ICES CM 2004/Q:21

Stock discrimination and temporal and spatial genetic variation of sardine (*Sardina pilchardus*) in northeastern Atlantic, with a combined analysis of nuclear (microsatellites and allozymes) and mitochondrial DNA markers.

Panagiotis Kasapidis, Serge Planes, Veronique Laurent, Ricardo Quinta, Ana Teia, Philippe Lenfant, Stelios Darivianakis, Vasso Terzoglou, Giorgos Kotoulas and Antonios Magoulas

P. Kasapidis, S. Darivianakis, V. Terzoglou, G. Kotoulas, A. Magoulas: Hellenic Centre for Marine Research (HCMR), Institute of Marine Biology and Genetics, Gournes Pediados, GR 71500 Heraklion, Greece [tel: +30 2810337854, fax: +30 2810337820, e-mail: kasapidi@imbc.gr, magoulas@imbc.gr]. S. Planes, V. Laurent, P. Lenfant: Ecole Pratique des Hautes Etudes – UMR CNRS 8046, Université de Perpignan, Avenue de Villeneuve, 66860 Perpignan cedex, France [tel: +33 4 68 66 20 55, fax: +33 4 68 50 36 86, e-mail: planes@univ-perp.fr]. R. Quinta, A. Teia: Instituto de Investigação Agraria e das Pescas (INIAP/IPIMAR), Avenida de Brasilia, s/n, Lisboa, 1449-006, Portugal [tel: +351 21 3027000, fax: +351 21 3015948, e-mail: rquinta@ipimar.pt].

ABSTRACT

Within the context of EU project SARDYN, we assess genetic variability of sardine in northeastern Atlantic and compare it with the extremes of the species distribution. Three different types of genetic markers are used: allozymes, microsatellites and mitochondrial DNA RFLP's. The spatial and temporal stability of genetic properties will be evaluated by comparing results from different adult samples from 2000 and 2003 spawning season, adult samples from 2003 feeding season, as well as egg and larvae samples from 2003 spawning season. For the present contribution adult samples from the 2000 and 2003 spawning seasons were scored for 5 microsatellite loci, while adult samples from the 2003 spawning and feeding seasons were scored for 27 allozyme loci (10 of them being highly polymorphic). The first results indicate that the sardine populations from the Gulf of Cadiz along the continental shelf till the Celtic Sea and the English Channel most likely comprise a single stock, which is distinct from the Azores and Madeira populations as well as the Mauritania population.

Keywords: *Sardina pilchardus*, Atlantic, microsatellite DNA, allozymes, molecular markers, stock structure, marine fisheries

INTRODUCTION

The definition of fish stock is an important issue for the management of marine resources. Stocks are arbitrary groups of fish large enough to be essentially self-reproducing, with members of each group having similar life history characteristics (Hilborn and Walters, 1992). Although the stock definition implies some degree of genetic differentiation, stock identification and delineation has mainly relied on phenotypic characters. Variation in phenotypic characters is now known to have both environmental and genetic components and many recent works acknowledge that morphological differences between fish groups do not necessarily indicate separation into genetically distinct stocks (e.g. Foote *et al.*, 1999). So, the critical question is to what extent "phenotypic" stocks represent "genetic" stocks. In many cases, patterns of genetic and morphological variation are concordant, but the degree of this variation can be spectacularly different (Swain *et al*., 1999).

The sardine (*Sardina pilchardus*, Walbaum, 1792) is a small pelagic clupeoid fish, whose distribution in northeastern Atlantic extends from the southern Celtic and North Seas to Mauritania and Senegal, with residual populations off the Azores, Madeira and the Canary islands (Parrish *et al*., 1989). It is an important resource in Atlantic with 85 000 tons landed in 2000 and 102 000 tons landed in 2001 (ICES, 2002). As with many pelagic fishes, sardine stocks are not very well defined and there is not much literature on this subject. Stock delimitation in sardine has been mainly based on phenotypic data. Parrish *et al*. (1989) described four stocks in the Atlantic, based on meristic characters: the septentrional Atlantic stock, distributed from the North Sea (57°N) to the Cantabric coast of Spain (43°N), the Iberian stock, distributed from Cantabric coast to the Strait of Gibraltar (36°N), the Moroccan stock, distributed from Cap Spartel (36°N) to Cap Juby (28°N) and the Saharian stock, distributed from Cap Juby to Levrier Bay (21°N). Silva (2003) distinguished two sardine morphotypes, one that includes sardine from southern Iberia (southern Portugal, Gulf of Cadiz) and northern Morocco, and the other that includes sardine from the northern Atlantic and the Mediterranean. Unlike Quero and Vayne (1997) who distinguished two morphotypes in the Bay of Biscay, Silva found no evidence of a discontinuity in sardine shape along northern Atlantic. Studies of genetic

variability have failed so far to conclusively clarify the population genetic structure of sardine in the Mediterranean and north eastern Atlantic (Spanakis *et al.*, 1989; Magoulas 1990; Tinti *et al*., 2002).

For management purposes, the sardines in European Atlantic waters have always been considered as a single stock. The geographical limits of this stock have changed over time. Since 1980 it is delimited by the French-Spanish border to the north, and by the Strait of Gibraltar to the south (ICES Divisions VIIIc and IXa), hence currently known as the Atlanto –Iberian stock of sardine (ICES, 1980).

Currently, the stock structure and dynamics of sardine in the northeastern Atlantic are being studied by a multi disciplinary approach, within the EU project "SARDYN", which aims to synthesize available information in order to perform a robust assessment of this valuable resource. In order to provide a biologically defensible definition of the stock boundaries, the genetic structure and variability of the Atlantic sardine populations is assessed with the use of three different genetic markers: allozymes, microsatellites and mitochondrial DNA (mtDNA) RFLP's. Samples from the Atlanto-Iberian stock will be compared with samples from the extreme of the species distribution (Celtic Sea, Azores, Morocco) and the spatial and temporal stability of genetic properties will be evaluated by comparing results between given locations from the spawning and feeding seasons from the same and from different years.

Microsatellite and mtDNA analysis of adult samples is carried out by the Hellenic Centre of Marine Research, the allozyme analysis by the Université de Perpignan, and microsatellite analysis of egg and larvae by the Instituto de Investigação Agraria e das Pescas (INIAP/IPIMAR).

MATERIAL AND METHODS

Sampling

The general sampling scheme for the genetic analysis within SARDYN project includes:
a) Adult samples from the 1999/2000 spawning season from a core area that extends from the Bay of Biscay to the north to the Gulf of Cadiz to the south and additional samples from the Celtic Sea, Azores, Morocco and Western Mediterranean.
b) Adult samples from the 2003 spawning season from the core area, and additional samples from Mauritania, Morocco, Madeira, Azores, and the English Channel.
c) Adult samples from the 2003 feeding season from the core area.
d) Egg and larvae samples from the 2003 spawning season from the core area and the English Channel.
In the present contribution, for microsatellites we analysed 11 adult samples from the Atlantic coast (Table 1, Fig. 1), of which 7 are from the 1999/2000 spawning season (from Celtic Sea, Bay of Biscay, Cantabrian Sea, north Portuguese coast, Gulf of Cadiz, Azores and Morocco-Casablanca) and 4 from the 2003 spawning season (from north French coast, north Portuguese coast, south Portuguese coast, and Gulf of Cadiz). Each sample had a mean size of about 100 individuals randomly collected in a single trawl. Total body length and total weight were measured and a piece of tissue from each individual was stored in ethanol until used for DNA extraction.
For allozymes, 13 samples were analysed (Table 1, Fig. 1) that are distributed on the Atlantic coast, 9 of which were from the 2003 spawning season (from Mauritania, Morocco-Larache, Azores, Madeira, 2 samples from the north Portuguese coast, south French coast, north French coast, Channel) and 4 from the 2003 feeding season (French coast, north Portuguese coast, south Portuguese coast, Gulf of Cadiz). Each sample consisted of about 50 individuals randomly collected in a single trawl. For each specimen, total body length and total weight were recorded. All samples were stored at –30°C until the dissection in the laboratory.
For eggs and larvae six samples were collected (from the Gulf of Cadiz, Algarve, south Portuguese coast, north Portuguese coast, Bay of Biscay and the Channel) and kept in 4%

formaldehyde immediately after catch.

Microsatellite DNA analysis

DNA has been successfully extracted from ethanol preserved samples using standard protocols. All samples were scored for 5 microsatellite loci, namely Sp2, Sp7, Sp8, SpI5 and SpIII93, which were developed in HCMR. Three of the loci (Sp2, Sp7, Sp8) contain dinucleotide repeats while the other two (SpI5, SpIII93) contain tetra-nucleotide repeats. Polymerase chain reaction (PCR) amplification was carried out on a PCT-100 machine (MJ-Research). For the dinucleotide loci, microsatellite DNA fragments were separated on a VISTRA automatic sequencer and genotypes were scored by eye and rechecked. Alleles were designated according to size (number of nucleotides), determined by comparison with marker-alleles of various sizes from sardine individuals, whose size had been determined by comparison with standard fragments. For the tetranucleotide loci, microsatellite DNA fragments were separated on a Base Station automatic sequencer (MJ Research) and genotypes were scored with the software Chartographer.

Allozyme analysis

Fish were dissected in order to isolate the liver and a piece of muscle. Each piece of tissue was homogenized at 4°C in an equal volume of Tris/EDTA/NADP buffer (pH 6.8). Homogenates were centrifuged at 15 000 g for 30 min at 4 °C and the supernatants were stored at -80 °C. Samples were then processed by horizontal starch gel electrophoresis following the technique of Pasteur *et al* (1987) using four buffers (TC 6.7; TC 8.0, TBE 8.6 and TG 9.0). Twenty seven loci were clearly scored (enzyme nomenclature according to Shaklee *et al*, 1990): 6 phosphogluconate isomerase EC 1.1.1.44. (*6PGD**, TC 8.0 on liver), aspartate aminotransferase, EC 2.6.1.1 (*AAT**, TBE 8.6 on liver), creatine kinase, EC 2.7.3.2. (CK*, TC 8.0 on muscle), esterase EC 3.1.1.1 (*EST**, TBE 8.6 on liver), glucose phosphate isomerase EC 5.3.1.9 (*PGI**, TC 8.0 on muscle), guanine deaminase EC 3.5.4.3. (*GDA**, TC 8.0 on liver), Hexose phosphate deshydrogenase EC 1.1.1.47. (*HPD-1** and *HPD-2**, TC 8.0 on muscle), Isocitrate deshydrogenase EC 1.1.1.42. (*IDH-*

*1** and *IDH-2**, TC 8.0 on muscle and liver, respectively), lactate deshydrogenase EC 1.1.1.27 (*LDH-1** and *LDH-2**, TC 8.0 on muscle), malate deshydrogenase EC 1.1.1.37 (*MDH-1** , *MDH-2**, TC 8.0 on muscle and *MDH-3**, TC 8.0 on liver), malic enzyme EC 1.1.1.40 (*ME-1* and ME-2**, TC 8.0 on muscle), peptidase using leucine-tyrosine substrate EC 3.4.11/13.* (*PEP LT**, TC 6.7 on liver), peptidase using leucine-glycine-glycine substrate EC 3.4.11/13.* (*PEP LGG-1** and *PEP LGG-2**, TG 9.0 on liver), peptidase using phenylalanine-proline substrate EC 3.4.11/13.* (*PEP PP-1** and *PEP PP-2**, TC 8.0 on muscle and TC 6.7 on liver respectively), peptidase using valine-leucine substrate EC 3.4.11/13.* (*PEP VL**, TC 8.0 on muscle), phosphoglucomutase EC 2.7.5.1 (*PGM-1** and *PGM-2**, TG 9.0 on liver), superoxide dimutase EC 1.15.1.1. (*SOD**, TG 9.0 on liver) and alpha-glycerophosphate deshydrogenase EC 1.1.1.8. (α*GPD**, TC 8.0 on muscle).

Alleles at polymorphic loci were assigned numerical designations expressing the mobility of their respective protein products relative to the mobility of the most common allele (designated 100) among the samples. Enzyme names and numbers follow the recommendations of the Commission on Biochemical Nomenclature (Shaklee *et al*, 1990).

Eggs and larvae genetic analysis

DNA extraction from eggs and larvae was performed using the commercial kit *High pure PCR Template Preparation* (Roche Diagnostics) with minor modifications. Before the addition of the first buffer the eggs were washed with PBS and were burst with a small plastic rod. After the washing the protocol was followed as recommended by the manufacturer. A total of 600 eggs (100 for each sampling area) and a number of larvae will be scored for at least 4 of the microsatellite loci. The actual analysis of egg and larvae samples has not been progressed considerably so far.

mtDNA analyses

For the mtDNA analyses, a 2000 bp part of the mtDNA will be amplified, which contains

the whole control region. The PCR product will be digested for three restriction enzymes. The digested fragments will be electrophoresed on an agarose gel and the produced patterns will be analysed. Fifty individuals from each sample will be analysed for this purpose. Actual analysis has not been progressed considerably so far.

Statistical analyses

The following statistical analyses were applied separately to each one of the microsatellite and allozyme data sets. Allelic and genotypic frequencies for polymorphic loci were obtained using the "GENETIX 4.05" package (Bonhomme *et al*. 1993) (available on: http://www.univ-montp2.fr/genome-pop/genetix.htm). Observed and expected heterozygosity values and the mean number of alleles per locus were computed for each sample for all loci. Deviations from Hardy-Weinberg equilibrium were estimated with the fixation index F_{IS} in each sample. The multilocus deviation from Hardy-Weinberg equilibrium was statistically tested using the Markov chain reaction implemented in Genepop 3.4 (Raymond & Rousset, 1995). Significance levels for statistical tests were adjusted, for each population separately, according to the sequential Bonferroni (Rice, 1989). Significant differences between allelic frequencies were tested using Fisher's exact test as implemented in Genepop 3.4 (Raymont and Rousset, 1995).
The level of genetic differentiation among samples was characterized using the Wright's standardized variance of allelic frequencies (*F*st, Wright, 1969) computed over all samples, with the Weir & Cockerham algorithm (1984), available in "GENETIX". Significance levels for statistical tests were adjusted, for each population separately, according to the sequential Bonferroni (Rice, 1989). A Factorial Correspondence Analysis for all populations for all loci was conducted using "GENETIX".

RESULTS

Microsatellites

In total there were found 52 alleles for Sp2 locus, 40 for Sp7, 48 for Sp8, 31 for SpI5 and 47 for SpIII93. The average number of alleles per locus ranged between 26.4 and 32.0 for the different samples. Observed heterozygosities were in general high, ranging between 0.84 and 0.89 (Table 2). The amount of genetic variability within samples, according to the average number of alleles per locus and the observed heterozygosity, was very similar among samples.

The probability tests for deviation from Hardy Weinberg equilibrium (HWE) for all loci combined showed that all populations were statistically significantly deviating from HWE expectations ($p < 0.05$) with heterozygote deficiencies. These deficiencies could possibly reflect a bias in genotyping more homozygotes than those actually existing on the gels (existence of null alleles), but other biologically meaningful explanations could also hold.

The Fisher's exact tests for allelic frequency differentiation across all populations revealed that the null hypothesis (no differentiation) is rejected for all loci (for Sp2 $p=0.0000$, for Sp7 $p<0.05$, for Sp8 $p<0.05$, for SpI5 $p=0.0000$ and for SpIII93 $p=0.0000$). When the test was applied to all pairs of samples across all loci, the Azoran sample (2000) was found significantly different ($p<0.0000$) from all the other samples. Also, some other of the pairwise comparisons showed statistically significant differences ($p<0.05$). For example, the sample from north French coast (2003) was different from all the samples of 2000 except from those from the Celtic Sea and Morocco, and the Cantabrian sample (2000) was different from the north Portuguese (2000) and the Bay of Biscay (2000) sample.

The Fst calculation showed an overall Fst estimation 0.0027, which points to a small degree of genetic subdivision in sardine populations in the study area. In the pairwise comparisons of samples, Azores sample was genetically distinct from all the other samples, while very few other pairs of samples showed statistically significant differentiation (Table 3).

The Factorial Correspondence Analysis, which depicts individuals on a space of n-dimensions according to their allelic composition, showed that the sample from the Azores was the most differentiated one (Fig. 2).

Allozymes

The average number of alleles per locus was much lower compared to microsatellites and ranged between 1.44 and 2.30 for the different samples. Observed heterozygosities ranged between 0.09 and 0.14 (Table 4).

Among the 13 samples, significant deviation from the Hardy-Weinberg equilibrium was observed for only 3 samples : Mauritania, Madeira, and Cadiz. The loci that are responsible for the deviation from the Hardy-Weinberg equilibrium are *SOD** and *PEP-LT** for Mauritania, *EST** and *PEP-LGG-2** for Cadiz and *PEP-LT**, *EST** and *GDA** for Madeira.

The Fisher's exact tests for allelic frequency differentiation across all populations revealed that the null hypothesis (no differentiation) is rejected for all the loci combined ($p<0.0001$) and for 10 loci out of the 27. The p-value was highly significant $p<0.001$ for 5 loci.

The test for allelic differentiation for all pairs of samples across all loci showed that four of the samples (Azores, Madeira, Mauritania and Morocco) are different from the others in most of the comparisons. At 5% level of significance ($p<0.05$) the Azoran sample differs from the rest except from Madeira, Central French coast (feeding season) and north Portuguese coast (spawning season), the Madeira sample differs from the rest except from Azores and north Portuguese coast (spawning season), the Mauritania sample is different from all the rest, and the Morocco sample is not significantly different from the north Portuguese samples from both the feeding and the spawning season. The Fst calculation among all pairs of samples (Table 5) confirms the genetic differentiation detected from the exact tests. The overall Fst was 0.050 ($P<0.001$).

The Factorial Correspondence Analysis showed that the sample from Mauritania was the more distinct one, and the samples from Azores, Madeira and Morocco occupied peripheral positions in the distribution of the cloud of the points (individuals) (Fig. 3).

DISCUSSION

Our analyses showed that there is a very high genetic variability within the sardine populations studied. This is probably due to the very large population sizes of sardine, a characteristic of many small pelagic fish, which allows the retaining of many different alleles within populations.

Both the microsatellite and allozyme genetic analyses showed that there were not significant or persistent genetic differences among samples from the area delimited from the Gulf of Cadiz in the south, along the coast to the English Channel to the north, between either different years (2000 and 2003) or the spawning and the feeding seasons. Microsatellite analysis showed some degree of significant genetic differentiation between the north French coast sample (spawning season 2003) and the rest of the samples to the south till the Gulf of Cadiz from the 2000 spawning season, but not with samples of those areas from 2003. For allozymes, the sample from south Portuguese coast (2003 feeding season) was significantly different from all the samples to the north till the English Channel, from both the 2003 feeding and spawning seasons, but not with the central French coast sample (2003 feeding season) and north Portuguese coast sample (2003 spawning season). These differences may reflect stochastic subtle changes in genetic constitution of the populations or sampling bias or may indeed indicate some persistent pattern of genetic differentiation. Further analyses of these samples for both markers will clarify whether those differences are persistent or stochastic.

On the contrary, the samples from the Azores for both the microsatellite and allozyme genetic analyses showed small but statistically significant differences with almost all the other samples. For microsatellites, F_{st} pairwise values between Azores and the other samples ranged from 0.0065 to 0.0164, while for allozymes from 0.04 to 0.22. Similarly, samples from Madeira, the south coast of Morocco and Mauritania, which where analyzed only for allozymes, were also different from the rest of the samples. The Azoran and Madeira samples had the greatest Fst values when compared with the Mauritania sample (Fst = 0.22 in both comparisons).

The reason for the low level of genetic differentiation among the sardine populations studied, as for many other marine organisms, is most likely a fair amount of gene flow

among populations (e.g. Ward *et al*, 1994; Waples, 1998). Gene flow could occur at either egg or larvae stage by passive drift along with ocean currents, by active migration of adult sardines, or both. Other studies of genetic variability have failed so far to clarify conclusively the genetic population structure of sardine in the Mediterranean and northeastern Atlantic. Only slight genetic differentiation was found between populations of Greek seas in an allozymic study (Spanakis *et al.*, 1989). Evidence has been obtained for genetically differentiated populations of sardine along the Almeria – Oran front in the western Mediterranean (Ramon and Castro, 1997), and separate subpopulations were also evidenced in the Adriatic and Ionian Seas (Carvalho *et al.*, 1994). On the contrary, no differences were found in mtDNA variation between samples from eastern Mediterranean and a sample collected from the area of Barcelona (Magoulas, 1990), and between the Adriatic and Ionian stocks (Tinti *et al*., 2002).

The preliminary results presented here, indicate that the sardine populations from the Gulf of Cadiz along the continental shelf till the Celtic Sea and the English Channel most likely comprise a single stock. The southern limits of this stock are difficult to be determined for the moment, since the Morocco sample (of 2000) analyzed for microsatellites does not differ from the rest samples of the north eastern Atlantic, while the Morocco sample (of 2003) analysed for allozymes shows statistically significant differences with about half of the north eastern Atlantic samples. The Azoran and Madeira populations seems to represent distinct stocks with restricted gene flow to both the north east Atlantic stock and to Morocco and Mauritania. The Mauritania sample most probably belongs to a quite different stock distributed southern of the Moroccan coasts. The analysis of more samples with both allozyme and microsatellite markers, as well as with mtDNA RFLP's, in combination with other types of data obtained from SARDYN project (morphometrics, tagging experiments, etc.) it will provide robust information for the delimitation of the sardine stock in the north eastern Atlantic.

ACKNOWLEDGEMENTS

This contribution was made under the European Union funded project SARDYN (QLRT-2001-00818). Authors wish to thanks all people involved in sampling and laboratory work during SARDYN project.

REFERENCES

Bonhomme, F., Belkhir, K., Borsa, P., Mathieu, E., Roux, M., 1993. Genetix: Logiciel d'analyse des données du groupe de génétique des populations de Montpellier. Ver 0.1. Montpellier : Université de Montpellier II.

Carvalho, G.R., Bembo, D.G., Carone, A. *et al.*, 1994. Stock discrimination in relation to the assessment of Adriatic anchovy and sardine fisheries. Final project report to the Commission of the European community, EC XIV-1/MED/91001/A.

Foote, C.J., Moore, K., Stenberg, K., Craig, K.J., Wenburg, J.K. and Wood, C.C., 1999. Genetic differentiation in gill raker number and length in sympatric anadromous and nonanadromous morphs of sockeye salmon, *Oncorhynchus nerka*. *Environ. Biol. Fishes* **54**, 263–274.

Hilborn, R., Walters, C.J., 1992. Quantitative Fisheries Stock Assessment. Choice, Dynamics and Uncertainty. Chapman & Hall, London, 570 pp.

ICES 1980. Rapport du Groupe de Travail pour l' évaluation des stocks de sardines dans les Divisions VIIIc et IXa. ICES C.M. 1980/H: 53. 41 pp.

Magoulas A., 1990. MtDNA variation in populations of sardine, *Sardina pilchardus* and anchovy, *Engraulis encrasicolus*, in the Greek seas and phylogenetic inferences. PhD dissertation, University of Crete (in Greek).

Parrish, R.H., Serra, R. and Grant, W.S., 1989. The monotypic sardines, *Sardina* and *Sardinops*: their taxonomy, distribution, stock structure, and zoogeography. *Canadian Journal of Fisheries and Aquatic Sciences* **46**, 2019–2036.

Pasteur, N., Pasteur, G., Bonhomme, F., Catalan, J., Britton-Davidian, J., 1987. Manuel de génétique par électrophorèses des protéines. Paris : Collection techniques et documentation, Lavoisier.

Quero, J.C., Vayne, J.J., 1997. Les poisons de mer des peches francaises. Delachaux et niestle (eds). Paris, 304 pp.

Ramon, M.M., and Castro, J.A., 1997. Genetic variation in natural stocks of *sardina pilchardus* (sardines) from the western Mediterranean Sea. Heredity, **78**, 520-528.

Raymond, M., Rousset, F., 1995. GENEPOP (vesrion1.2): population genetics software for exact tests and ecumenism. Journal of Heredity **86**: 248-249.

Rice, W.R., 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223-225.

Shaklee, J.B., Allendorf, F.W., Morizot, D,C., Whitt, G.S., 1990. Gene nomenclature for protein-coding loci in fish. *Transactions of the American Fisheries Society* **119,** 2-15.

Spanakis, E., Tsimenides, N., Zouros, E., 1989. Genetic differences between populations of sardine, *Sardina pilchardus*, and anchovy, *Engraulis encrasicolus*, in the Aegean and Ionian seas. *Journal of Fish Biology* **35**, 417-437.

Tinti, F., Di Nunno, C., Guarniero, I., Talenti, M., Tommasini, S., Fabbri, E., Piccinetti, C., 2002. Mitochondrial DNA sequence variation suggests the lack of genetic heterogeneity in the Adriatic and Ionian stocks of Sardina pilchardus. *Marine Biotechnology*, **4**, 163-172.

Table 1: Sample code and locality of the samples used in the present study for microsatellite and allozyme analyses. As core samples are characterized the samples collected from the Gulf of Cadiz to the south till the Celtic Sea to the north.

Samples for microsatellite analysis		**Samples for allozyme analysis**	
Sample code	Locality	Sample code	Locality
Core samples 2000 (Spawning season)		**Core samples 2003 (Spawning season)**	
1-CELT	Celtic Sea	1-SCNFRA	North French coast
4-PB	Bay of Biscay	2-SCSFRA	South French coast
5-CAN	Cantabrian Sea	10-SAOCNa	North Portuguese coast
7-NP	North Portuguese coast	10-SAOCNb	North Portuguese coast
9-CAD	Gulf of Cadiz		
		Extra samples 2003 (spawning season)	
Extra samples 2000 (Spawning season)		14-SCCHA1b	English Channel
11-MOR	Morocco, Casablanca	21-SEAZO	Azores
14-AZOR	Azores	22-SEMAD	Madeira
		24-SEMOR	Morocco, Larache
Core samples 2003 (Spawning season)		25-SEMAU	Mauritania
1-SCNFRA	North Central France		
4-SCOCN	North Portuguese coast	**Core samples 2003 (Feeding season)**	
5-SCOCS	South Portuguese coast	26-FCFRA	Central France
7-SCCAD	Gulf of Cadiz	27-FCOCN	North Portuguese coast
		28-FCOCS	South Portuguese coast
		30-FCCAD	Gulf of Cadiz

Table 2: Expected and observed heterozygosities (Hexp. and Hobs., respectively) and mean number of alleles per locus, for the samples analyzed for microsatellites.

	Hexp.	Hobs.	Mean mumber of alleles per locus
1CEL	0.92	0.86	28.4
4PB	0.92	0.87	32.0
5CAN	0.92	0.84	28.6
7NP	0.92	0.85	29.4
9CAD	0.92	0.86	26.4
11MOR	0.92	0.85	28.8
14AZOR	0.93	0.85	28.6
2-SCNFRA	0.91	0.89	28.0
4-SCOCN	0.92	0.88	28.6
5-SCOCS	0.92	0.87	30.0
7-SCCAD	0.93	0.88	28.8

Table 3: Fst values and p-values in parentheses obtained by 1000 permutations for the populations scored for 5 microsatellite loci. F-st values significant at 5% level ($p<0.05$) are in bold.

	4PB	**5CAN**	**7NP**	**9CAD**	**11MOR**	**14AZOR**	**2-SCNFRA**	**4-SCOCN**	**7-SCCAD**	**5-SCOCS**
1CEL	0.0007 (0.251)	0.0009 (0.184)	-0.0010 (0.830)	-0.0007 (0.719)	-0.00107 (0.823)	**0.0087 (0.000)**	0.0015 (0.103)	0.0001 (0.432)	0.0002 (0.383)	-0.0005 (0.625)
4PB		0.0018 (0.062)	0.0007 (0.219)	0.00065 (0.269)	0.0014 (0.086)	**0.0124 (0.000)**	**0.0034 (0.001)**	0.0015 (0.086)	0.0021 (0.039)	0.0008 (0.202)
5CAN			**0.0022 (0.036)**	0.00102 (0.187)	0.0004 (0.328)	**0.0101 (0.000)**	**0.0030 (0.015)**	0.0004 (0.306)	0.0012 (0.152)	-0.0009 (0.791)
7NP				0.00002 (0.450)	-0.0003 (0.544)	**0.0081 (0.000)**	**0.0026 (0.018)**	0.0018 (0.072)	0.0002 (0.346)	0.0002 (0.398)
9CAD					-0.0004 (0.629)	**0.0065 (0.000)**	**0.0042 (0.001)**	**0.0021 (0.024)**	0.0007 (0.201)	0.0002 (0.398)
11MOR						**0.0083 (0.000)**	0.0007 (0.251)	-0.0005 (0.697)	-0.0006 (0.675)	-0.0005 (0.625)
14Az							**0.0164 (0.000)**	**0.0146 (0.000)**	**0.0100 (0.000)**	**0.0074 (0.000)**
2-SCNFRA								0.0001 (0.459)	0.0016 (0.092)	0.0011 (0.166)
4-SCOCN									0.0012 (0.114)	0.0006 (0.294)
7-SCCAD										0.0002 (0.387)

Table 4: Expected and observed heterozygosities (Hexp. and Hobs., respectively) and mean number of alleles per locus, for the samples analyzed for allozymes.

	Hexp.	Hobs.	Mean mumber of alleles per locus
1-SCNFRA	0.13	0.13	2.11
2-SCSFRA	0.13	0.12	1.89
10-SAOCNa	0.13	0.12	2.04
10-SAOCNb	0.11	0.09	1.85
14-SCCHA1b	0.12	0.12	1.96
21-SEAZO	0.09	0.08	1.44
22-SEMAD	0.11	0.09	1.89
24-SEMOR	0.14	0.11	1.96
25-SEMAU	0.12	0.09	2.30
26-FCFRA	0.12	0.11	1.78
27-FCOCN	0.13	0.14	2.11
28-FCOCS	0.14	0.14	1.93
30-FCCAD	0.13	0.10	1.89

Table 5: Fst values and p-values in parentheses obtained by 1000 permutations for the populations scored for 27 allozyme loci. F-st values significant at 5% level ($p<0.05$) are in bold.

	1-SCNFRA	**25-SEMAU**	**24-SEMOR**	**27-FCOCN**	**10-SAOCNa**	**10-SAOCNb**	**28-FCOCS**	**30-FCCAD**	**26-FCFRA**	**14-SCCHA**	**21-SEAZO**	**22-SEMAD**
2-SCSFRA	**0.0143 (0.0233)**	**0.0917 (0.000)**	**0.0390 (0.0006)**	0.0193 (0.1297)	0.0147 (0.4734)	-0.0035 (0.4166)	**0.0257 (0.0016)**	0.0046 (0.2749)	**0.0116 (0.0349)**	0.0073 (0.7134)	**0.0384 (0.000)**	**0.0692 (0.000)**
1-SCNFRA		**0.0753 (0.000)**	**0.0472 (0.000)**	0.0122 (0.2729)	**0.0295 (0.0053)**	-0.0017 (0.9193)	**0.0299 (0.000)**	0.0098 (0.1793)	**0.0221 (0.0188)**	**0.0185 (0.0347)**	**0.0654 (0.000)**	**0.0533 (0.000)**
25-SEMAU			**0.0469 (0.0055)**	**0.0632 (0.000)**	**0.0663 (0.000)**	0.0960 (0.3351)	**0.1064 (0.000)**	**0.0631 (0.000)**	**0.1169 (0.000)**	**0.1123 (0.000)**	**0.2230 (0.000)**	**0.2220 (0.000)**
24-SEMOR				0.0206 (0.1445)	0.0043 (0.5150)	**0.0513 (0.000)**	**0.0217 (0.0302)**	**0.0116 (0.0372)**	**0.0381 (0.000)**	**0.0472 (0.000)**	**0.1395 (0.000)**	**0.1679 (0.000)**
27-FCOCN					0.0178 (0.2088)	0.0192 (0.0542)	**0.0246 (0.0092)**	0.0144 (0.4169)	0.0145 (0.0525)	0.0204 (0.2463)	**0.0765 (0.000)**	**0.0887 (0.000)**
10-SAOCNa						0.0243 (0.5088)	0.0195 (0.4071)	-0.0038 (0.3759)	0.0129 (0.2650)	0.0179 (0.4680)	**0.1021 (0.000)**	**0.1380 (0.000)**
10-SAOCNb							0.0192 (0.1029)	-0.0034 (0.3813)	0.0021 (0.9646)	-0.0040 (0.5798)	0.0312 (0.9228)	0.0658 (0.0528)
28-FCOCS								0.0102 (0.0886)	0.0197 (0.0663)	**0.0289 (0.0034)**	**0.0809 (0.000)**	**0.0940 (0.000)**
30-FCCAD									0.0071 (0.3890)	0.0073 (0.1683)	**0.0739 (0.000)**	**0.1062 (0.000)**
26-FCFRA										-0.0011 (0.3051)	0.0378 (0.1823)	**0.0884 (0.000)**
14-SCCHA											**0.0401 (0.0043)**	**0.0757 (0.000)**
21-SEAZO												0.0455 (0.2323)

Figure 1: Samples from the spawning season of 1999/2000 analyzed for microsatellites (open triangles), samples from the spawning season of 2003 analyzed for microsatellites (black triangles), samples from the spawning season 2003 analyzed for allozymes (empty circles), and samples from the feeding season 2003 analyzed for allozymes (black circles).

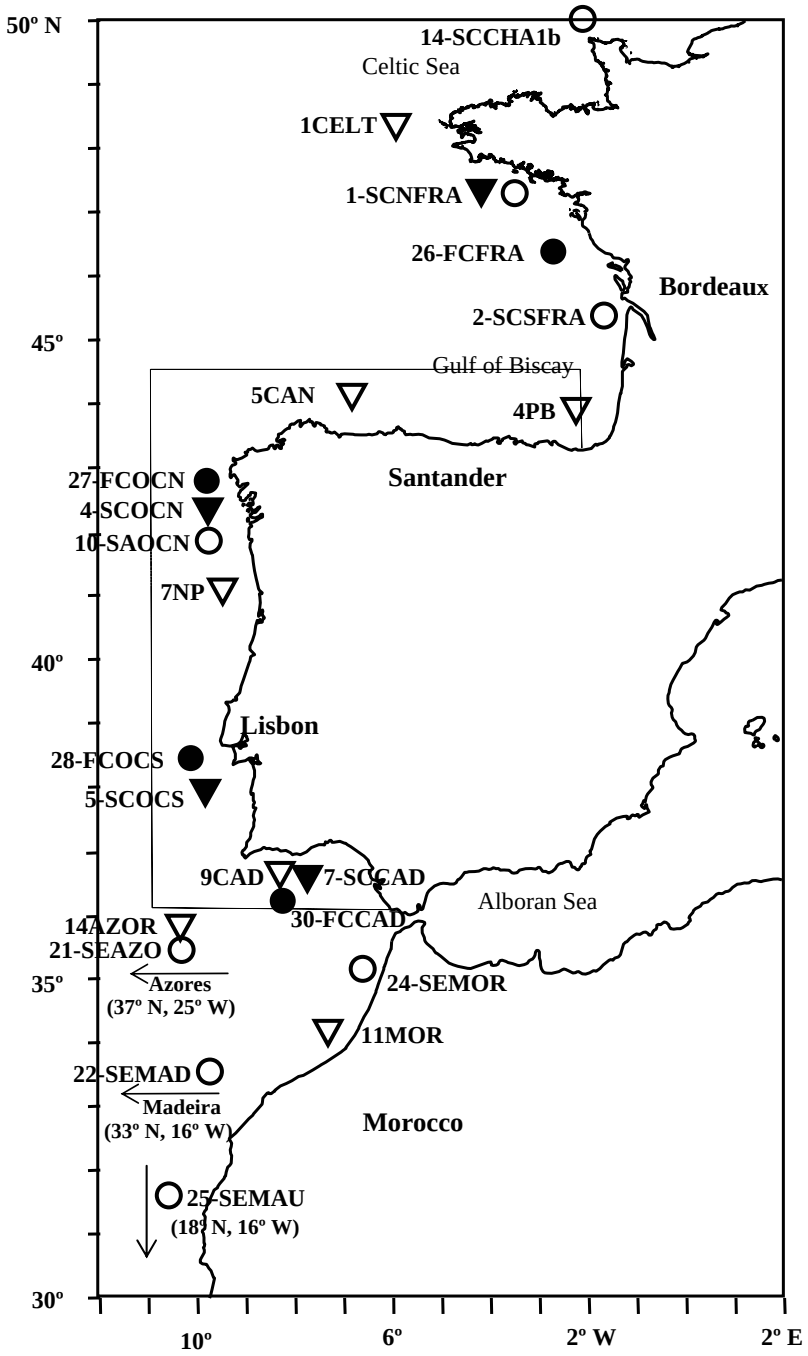

Figure 2: . Factorial Correspondence Analysis for all the populations analyzed for five microsatellite loci. The individuals of the Azoran sample (14AZOR) are indicated with different color and encircled with an oval line.

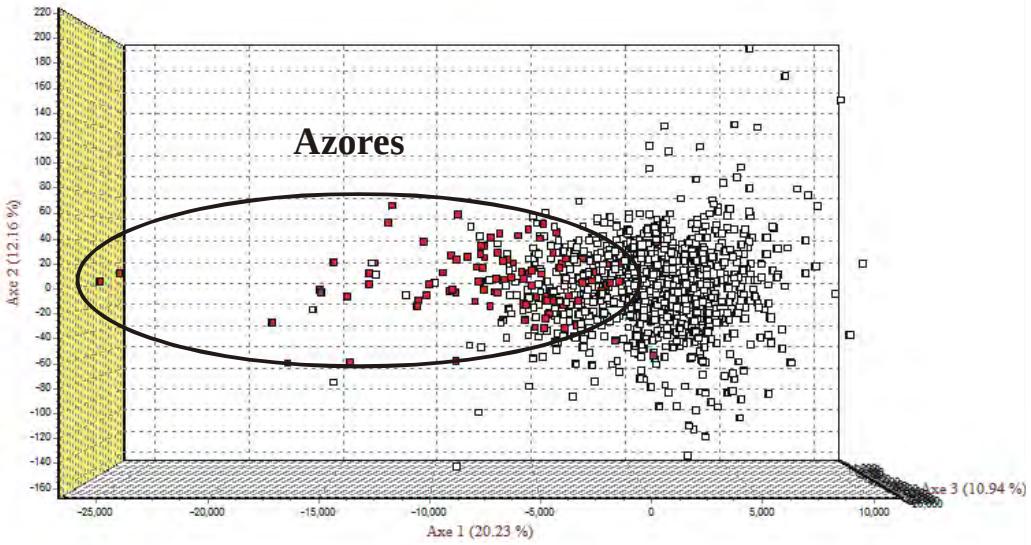

Figure 3: Factorial Correspondence Analysis for all the populations analyzed for 27 allozyme loci. The individuals of the Mauritania, Morocco, Madeira and Azores samples are encircled with an oval line. The individuals of the Azoran sample are indicated with different color.