y separar capturas pertenecientes al Atlántico y al Mediterráneo. En relación al locus *GPI-2 ** también presenta una variante alélica lenta, *GPI-2 ** (90*),

sólo en las poblaciones atlánticas, por lo que podemos considerar también este alelo como un posible marcador genético que permitiría separar capturas de las dos zonas.
El valor de la distancia genética encontrado entre las localidades atlánticas y las mediterráneas no nos permite hablar de especies diferentes de merluza. El análisis de la diversidad genética muestra que el porcentaje más elevado de variación genética se debe a los individuos dentro de las poblaciones y entre las áreas mediterránea y atlántica, por lo que deducimos que dentro de éstas áreas existe un flujo de genes que reduce considerablemente la diferenciación genética entre las diversas localidades muestreadas. La menor diferenciación genética existente entre las localidades atlánticas en comparación con las mediterráneas puede ser explicada en base a mayores tamaños poblacionales y/o mayores tasas de migración en la población atlántica de merluza. Este resultado es de gran interés para la futura gestión y conservación de este recurso biológico en las zonas estudiadas.

PALABRAS CLAVE: *Merluccius merluccius*, océano Atlántico, mar Mediterráneo, electroforesis, polimorfismo, estocs de peces, marcadores genéticos, gestión pesquera.

Introduction

The European hake, *Merluccius merluccius*, can be found over an area extending from the Norvegian coasts to Marocco and from the Black Sea to Strait of Gibraltar (Svetovidov, 1986). Up untill now, there are no reports on quantification of the actual amount of genetic divergence between North-Atlantic hake stocks. Management of the european hake has been based on the assumption that the species comprises almost two populations in this area, the Atlantic Ocean stock and the Mediterranean stock. This strategy arose from the assumption that the Gibraltar channel represents a very important ecological barrier which considerably reduces the migration of individuals from the Atlantic Ocean to the Mediterranean Sea and vice versa. Very early morphological studies had already showed significant differences between the individuals from the Atlantic Ocean and those from the Mediterranean Sea (Cadenat, 1952; Maurin, 1968; Lleonart & Agell, 1980).

In addition, the «Working group on the Assesment of the stocks of Hake» considers, in management terms, the existence of two different stocks of european hake in the Atlantic Ocean: the Northern stock, which is distributed from the Norwegian coastline to the Cantabric Sea; and the Southern stock, which is found from the North of Peninsula Iberica to the most meridian limit of distribution of the species, both with different areas of laying (STCF, 1986). However, Mangaly & Jamieson (1978) failed to detect any ellectrophoretical genetic variation among the North-Atlantic hake populations. Similar results were obtained on other hake species, *M. australis* (Smith *et al*., 1979) or *M. capensis* and *M. paradoxus* (Grant *et al*., 1987). Nevertheless, one electrophoretic study on the Pacific hake (*M. productus)* demonstrated the presence of allele frequency differences between populations located in Puget Sound, a large embayment, and offshore oceanic populations (Utter & Hodgins, 1969, 1971; Utter *et al*., 1970).

The species is highly favored in Spanish restaurants and residences and sustains an intensive fishery in both the Atlantic Ocean and the Mediterranean Sea (Sardà & Martin, 1986; FAO, 1989). Thereby an effective management of this renewable biological resource requires knowledge of genetic substructure. The purpose of this paper is, firstly, to present the genetic interpretations for the patterns of ellectrophoretically detectable proteins in the european hake *(M. merluccius)* and secondly, to estimate the amount of intraspecific genetic differenciation in the European hake, based on samples collected from some Spanish locations distributed all over the North-Atlantic Ocean and Mediterranean Sea coastlines.

Material and methods

A total of 416 North-Atlantic hake were collected from 7 Spanish locations, two on the Atlantic coast and five on the Mediterranean one (fig. 1; table 1).

All the fishes were captured with the aid of fishing boats. The samples were kept in a cool place aboard until they were brought to the laboratory, where they were stored in freezers (– 80 ºC) and eventually electrophoretically analysed.

Eye, liver, kidney and muscle tissue extracts were analysed by horizontal starch gel electrophoresis following the methods of Aebersold *et al.* (1987). All four tissues were tested on all combinations of buffer and stain in the search for optimal electrophoretical activity and resolution. We have used the buffers and specific stains that are shown in Table 2.

The genetic interpretation of electrophoretic banding pattern follow the principles which have been previously outlined for other species (Allendorf *et al*., 1977; Utter *et al.*, 1979; Grant & Utter, 1980). A single invariant band is assumed to represent the gene product of a single locus (Harris & Hopkinson, 1976). When more than one zone of activity was resolved for an enzyme, tissue distribution of activity and information on the quaternary structure (Darnall & Klotz, 1975) were used to interpret the

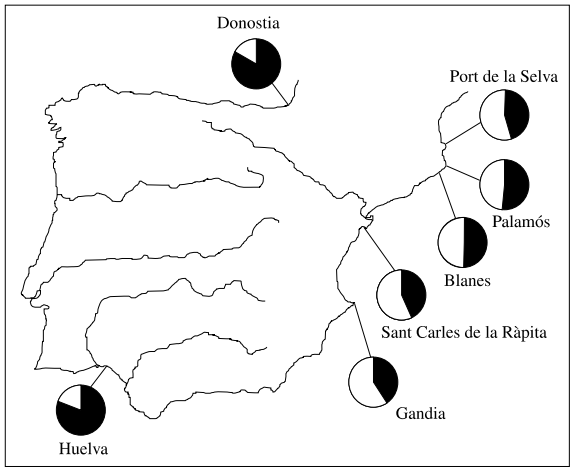

FIGURE 1. Geographic locations where European hake where sampled and diagrammatic representation of allele-frequencies of *GAPDH-1**. In black, the frequency of the *100 allele.

Localització dels diferents punts de mostreig del lluç europeu i representació de les freqüències al·lèliques del locus *GAPDH-1** en les mostres de *Merluccius merluccius*. En negre, freqüència de l'al·lel *100.

TABLE 1. Regions, localities, dates and sizes of samples used for electrophoretic analyses.
Regions, localitats, dates i grandària de les mostres utilitzades en les anàlisis electroforètiques.

Region	Zone	Locality	Date	Sample size
Western Mediterranean	North	Port de la Selva	21 Jun 88	35
			21 Sep 88	36
		Palamós	16 Jun 88	25
			23 Sep 88	25
		Blanes	07 Jun 88	10
			11 Jul 88	28
			16 Set 88	25
		St. Carles Ràpita	20 Set 88	52
	South	Gandia	04 Oct 88	55
Noth-Atlantic	South	Huelva	28 Nov 88	65
	North	Donostia	20 Jul 88	30
			20 Set 88	30

number of loci coding for the enzyme in question. The designation of loci, alleles and genotypes follows the system of nomenclature proposed by Allendorf & Utter (1979) and Shaklee *et al.* (1990), in which an italised abbreviation designated the locus coding for a protein and, for each locus, alleles were designated by their mobilities relative to the mobility of the most common allele which is arbitrarily designated 100. Where more than one locus coded for the same functional enzyme, loci were designated numerically from the cathodal end of the gel.

All analyses were carried out through the BIOSYS-1 computer program (Swofford & Selander, 1981). Statistical tests included exact significance probabilities test (analogous to Fisher's exact test) for Hardy-Weinberg equilibrium and contingency chi-square analysis to measure heterogeneity of allele frequencies among all samples.

Results

–Interpretation of electrophoretic patterns

The names of enzymes examined, locus abbreviations, tissue distributions and the buffer system used to obtain the results are compiled in Table 2.

We detected the gene products of 35 loci which are distributed among various tissues. Notations of the structure and migration directions for proteins refer, in the following descriptions, to the results obtained when the recommended buffer system was used.

The 35 loci under study were grouped according to the degree of resolution of the bands obtained in the electrophoresis and to the level of genetic variation that they presented.

–Loci with bad resolution or no activity

• Peroxidase *(POD)* and N-Acetyl-B-glucosaminidase *(BGA)*. No activity was

TABLE 2. Enzymatic proteins, locus abbreviation, tissues with strongest expression, and electrophoretic buffers used in the study of european hake. Tissues: M, skeletal muscle; L, liver; E, eye fluids; K, kidney. Buffer components: (1) Gel: 0.002M citric acid (pH 7.1); electrode: 0.04M citric acid (pH 7.1). (2) Gel: 0.076M Tris-0.05M citric acid; electrode: 0.06M lithium hydroxide, 0.03M boric acid. (3) Gel: 1:4 dilution of electrode buffer; electrode: 0.18M Tris, 0.1M boric acid, 0.004M EDTA, with addition of NAD to the gel and to the cathodal electrode compartment. (4) Gel: Buffer 2 but without NAD; electrode: (3). (5) Gel: 1:9 dilution of electrode buffer; electrode: 0.1M Tris (pH 8.0). «a» indicates that the resolution in insufficient.
Proteïnes enzimàtiques, abreviacions dels loci, teixits emprats i tampons electroforètics utilizats en l'estudi del lluç europeu.

Protein	**Locus**	**Tissue(s)**	**Buffer**
Asapartate Amino Transferase	*AAT-1**	M,L,E	all
	*AAT-2**	M,L,E	all
Acid Phospatase	*ACP-1**	M	1
	*ACP-2**	M	1
Adenosine desaminase	*ADA-1**	L	5
	*ADA-2**	L	5
Alcohol Deshydrogenase	*ADH**	L	2
N-Acetyl-B-glucosaminidase	no activity	L	5
Catalase	*CAT**	K,L	5,2,1
Creatine Kinase	*CK-1**	M,E	2
Esterase D	*ESTD-1**	L,M	2
	*ESTD-2**	L,M	2
Fructose-biphosphate aldolase	no activity	M	4
Glyceraldehidephosphate Deshydrogenase	*GAPDH-1**	M	3
	*GAPDH-2**	E	3
	*GAPDH-3**	E	3
Glycerol-3-phosphate Desheydrogenase	*G3PDH**	M	4
Glucosephosphate Isomerase	*GPI-1**	M,E	2
	*GPI-2**	E,M	2
Guanidine desaminase	*GDA-1**	L	5
Isocitrate Desheydrogenase	*IDH-1**	M	1
	*IDH-2**	L	1
Lactate Desheydrogenase	*LDH-1**	M	2
	*LDH-2**	M	2
	*LDH-3**	L	2
Malate Desheydrogenase	*MDH-1**	M	1
	*MDH-2**	M	1
Malic Enzyme	*ME-1**	L,M	1
Mannose-phosphate Isomerase	*MPI-1**	L	1
Phosphoglucomutase	*PGM-1**	M	2
Phosphogluconate Desheydrogenase	*PGDH**	M,L	1
Superoxyde Dismutase	*SOD-1**	L	2
	*SOD-2**	L	2[a]
Peptidase			
Substratum Leucil.glycil-glycine	*PEP1**	M	1
Substratum Glycil-leucine	*PEP2**	M	1
Substratum Leucil-tyrosine	*PEP3**	M	1
Substratum Phenylalanil-proline	*PEP4**	M	1
Peroxidase	no activity	K,L	all

obtained for these proteins in this study.

• Fructose biphosphate aldolase *(FBALD)*. The resolution for this enzyme was unsatisfactory in the present study but the patterns of bands obtained seemed to coincide with the expression of the enzyme, *GAPDH*.

–Monomorphic loci

• Adenosine deaminase *(ADA)* (EC 3.5.4.4). Two zones of enzyme activity appeared on gels stained for *ADA* and expressed in liver. Both zones representing the monomeric products of *ADA: ADA-1** and *ADA-2**.

• Alcohol dehydrogenase *(ADH)* (EC 1.1.1.1). This liver-specific dimeric enzyme has shown to be encoded by a single cathodal zone in the present study.

• Creatin phosphokinase *(CPK)* (EC 2.7.3.2). In muscle tissues, a single zone of activity showing a single invariant band which has migrated anodally from the origin. We observed the same results using eye-fluids but the resolution was worse.

• Esterase D *(EST D)* (EC 3.1.1). Two anodal zones of Esterase activity were observed in the present study. They were assumed to represent the expression of two loci designated *ESTD-1** and *ESTD-2**. Both loci were represented by a single invariant zone expressed in liver and muscle extracts.

• Guanine deaminase *(GDA)* (EC 3.5.4. 3). For this enzyme a single-invariant-anodal zone was obtained in liver extracts. A single locus is expressed for this enzyme.

• Malate dehydrogenase *(MDH)* (EC 1.1.1.37). We observed the products of two loci in skeletal muscle tissue. No variant phenotypes were observed in both loci designated as *MDH-1** and *MDH-2**.

• Malic enzyme *(ME)* (EC 1.1.1.40). One anodal zone of activity was resolved for this enzyme. No variability was observed for *ME** in liver and skeletal muscle tissues.

• Phosphogluconate dehydrogenase *(PG DH)* (EC 1.1.1.44). A single locus appears to code for this enzyme in hake. No variability was observed in liver and skeletal muscle tissues.

–Loci showing some degree of genetic variation (P < 0.5, P based on the 95 % criterion for polymorphism).

• Aspartate aminotransferase *(AAT)* (EC 2.6.1.1). In hakes we have found two duplicate loci to code for *AAT* -activity in muscle, liver and eye. At both loci, *AAT-1** and *AAT-2**, rare three-banded heterozygous phenotypes were observed. *AAT-1** showed a past allele (*157) in some individuals from Blanes, Donostia, Port de la Selva and Gandia and *AAT-2** fast and presented allelic variants restricted to individuals from Huelva. The resolution for this enzyme was much better when very fresh samples were used.

• Isocitrate dehydrogenase *(IDH)* (EC 1.1.1.42). Staining for this dimeric enzyme revealed two zones of activity with different tissue expressions. The most anodal zone was predominantly expressed in muscle *(IDH-1*)* whereas the less anodal zone was obtained from liver extracts *(IDH-2*)* . No variability was observed in *IDH-1** but in *IDH-2** we found a slow allelic variant (*72) in some heterozigote individuals belonging to the localities of Port de la Selva, Palamós, Blanes and Donostia. An heterozigote individual for fast allele (*120) appears as well in the samples from Gandía, which has not been detected in any other of the sampled localities.

• Lactate dehydrogenase *(LDH)* (EC 1.1.1.27). Three loci code for this tetrameric enzyme: *LDH-1** and *LDH-2** were predominantly expressed in muscle tissue and *LDH-3** in liver tissue. *LDH-1** and *LDH-3** were considered monomorphic but *LDH-2** showed genetic variation for a slower allele (*80) in one heterozygote from Blanes.

• Mannose phosphate isomerase *(MPI)* (EC 5.3.1.8). We detected the product of a single locus for this enzyme. We found a slower allel (*80) in two heterozygotes from Gandía.

• Phosphoglucomutase *(PGM)* (EC 5.4.2.2). One locus was assumed to code for the two anodal bands predominantly expressed in muscle extracts. For this enzyme we found two heterozygotes from Donostia and Huelva with a faster allel (*117) and two heterozygotes from Gandía and Huelva with a slower allel (*85).

• Peptidase *(PEP)* (EC 3.4). Several loci were detected more strongly in liver and muscle extracts using four different polypeptides as substrates:

– Glycil-leucine (dipeptid). We observed one anodal zone of activity designated as *PEP-2**. We found some heterozygotes from Huelva with a fast and slow allelic variation.

– Leucil-glycil-glycine (tripeptid). Two areas of activity are observed, the fastest of which presents an allelic variant (*117) in heterozigote individuals belonging to the populations of Port de la Selva, Palamós, Gandia, Sant Carles de la Ràpita and Huelva. The other area of activity, with a slower mobility, presents variation in a heterzigote individual from the Donostia population. This area is designated as *PEP-1** locus.

– Leucil-tyrosine (dipeptid). We found two anodal areas of activity which seem to coincide with those that can be observed using the Glycil-leucine substrate. The one with a lowest mobility was designate as *PEP-2**, as we pointed out above, whereas the one with a highest mobility was designate as *PEP-3**. It is worth noting that the *PEP-2** locus presents a better resolution using Glycil-leucine as substrate, and the *PEP-3**, on the contrary, gets a better resolution using the Leucil-tyrosine substrate.

– Phenylalanil-proline (dipeptid). A single anodal area of activity, with a fast allele variant in a heterozigote individual from the locality of Huelva. This locus is designated as *PEP-4**.

–Polimorphic loci. (P > 0.95, P based on the 95 % criterion for polymorphism).

Out of the 33 loci considered proper for population surveys, a total of 6 loci were polymorphic by the 95 % criterion. The electrophoretic variability pattern for each of the following enzymes was consistent with that expected for allelic segregation at a locus coding for an enzyme of that particular quaternary structure. In addition, in those loci that were expressed in more than one tissue, our genetic interpretations were supported by the consistency of electrophoretic banding patterns among tissues.

• Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* (EC 1.2.1.12). Three different loci coding for this enzyme; two of these *(GAPDH-2** and *GAPDH-3*)* were represented by two single invariant bands, only expressed in eye-fluids; but the other one, *GAPDH-1**, is only present in skeletal muscle tissue and showed allelic variation in some individuals. This allelic variation (*300) was observed in all of the seven localities analysed.

• Glucosephosphate isomerase *(GPI)* (EC 5.3.1.9). This enzyme has been shown to be encoded by two loci in all samples examined. *GPI-1** was predominantly expressed in muscle extracts and *GPI-2** in eye-fluids. Under the conditions used in the present study the gene products of the two loci migrated anodally. Both loci were

polymorphic in our material. The polimorphism in *GPI-1** was determined by three codominant alleles, *GPI-1**100, *GPI-1**200 and *GPI-1**240. The last one appears in a very low frequency only in Palamós. In *GPI-2** locus we have found four codominant alleles, *GPI-2**100, *GPI-2**125, *GPI-2**140 and *GPI-2**90. The fastest allele (*140) only appears in low frequency in samples from Blanes and the slowest allele (*90) has only been found, also in low frequency, in Atlantic localities.

• Superoxide dismutase *(SOD)* (EC 1.15.1.1). The superoxide dismutase appeared in two zones in zymogram with liver extracts. The slower one was polymorphic with three alleles, *SOD-1**100, *SOD-1**20 and *SOD-1**125. The *125 allele was only seldom observed in samples from Gandía. All homozygotes showed a double-banded pattern with distinct migration rates. All heterozygotes showed five bands. One part of the pattern attributed to the slower allele moved in a cathodal direction relative to the place of origin. The other zone of *SOD*-activity *(SOD-2*)* was monomorphic and appeared to be less active. This locus was not used to analyse the genetic variability because it showed a bad resolution.

• Catalase *(CAT)* (EC 1.11.1.6). One zone of activity was found for this tetrameric enzyme, predominantly expressed in liver and kidney tissues. Allelic variation was observed in samples analysed from Palamós, Port de la Selva, Gandía and Huelva. We only analysed samples from these localities because those were the only places from which we had samples at the moment of analysing this enzyme. Two allelic variants, designated as *CAT**100 and *CAT**90, appeared in all of these samples. This enzyme was not used in the eventual analysis of genetic variability.

• Glycerol-3-phosphate dehydrogenase *(G3PDH)* (EC 1.1.1.8). A single area of activity was obtained, predominantly expressed in muscle extracts. This enzyme appears to be encoded by a single locus, designated as *G3PDH**. Allelic variation, *G3PDH**100 and *G3PDH**88-95, was observed for this dimeric enzyme in all samples under study. The genetic basis for this polymorphism was supported by consistency of banding patterns among all tissues examined. The faster homozygotes showed a single band pattern, the heterozygotes showed three bands and the slower homozygotes two bands. We supose that the double-banded pattern in slow homozygotes was due to a post-transductional processes but it is not a sure fact in the present state of affairs. This is the reason why we expressed the slower allel as *88-95. In other congeneric species, *M. capensis* and *M. paradoxus* for example, this enzyme appeared in muscle tissue encoded by three different loci: *G3PDH-A** and *G3PDH-B** showed invariant bands on the gels, but *G3PDH-C** had triple-banded heterozygotes typical of dimeric enzymes (Grant *et al.*, 1988b).

–Allele frequencies

The total amount of enzymes analysed has been of 24 and 34 loci have been detected (Table 2). Sixteen of these loci presented genetic variability *(AAT-1*, AAT-2*, CAT*, GAPDH-1*, G3PDH*, GPI-1*, GPI-2*, IDH-2*, LDH-2*, MPI-1*, PGM-1, PEP-1*, PEP-2*, PEP-3*, PEP-4** and *SOD-1*)*, but only six *(GAPDH-1*, G3PDH*, GPI-1*, GPI-2*, SOD-1** and *CAT*)* can be considered polymorphic at the 5 % level, which is the criterion used in the present paper. The allelic frequencies calculated in all samples, for each different polymorphic loci, except for the *CAT**, are summaryzed in Table 3.

For the 12 samples analysed, belonging to the 7 sampled localities, only 3 *(GPI-2** in

TABLE 3. Frequencies of most common alleles based on 5 polymorphic loci of *M merluccius*. Locality samples: PS1 = Port de la Selva 1, PS2 = Port de la Selva 2, B1 = Blanes 1, B2 = Blanes 2, B3 = Blanes 3, P1 = Palamós 1, P2 = Palamós 2, SC1 = Sant Carles de la Ràpita, G1 = Gandia, H1 = Huelva, D1 = Donostia 1, D2 = Donostia 2.

Freqüències dels al·lels més comuns dels 5 loci polimòrfics de *M. merluccius*.

	Locality samples											
Loci	PS1	PS2	B1	B2	B3	P1	P2	SC1	G1	H1	D1	D2
GAPDH.1*												
N	35	36	10	28	25	25	25	52	55	65	30	30
100	.443	.472	.600	.518	.400	.500	.520	.442	.418	.808	.867	.783
300	.557	.528	.400	.482	.600	.500	.480	.558	.582	.192	.133	.217
GPI-1*												
N	35	36	10	28	25	25	25	52	55	65	30	30
100	.529	.528	.650	.536	.580	.580	.400	.529	.573	.485	.583	.467
200	.471	.472	.350	.464	.420	.420	.580	.471	.427	.515	.417	.533
240	.000	.000	.000	.000	.000	.020	.000	.000	.000	.000	.000	.000
GPI-2*												
N	35	36	10	28	25	25	25	51	55	65	30	30
100	.357	.319	.300	.304	.320	.400	.300	.363	.300	.292	.267	.183
125	.643	.681	.700	.679	.660	.600	.700	.637	.700	.700	.700	.800
140	.000	.000	.000	.018	.020	.000	.000	.000	.000	.000	.000	.000
90	.000	.000	.000	.000	.000	.000	.000	.000	.000	.008	.033	.017
G3PDH*												
N	35	30	10	28	24	25	25	52	55	65	30	30
100	.671	.800	.450	.714	.646	.600	.700	.673	.718	.677	.750	.783
90	.329	.200	.550	.268	.354	.400	.300	.327	.282	.323	.250	.217
80	.000	.000	.000	.018	.000	.000	.000	.000	.000	.000	.000	.000
SOD-1*												
N	35	36	10	28	25	25	25	52	55	65	30	30
100	.557	.750	.700	.536	.720	.760	.720	.625	.655	.800	.800	.783
20	.443	.250	.300	.464	.280	.240	.280	.375	.345	.200	.200	.217

Blanes 2, *SOD-1** in Palamós 2 and Huelva) out of the 60 tests of adjustment from the genotypic frequencies observed to the expected, according to Hardy-Weinberg, have provided significant differences. This only represents the 5 % of the tests carried out, for which reason we consider this a casual fact and without any biological significate. These results indicate that no selection has taken place due to the sampling method and that no genetic migration (arrival of genetically different individuals) has occurred either all along the period of sampling.

The application of the statistical test of heterogeneity among samples of one single locality, for the allelic frequencies, does not result in relevant differences, at the 5 % level, for different loci among the samples from Blanes, Palamós or Donostia. Among the samples from Port de la Selva significant

differences appear for the *SOD-1** locus, but the χ^2 for all loci is not significant. This allows us to consider the various samples from a single locality as belonging to same biological population and therefore, we can group them in a single one corresponding to each locality of sampling for further analysis. The allelic frequencies of the polymorphic loci corresponding to this localities are shown in Table 4.

After applying the adjustment tests again at the genotypic frequecies of the Hardy-Weinberg balance, only the locus *SOD-1** perdures, with significant differences, in the locality of Huelva. This represents less than 5 % of the carried out tests, which reinforces

TABLE 4. Pooled frequencies of variant alleles based on 5 polymorphic loci of *M. merluccius* indifferent sampling localities. Key of localities: PS = Port de la Selva, B = Blanes, P = Palamós, SC = Sant Carles de la Ràpita, G = Gandia, H = Huelva, D = Donostia.

Freqüències al·lèliques totals dels al·lels més comuns dels 5 loci polimòrfics de *M. merluccius* en cadascuna de les localitats mostrejades.

	Localities						
	PS	B	P	SC	G	H	D
GAPDH-1*							
(N)	71	63	50	52	55	65	60
100	0.458	0.484	0.510	0.442	0.418	0.808	0.825
300	0.542	0.516	0.490	0.558	0.582	0.192	0.175
GPI-1*							
(N)	71	63	50	52	55	65	60
100	0.528	0.571	0.490	0.529	0.573	0.485	0.525
200	0.472	0.429	0.500	0.471	0.427	0.515	0.475
240	0.000	0.000	0.010	0.000	0.000	0.000	0.000
GPI-2*							
(N)	71	63	50	51	55	65	60
100	0.338	0.310	0.350	0.363	0.300	0.292	0.225
125	0.662	0.675	0.650	0.637	0.700	0.700	0.750
140	0.000	0.016	0.000	0.000	0.000	0.000	0.000
90	0.000	0.000	0.000	0.000	0.000	0.008	0.025
G3PDH*							
(N)	65	62	50	52	55	65	60
100	0.731	0.645	0.650	0.673	0.718	0.677	0.767
90	0.269	0.347	0.350	0.327	0.282	0.323	0.233
80	0.000	0.008	0.000	0.000	0.000	0.000	0.000
SOD-1*							
(N)	71	63	50	52	55	65	65
100	0.655	0.635	0.740	0.625	0.655	0.800	0.792
20	0.345	0.365	0.260	0.375	0.345	0.200	0.208

the previously mentioned idea about the lack of biological signification of this fact. Likewise, the fact that no significant differences were obtained within the analysed localities from the later grouping of samples supports the idea that the various samplings for a single locality were affecting a single biological population.

Discussion

–Genetic variation within populations

A total of 33 loci were identified and were considered suitable for population surveys using the techniques employed in the present investigation. This number could be increased by modifications in the electrophoretic and staining conditions; in particular, *POD*, BGA** and *FBALD** could be further analysed.

The proportion of polymorphic loci (referred to the 95 % criterion for polymorphism) and the average heterozigosity (H), calculated on the basis of the expected allele frequencies in each sample, are given in Table 5. The values of heterozigosity obtained are very similar to the average for bony fishes in general (Nevo, 1978; H= 0.051), similar to the average for 161 species

TABLE 5. Proportion of polymorphic loci (with *CAT**) and average heterozygosity (H) on *M. merluccius* and other species.

Proporció de loci polimòrfics (incloent-hi *CAT**) i heterozigositats mitjanes (H) en *M. merluccius* i altres espècies.

	Proportion of polymorphic loci	Heterozygosity (H)
Port de la Selva	14.7	0.068
Blanes	14.7	0.070
Palamós	14.7	0.068
Sant Carles de la Ràpita	14.7	0.070
Gandia	14.7	0.067
Average Mediterranean	14.7	0.068
Huelva	14.7	0.059
Donostia	14.7	0.055
Average North-Atlantic	14.7	0.057
Merluccius merluccius	14.7	0.0625
Merluccius capensis	22.6	0.063
Merluccius paradoxus	25.8	0.065
Teleosts Marine		0.055
Teleosts		0.051

TABLE 6. Results of the contingency chi-squares tests.
* = p < 0.05, ** = p < 0.01, *** = p < 0.001; df = degrees of freedom.
Valors de la khi-quadrat dels tests d'homogeneïtat de freqüències de l'al·lel *100.

Polymorphic loci

(χ^2, df)	*GAPDH-1**	*GPI-1**	*GPI-2**	*G3PDH**	*SOD-1**	Total χ^2
Among samples	(91.54, 6) ***	(10.57, 12)	(30.56, 18) *	(12.12, 12)	(19.56, 6) **	(164.36, 54) ***
Within Medit. Sea	(2.197, 4)	(6.739, 8)	(5.515, 8)	(6.731, 8)	(3.803, 4)	(27.98, 32)
Within North-Atl. Ocean	(0.124, 1)	(0.407, 1)	(2.471, 2)	(2.493, 1)	(0.027, 1)	(5.522, 6)
Between Medit. and Noth-Atlantic	(89.238, 1) ***	(1.363, 2)	(13.864, 3) **	(1.386, 2)	(15.471, 1) ***	(121.32, 9) ***

of marine teleosts (Smith & Fujio, 1982; H= 0.055) and for other species of *Merluccius (M. capensis,* H= 0.063; *M. paradoxus*, H= 0.065; Grant *et al.*, 1987).

Considering the absence of recent bottlenecks, it should be expected that those marine organisms which present large populations should have a high percent of heterozigosity, as opposed to organisms which generally present more reduced populations (Fuerst *et al.*, 1977; Gyllensten, 1985). A high heterozigosity value would imply a longer history of the population, without drastical changes in size or structure of the latter (Grant & Stahl, 1988a). If we consider other species of the same order as the Gadiphorms, such as *Gadus morhua and G. macrocephalus*, we find that the first species presents a high heterozigosity percent (12.5 %), as opposed to *G. macrocephalus,* which presents a very low percent of heterozigosity (2.5 %). This could be explained by reference to the fact that *G. macrocephalus* is in a bottleneck at the present, since the geological history of the North-Atlantic oceans suggests that the Pacific Ocean cod *(G. macrocephalus)* differentiated itself not too long ago and that it has not been long enough yet for this secies to reach the equilibrium and present higher heterozigosity levels (Grant & Stahl, 1988c). Thus, the clearly superior values obtained in the case of the hake should indicate the absence of recent bottlenecks in this species, both in the case of Atlantic and Mediterranean populations. That would signify a contrast with the prenous mentioned fact of the over-exploitation that this species is subject to, out of which we deduce that it is only their considerably large size which has allowed the Atlantic and Mediterranean populations of hake to avoid a decrease in heterozigosity as a consequence of the intensive exploitation.

–Genetic variation between populations

Marine fish in general tend to show very little genetic subdivision among geographic stocks (Ryman *et al.*, 1984; Shaklee, 1984) because there is a very large potential for gene flow between areas by the passive drift of larvae in ocean currents and by active migration of adults. Gene flow, when it occurs, acts as a strong homogenizing force on geographically separated stocks or populations (Waples, 1987). Another reason why marine fishes are characterized by a severe lack of population subdivision is that a genetic drift is negligible at very large population sizes that are typical of marine fishes. Even for populations that become completely isolated from one another, at least N generations must pass before any substantial divergence is likely (Kimura, 1955). Nonetheless, genetic subdivisions among regional stocks of marine fishes have been reported, which appear to be the result of ancient allopatric subdivision rather than the result of differenciation in situ (Grant, 1987).

Comparing the allele frequency distributions for the polymorphic loci among all samples, our most striking observation concerns the large allele frequency differences (χ^2 = 164.368, df = 54, p < 0.001), basically due to differences in loci *GAPDH-1*, GPI-2** and *SOD-1** (Table 6). These results indicated that the samples did not represent a single homogeneous gene pool. If we compare the allele frequency distributions within regions we find an obvious lack of allele frequency differences between localities. But if the statistical analysis were performed between regions, Mediterranean Sea and North-Atlantic Ocean, important differences in allelic frequency distributions were observed (χ^2 = 121.32, df = 9, p < 0.001). These differences were due to loci *GAPDH-1*, GPI-2** and *SOD-1* (GAPDH-1*,* χ^2 = 89.238, df = 1, p < 0.05; *GPI-2**, χ^2 = 13.864, df = 3, p < 0.05; *SOD-1*,* χ^2 = 15.471, df = 1, p < 0.05). Three of the six polymorphic loci showed significant allele frequency differences between North-Atlantic

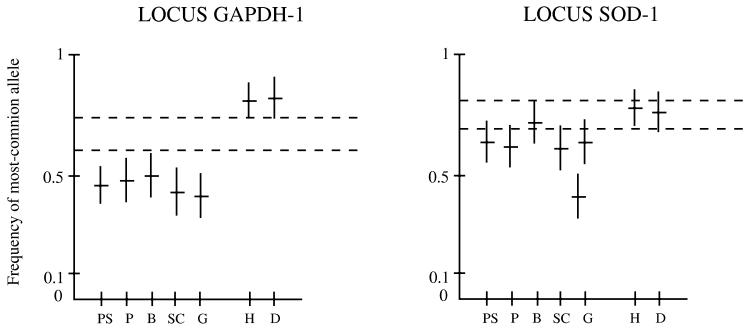

FIGURE 2. Common-allele frequencies of *GAPDH-1** and *SOD-1** in samples of *M. merluccius*. Vertical bars represent the standard error (aproximate a 95 % confidence interval). Key of localities corresponds to those in Table 4.
Freqüències de l'al·lel més comú dels loci *GAPDH-1** i *SOD-1** en les mostres de *M. merluccius*. Les barres verticals representen l'error estàndard amb un interval de confiança del 95 %. La clau de localitats es correspon amb la de la taula 4.

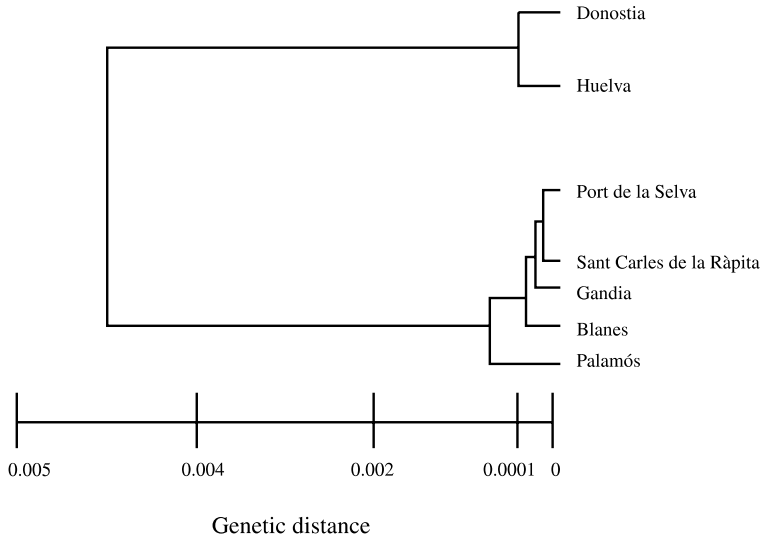

FIGURE 3. Dendrogram showing results of UPGMA cluster analysis of genetic distance (Nei, 1972) between samples based on 33 protein-coding loci.

Dendrograma corresponent a l'anàlisi de *cluster* (UPGMA) utilizant la distància genètica de Nei (1972) entre mostres, basat en 33 loci que codifiquen proteïnes.

and Mediterranean Sea samples. Data for *GAPDH-1** and *SOD-1** suggested that the North-Atlantic population was genetically distinct from the Mediterranean population. Apart from these differences, the remaining loci were largely homogeneous, even the locus *GPI-2*,* in which the allele frequency differences were due to the presence of rare alleles.

Most of the genetic heterogeneity in the North-Eastern Atlantic hake is due to differences in the loci *GAPDH-1** and *SOD-1**. Within the samples from the North-Atlantic Ocean the allelic frequency of *GAPDH-1** was very similar but changed all fashion in the Mediterranean Sea samples (Fig. 1; Fig. 2). Likewise, data for *SOD-1** showed that the frequency of the most common allele declined from the North-Atlantic Ocean samples to Mediterranean Sea samples (fig. 2; table 7). One possible explanation for this is that the Gibraltat Strait could represent an ecological barrier which would tend to reduce the gene flow between these two areas. A similar biogeographic model appears to explain the distinct nature of the Baltic Sea samples of North-Atlantic cod in which the genetic differences to the Atlantic samples were consistent to isolation due, perhaps, to ecological barriers such as salinity differences (Mork *et al.*, 1985).

If the regional differenciation in *M. merluccius* was due to the barrier effects of

TABLE 7. Common-allele frequencies of *SOD-1** in samples of *M. merluccius* from several localities and the contingency chi-squares test. N = number of fish analysed, n.s. = nonsignificant, df = degrees of freedom.

Freqüències dels al·lels més comuns de la *SOD-1** en mostres de *M. merluccius* de diferents localitats. La khi-quadrat es refereix als tests d'homogeneïtat de freqüències de l'al·lel *100.

Localities	**N**	**SOD-1* (100)**	**Region**	χ^2	χ^2
Norway (SE of Viking Bank)	27	0.889	North-Atlantic		
NW of Muckle Flugga	39	0.846	North-Atlantic		
W of Sulisker	24	0.854	North-Atlantic		
Stornoway Ground E.	126	0.837	North-Atlantic	10.5	
Flannan Islands	21	0.762	North-Atlantic		
St. Kilda Ground	45	0.778	North-Atlantic	df = 11	
Barra Head Ground	108	0.819	North-Atlantic		
Tory Island Ground	109	0.862	North-Atlantic	n.s.	
Dinglebay Ground	10	0.750	North-Atlantic		84.4
Coral Bank (Great Sole bank)	16	0.750	North-Atlantic		
Donostia	60	0.792	North-Atlantic		df = 17
Huelva	65	0.800	North-Atlantic		
					p < 0.001
Gandia	55	0.655	Mediterranean		
Sant Carles de la Ràpita	52	0.625	Mediterranean	4.1	
Blanes	50	0.740	Mediterranean		
Palamós	63	0.635	Mediterranean	df = 5	
Port de la Selva	71	0.655	Mediterranean		
Thyrrenian Sea	50	0.632	Mediterranean	n.s.	

the Gibraltar Strait, we should expect, first of all, that all of the loci in a species would be affected to the same extent and that each locus would show a similar amount of divergence (Allendorf & Phelps, 1981) and would have similarly affected other species in the same area (Grant *et al.*, 1987). Till now, there are no reports on genetic data from the same area, so as to compare the results. Our observation that *GAPDH-1** and *SOD-1** show a significantly greater degree of geographic differenciation than other loci can suggest also that these loci may be under selection. It is, however, notoriously difficult to check that with allele frequency alone (Ewens & Feldman, 1976); and no disequilibrium with the Hardy-Weinberg expectations have been observed.

Population genetic data for the european hake have previously been described:

Mangaly & Jamieson (1978) and Presciuttini *et al.* (1987, oral communication). The polymorphisms of *SOD-1** reported in these studies are strikingly similar to those of the present investigation, both with regard to the relative electrophoretic mobility of alleles (as judged from the zymograms) and the frequencies of variant alleles. Assuming, (with the reservation that the allozymes have not been compared on the same gels), that the alleles at *SOD-1** described in these studies are electrophoretically identical to ours, the lack of spatial differenciation of allele frequencies is conspicuous by comparing allele frequencies from the same area: the North-Atlantic Ocean and the Mediterranean Sea (Table 7). But if we compared by pooling all the allelic frequencies that we had, we found significant differences in this locus due to genetical differences between the North-Atlantic and Mediterranean populations of hake (Table 7).

This genetic population structure is also reflected in the gene diversity analysis. The greatest amount of subdivision (2.64 %) was due to differences between the North-Atlantic and Mediterranean samples. Only a small amount of the total was due to differences among localities within regions (0.23 %). The remaining gene diversity, 96.92 %, was contained within localities.

The observation of that low amount of genetic heterogeneity, with conspicuous allele frequency differences found in three loci *(GAPDH-1*, SOD-1** and *GPI-2*),* is also clearly reflected in the little genetic distances (Nei, 1972), averaged from 0.002 to 0.006, and the resulting dendrogram (Fig. 3). The major observation from the dendrogram includes the distinct subclustering of samples from North-Atlantic Ocean and Mediterranean Sea, respectively. The distance values were very low considering that the samples were being collected over a big range of the species distribution. But these were ten times higher than the ones found in other congeneric species, *M. capensis and M. paradoxus,* which averaged from 0.0006 to 0.0007 (Grant *et al*., 1987).

All results indicate that the genetic differenciation of european hake is weak. This characteristic is particularly reflected in the very low genetic distance between the most diverse samples (0.006), and the small fraction of the total gene diversity explained by differences among samples (2.64 %). These characteristics persist amidst the apparent distinction of the samples from the North-Atlantic Ocean and Mediterranean Sea, respectively. Considering the possibility of natural selection affecting the genotypic distributions of the two most variable loci *(GAPDH-1** and *SOD-1*)* does not change the impression of a general amount of genetic differenciation among the sampled populations. Although we lack information on the type of selection that may occur, it is reasonable to assume that environmental heterogeneity would tend to amplify allele frequency differences in selected loci and, thus, inflate the estimates of genetic divergence (Grant *et al.*, 1987). Under a neutral model, the low amount of genetic differenciation may be explained as a result of a substantial gene flow between populations. In contrast, the distinct nature of the North-Atlantic samples from the Mediterranean is more consistent with a model implying restricted gene flow between these areas due to the barrier effect of the Gibraltar Strait. In Atlantic cod *(G. morhua),* the Baltic Sea population exhibits a similar genetic divergence from the Atlantic populations due, perhaps, to ecological barriers such as salinity differences (Mork *et al.*, 1985).

Management decisions for the heavily exploited populations in the North-Atlantic

ocean are currently based on demographic data on «stocks» identified by means of ecological and morphological characters (Cushing, 1975). From a management perspective, an important observation in the present study concerns the existence of genetically distinct geographic populations within a european hake: West-Mediterranean and North-Atlantic populations, which have previously been described on the basis of morphological differences (Cadenat, 1952; Maurin, 1968). That fact allows us to think of the possibility of two different stocks of hake corresponding to these areas. Furthermore, the lack of genetic differenciation found within these areas confirms the unit stock interpretation for the North-Atlantic population (Jones & Mackie, 1970; Guichet, 1973 and Mangaly & Jamieson, 1978). This stock structure is similar to that of the Atlantic cod *(G. morhua),* which appears to be a single species with apparently sufficient gene flow to prevent substantial genetic divergence among stocks over broad geographic areas (Mork *et al.*, 1985). These patterns were also observed in several other marine species such as Pacific herring *(Clupea pallasi)* and Atlantic herring *(C. harengus)* (Grant, 1984; Grant & Utter, 1984; Ryman *et al.*, 1984). These findings and interpretations are important and require further attention, since the possibilities of formulating efficient management programs are dependent on the identification of biologically meaningful management units.

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