

# Shallow genetic structure of three species of the genus *Trachurus* in European waters

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**ABSTRACT:** Pelagic marine fishes generally show little geographical population differentiation. Of special interest are marine species with Atlantic–Mediterranean distribution because of the possible effect of past and present day restrictions to gene flow between the 2 seas. Thus, restriction analysis of the whole control region (D-loop) of mitochondrial DNA (mtDNA) was used to extensively investigate the genetic structure of the 3 *Trachurus* species present both in Atlantic and Mediterranean waters: *T. trachurus*, *T. mediterraneus* and *T. picturatus*. In total, 666 individuals were screened from 5 Mediterranean and 6 Atlantic areas. Two African areas were also sampled for *T. trachurus*. No differentiation was revealed among European populations within the 3 studied species. Additionally, no sign of Atlantic–Mediterranean division was found, probably due to the fact that specific life history traits provide the *Trachurus* species with flexibility to oceanographical constraints and give them the ability to migrate long distances. Differentiation was only revealed between European and African populations of *T. trachurus*, reinforcing the existence of the *T. trachurus capensis* subspecies in African waters. Populations are probably under long and stable demographic equilibrium conditions as indicated by high haplotypic and nucleotide diversity values for all 3 species. Thus, mtDNA could be a powerful tool in determining the taxonomic distinctiveness of individual populations and species, and therefore, aid in setting priorities for future management policies. The present results are also discussed in comparison with the results of previous cytochrome b sequence analysis of the 3 *Trachurus* species.

**KEY WORDS:** *Trachurus* spp. · Mitochondrial DNA · D-loop · RFLP · Marine species · Mediterranean

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## INTRODUCTION

Many marine organisms have exceptional dispersal and migratory capabilities; species ranges can be vast and their life history may include high fecundities and explosive reproductive potentials (e.g. Graves 1998, Grewe & Hampton 1998). Although these features lead marine fishes to display a population structure that is characterized by a lack of significant heterogeneity even between ocean samples (e.g. Graves et al. 1984), striking differences have been found in certain cases (e.g. Kotoulas et al. 1995, Magoulas et al. 1996, Nesbø et al. 2000). These differences underscore the need for a thorough understanding of the genetic basis of population structure of marine species.

The degree of genetic differentiation between Atlantic and Mediterranean populations has been the

subject of study for many marine organisms. In many cases, the Mediterranean and the adjacent Atlantic coasts support very similar faunas (reviews in Borsa et al. 1997b, Bargelloni et al. 2003). However, for a growing number of species, the Strait of Gibraltar has been proposed to be the geographical constraint between these 2 biogeographical regions. Evidence for this hypothesis has recently been provided by moderately strong to strong intraspecific genetic variation in a variety of species, e.g. the swordfish *Xiphias gladius* (Kotoulas et al. 1995), 75% of the species studied in a review by Borsa et al. (1997b), *Platichthys flesus* and *P. stellatus* (Borsa et al. 1997a), the mussel *Mytilus galloprovincialis* (Quesada et al. 1998), the sea bass *Dicentrarchus labrax* (Naciri et al. 1999), the krill *Meganycitiphanes norvegica* (Zane et al. 2000) and a number of sparid fishes (Bargelloni et al. 2003). These studies

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have shown restricted gene flow between Atlantic and Mediterranean populations. Hypotheses for the observed patterns of differentiation have included various models such as isolation by distance with limited gene flow and, alternatively, secondary contact of previously isolated and divergent populations (Perez-Losada et al. 2002).

Three species of the genus *Trachurus*: *T. trachurus*, *T. mediterraneus* and *T. picturatus*, are found together in the Northeast Atlantic and Mediterranean waters and are commercially exploited in different water areas (following the International Council for the Exploration of the Sea [ICES] area divisions.) The species *T. trachurus* is commonly found in the waters off West Africa/Cape Verde Islands to the Norwegian Sea, including Iceland as well as the Mediterranean and Black Seas. It is the most northerly representative of the trachurid sub-family, which is widely distributed in the world's seas and often supports important fisheries. The other 2 species have a more restricted distribution expanding from the Bay of Biscay (France) southwards to Moroccan waters and eastwards in the Mediterranean Sea. *T. mediterraneus* is even found in the Marmara and the Black Seas (Froese & Pauly 2001). The name horse mackerel is also used to describe the subspecies *Trachurus trachurus capensis* that is distributed in the eastern Atlantic from the Gulf of Guinea to South Africa. However, there are researchers that suggest that this is not a valid taxonomic group (Froese & Pauly 2001).

Several studies have explored the biology and fisheries throughout the geographic range of the 3 *Trachurus* spp., especially of *T. trachurus*, with regard to reproduction and growth (e.g. Massé et al. 1996, Viette et al. 1997, Waldron & Kerstan 2001, Abaunza et al. 2003). However, there are extremely few data on their genetic structure. The first published attempt (Karaiskou et al. 2003) was based on sequence data of the cytochrome b gene of mtDNA. This work shed light mostly on the phylogenetic relationships of the 3 *Trachurus* species and provided a possible scenario of the divergence time of the 3 species related to the closure of the Gibraltar Strait. Global and pairwise tests of population differentiation (based on restricted sampling) did not reveal significant differentiation for the 9 European studied areas of the 3 *Trachurus* species.

Thus, the present study attempts to thoroughly investigate the genetic structure of the 3 *Trachurus* species populations (in an extensive sampling scheme) from the Mediterranean and Atlantic Seas, by means of restriction fragment length polymorphism (RFLP) analysis of a PCR-amplified mitochondrial DNA (mtDNA) region. Since different regions of mtDNA evolve at different rates, specific mtDNA genes have been targeted for phylogeny reconstruction (Hillis et

al. 1996), species identification and assays of intraspecific variation (Chow et al. 1993, Bembo et al. 1995). One of the mtDNA regions that has often been used is the control region (D-loop region). The rate of evolution of the D-loop region is reputedly 2 to 5 times higher than that of mitochondrial protein coding genes (Meyer 1993) and for this reason is the molecule of choice for the study of population level phenomena.

The primary objective of this study is to test whether the Strait of Gibraltar constitutes a constraint in the free movement of the 3 *Trachurus* species (*T. trachurus*, *T. mediterraneus* and *T. picturatus*) leading to the accumulation of genetic differences among Atlantic and Mediterranean populations. A further and more general objective is to see if genetic differences can even be detected among local *Trachurus* populations within the Mediterranean and the Atlantic Seas. Furthermore, this study aims to determine the genetic divergence and the phylogenetic relationships of the 3 *Trachurus* species. These objectives have obvious implications for the understanding of the population biology of the 3 species and their proper management.

## MATERIALS AND METHODS

**Sample collection.** Samples of the 3 *Trachurus* species were collected from 5 Mediterranean regions and 6 regions from the Atlantic Sea (Table 1 & Fig. 1). Samples of *T. trachurus* were also obtained from Namibia and South Africa, and samples of *T. trecae* from the area of Cadiz (Portugal) were also included in the analysis as an outgroup.

**DNA extraction, PCR amplification and restriction enzyme digestion.** White muscle tissue was taken from each individual and either stored at  $-20^{\circ}\text{C}$  or kept in absolute ethanol. DNA was extracted according to the protocol of Taggart et al. (1992). Overall, 12 geographic samples of *Trachurus trachurus* (344 individuals), 6 samples of *T. mediterraneus* (182 individuals) and 5 samples of *T. picturatus* (140 individuals) were screened for polymorphism in the D-loop region. In total, 666 individuals were analyzed. In addition, 12 individuals of *T. trecae* were scored.

The whole control region (D-loop) was amplified using primers that were designed based on the published sequence of the whole control region of *T. trachurus* under the Accession number AF271658. Primers were designed using the programme Primer3 (Rozen & Skaletsky 2000). PCR amplifications were performed using the Dloop-H (5'-CCAGTCTTG-TAAACCGAATGC-3') and Dloop-L primers (5'-GGGCCCCATCTTAACATCTTC-3') that bind at the tRNA<sup>Thr</sup> and at the tRNA<sup>Phe</sup> genes, respectively. They amplify the tRNA<sup>ProI</sup> and the whole control region,

Table 1. *Trachurus* spp. Sampling sites, sampling date and number of individuals (N) analyzed from every region

Species	Sampling area	ICES division	Sampling date	N
<i>T. mediterraneus</i>				
	N Aegean	–	05/00	37
	S Aegean	–	06/00	32
	Ionian	–	09/00	32
	W Italy	–	10/02	16
	E Spain	–	09/00	35
	Portugal	(IXa)	06/01	30
<i>T. trachurus</i>				
	N Aegean	–	08/01	30
	S Aegean	–	10/01	63
	Ionian	–	05/01	29
	W Italy	–	10/02	18
	Portugal	(IXa)	06/02	29
	N Spain	(VIIIc)	08/00	30
	NW Spain	(VIIIb)	08/02	32
	NW France	(VIIe)	04/00	12
	SE England	(IVb–c)	08/02	29
	W Norway	(IVa)	05/02	30
	South Africa	–	08/02	20
	Namibia	–	08/02	22
<i>T. picturatus</i>				
	N Aegean	–	05/00	30
	S Aegean	–	09/00	31
	Ionian	–	06/01	31
	W Italy	–	10/02	19
	Portugal	(IXa)	06/01	29
Total				666

giving a 1030 bp PCR product. Double-stranded DNA amplification was performed in 50 ml reaction volumes containing 2 units of Taq polymerase, 5 ml of 10× Reaction Buffer, 2.5 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 40 pmol of each primer and approximately 50 to 100 ng of template DNA.

Thermal cycling amplification conditions were as follows: initial denaturation at 95°C for 4 min, followed by 31 cycles of strand denaturation at 94°C for 45 s, annealing at 51°C for 45 s and primer extension at 72°C for 45 s, and a final 5 min elongation time at 72°C. The size of the PCR products was checked against a 100-bp DNA ladder (Gibco BRL) in 1.5% agarose gel, run in 1× TBE buffer and stained with 0.5 mg ml<sup>-1</sup> ethidium bromide.

Mitochondrial DNA variation was analyzed by restriction fragment length polymorphism (RFLP) performed on PCR-amplified products. The amplified fragments were initially screened for polymorphism with the following 12 restriction endonucleases: *Bst*UI, *Bst*NI, *Hae*III, *Msp*I, *Rsa*I, *Ssp*I, *Dde*I, *Hha*I, *Hinc*II, *Hinf*I, *Nla*III and *Taq*I. For each sample, 5–10 ml of the PCR product was digested with the appropriate restriction enzyme. The digested samples were then electrophoretically separated on 2% agarose gels in 1×

TBE buffer, stained with ethidium bromide, visualized under UV light and the size of the fragments was estimated relative to a standard 100 bp DNA ladder (Gibco BRL).

**Scoring of variation.** The raw data were fragment profiles, but we inferred site differences among haplotypes from changes in fragment profiles and based on a limited number of obtained sequences (not shown). All RFLPs could be explained by the gain or loss of particular restriction sites. For each RFLP pattern observed for each enzyme, a presence/absence restriction site matrix was created. Distinct single endonuclease patterns were identified by a specific letter in order of appearance. Each specimen was assigned a multiletter code that described its composite mtDNA genotype. Restriction patterns are available upon request.

**Data analysis.** Haplotype diversity (Nei 1987) and nucleotide diversity (Nei & Tajima 1981) values within samples, as well as nucleotide divergence (Nei & Tajima 1981) values between haplotypes and samples were computed using the statistical package REAP 4.0 (McElroy et al. 1991). The global and pairwise sample differentiation was assessed using exact tests (Raymond & Rousset 1995) included in the ARLEQUIN package (Schneider et al. 2000). Corrections for simultaneous multiple comparisons were applied using sequential Bonferroni correction (Rice 1989). Analysis of molecular variance (AMOVA, Excoffier et al. 1992) was used to estimate the amount of genetic structuring at different

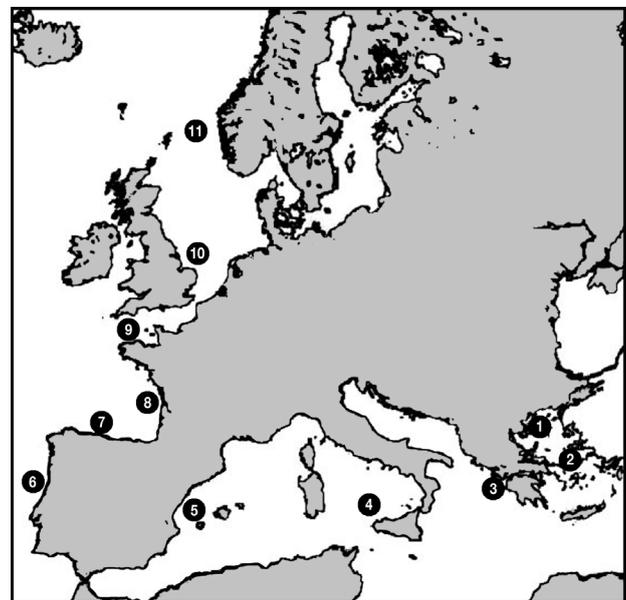


Fig 1. European waters where the different sampling areas (some with ICES divisions) are shown. 1: N Aegean Sea; 2: S Aegean Sea; 3: Ionian Sea; 4: W Italy; 5: E Spain; 6: IXa (Portugal); 7: VIIIe (N Spain); 8: VIIIb (NW Spain); 9: VIIe (NW France); 10: IVb–c (SE England); 11: IVa (N Norway)

hierarchical levels by quantifying the inter- and intra-group component of total variance by *F*-statistics in the ARLEQUIN package (Schneider et al. 2000). The significance of the resultant *F*-statistics and variance components were tested with 10 000 permutations.

Both distance and character-based approaches included in PHYLIP version 3.57c (Felsenstein 1995) were used to find relationships among haplotypes. Genetic distances among haplotypes from restriction site data were used to construct a UPGMA tree (Saitou & Nei 1987) using the NEIGHBOR program. A FITCH (distance) phenogram was also constructed using the FITCH program. Wagner parsimony analysis was also performed using the MIX program contained in the PHYLIP program. The topology of the consensus tree constructed with the CONSENSE program was evaluated by 1000 bootstrap replications using the SEQBOOT program. In all cases, trees were rooted using as

an outgroup the species *Trachurus trecae*. UPGMA phenograms that cluster samples according to nucleotide divergence were constructed with the NEIGHBOR programme included in PHYLIP.

In order to test for isolation by distance, a Mantel test (Mantel 1967), with 10 000 random permutations, was carried out with the GENETIX package (Belkhir 1999).

## RESULTS

### Initial experiments with 12 restriction enzymes

The amplified segments of the tRNA<sup>Pro</sup> and the whole control region had an approximate size of 1030bp. All the restriction enzymes used to screen the 23 samples have at least 1 recognition site on the

Table 2. *Trachurus trachurus*. Composite haplotypes, haplotype frequency, nucleotide and haplotype diversity values (%) including standard error (SE), and sample sizes of the studied populations (species-specific haplotypes of African *T. trachurus* are marked in bold). Composite haplotypes are denoted by capital letters in the following order: *DdeI*, *HhaI*, *HinfI*, *NlaIII*, *HincII*, *TaqI*. Roman numerals refer to the following areas: IXa (Portugal), VIIIc (N Spain), VIIIb (NW Spain), VIIe (NW France), IVb–c (SE England), IVa (W Norway); see also Fig. 1. Capital letters of composite haplotypes refer to different patterns obtained for each restriction enzyme

Haplo- type	<i>Trachurus trachurus</i> composite haplotype	Sample locality											
		North Aegean	South Aegean	Ionian	West Italy	IXa	VIIIc	VIIIb	VIIe	IVa	IVb–c	South Africa	Namibia
T.t. 1	C B B C D A	2	2										1
T.t. 2	C B A C D A	1	7	3	3		1	3	2				
T.t. 3	B B B A D A	10	13	9	4	11	8	6	3	9	6		
T.t. 4	B B A C D A	2	2	1	1			1	1				
T.t. 5	C B B A D A	4	5	3	4	4	8		2	5	4		
T.t. 6	C B A A D B		1										
T.t. 7	B B A A D A	6	18	8	2	7	7	10	2	7	14		
T.t. 8	C B A A D A	4	8	4	2	5	4	11	2	8	3		
T.t. 9	I B A A D A	1					1				1		
T.t. 10	B B B C D A		3	1		1							
T.t. 11	G B A C D A		1										
T.t. 12	B B A G D A		2										
T.t. 13	B B A F D A		1										
T.t. 14	P B B A D A							1					
T.t. 15	K B A A D A									1			
T.t. 16	B B A C D B						1						
T.t. 17	G B A A D A				1	1							
T.t. 18	C B A F D A				1								
<b>T.t. 19</b>	<b>M A A C D A</b>												1
<b>T.t. 20</b>	<b>N A B A D A</b>											3	13
<b>T.t. 21</b>	<b>N A A A D A</b>											7	2
<b>T.t. 22</b>	<b>M A A A D A</b>											6	1
<b>T.t. 23</b>	<b>M A B C D A</b>												3
<b>T.t. 24</b>	<b>M A B A D A</b>											2	1
<b>T.t. 25</b>	<b>N A A C D A</b>												1
<b>T.t. 26</b>	<b>N A B C D A</b>											1	
<b>T.t. 27</b>	<b>M C A A D A</b>											1	
Nucleotide diversity (%)		0.98	1.15	0.93	1.18	0.80	0.93	0.84	1.08	0.78	0.75	0.89	0.88
Haplotype diversity (%)		82.99	84.84	81.28	88.89	77.34	80.92	76.21	89.39	78.16	71.67	78.95	64.50
±SE (%)		4.27	2.51	4.09	4.16	4.58	3.35	4.02	5.37	2.98	6.75	5.71	10.87
Total sample size		30	63	29	18	29	30	32	12	30	29	20	22

amplified segment. A total of 132 individuals of the species *Trachurus trachurus* (41 European and 12 African specimens), *T. mediterraneus* (41) and *T. picturatus* (38) were initially scored with all 12 enzymes. Two of the restriction enzymes used for the RFLP analysis (*Bst*UI and *Bst*NI) did not reveal polymorphism, 4 of them (*Hae*III, *Msp*I, *Rsa*I and *Ssp*I) revealed very low levels of polymorphism and the remaining 6 (*Dde*I, *Hha*I, *Hinc*II, *Hinf*I, *Nla*III and *Taq*I) discovered high levels of polymorphism. Additionally, 12 individuals of *T. trecae* were scored that generated totally different restriction patterns in 10 out of the 12 enzymes used (except for *Bst*NI and *Hinf*I).

**RFLP analysis with 6 enzymes**

The 6 enzymes that discovered high levels of polymorphism were used to screen more individuals from all 3 studied species. An additional 534 individuals

were scored, and 27, 25 and 25 haplotypes were generated in total for *Trachurus trachurus*, *T. mediterraneus* and *T. picturatus*, respectively (Tables 2, 3 & 4). Nine of the 27 *T. trachurus* haplotypes were present in *T. trachurus* from Namibia and South Africa and were totally different from the haplotypes present in European *Trachurus* samples (Table 2).

There were restriction enzymes that produced species-specific patterns. Specifically, *Hha*I produced patterns in European *Trachurus trachurus* that were never present in the African *T. trachurus* or in the other 2 species and thus, produced composite haplotypes that could be used as diagnostic between European and African samples of *T. trachurus* as well as between the 3 studied species. Also, *Dde*I could be characterized as species-specific for *T. trachurus* as it could discriminate it from the other 2 species. The composite haplotypes with the absolute haplotype frequencies within each sampling site and the different restriction patterns that could be used to discriminate the 3 species are presented in Tables 2, 3 & 4.

Table 3. *Trachurus mediterraneus*. Composite haplotypes, haplotype frequency, nucleotide and haplotype diversity values (%) including standard error (SE), and sample sizes of the studied populations. Composite haplotypes are denoted by capital letters in the following order: *Dde*I, *Hha*I, *Hinf*I, *sNla*III, *Hinc*II, *Taq*I. Roman numerals refer to the following area: IXa (Portugal); see also Fig. 1. Capital letters of composite haplotypes refer to different patterns obtained for each restriction enzyme

Haplotype	<i>Trachurus mediterraneus</i> composite haplotype						Sample locality					
							N Aegean	S Aegean	Ionian	W Italy	E Spain	IXa
T.m. 1	A	A	A	E	A	A	5	6	5	2	8	4
T.m. 2	A	A	A	A	A	A	3	3	2		2	2
T.m. 3	A	A	A	B	A	A	17	18	12	9	14	15
T.m. 4	A	A	A	B	A	B	2	2	1	1	1	
T.m. 5	H	A	A	B	A	A	1		1			
T.m. 6	A	A	A	B	B	B	2					
T.m. 7	A	A	A	G	A	A	1					
T.m. 8	D	C	A	B	A	A	1					
T.m. 9	A	A	A	C	A	B	1					1
T.m. 10	A	A	A	B	B	A	1					
T.m. 11	A	A	A	E	A	B	1	1			3	
T.m. 12	D	A	A	E	A	A	1	1			1	1
T.m. 13	D	A	A	B	A	A	1	1	3	1	4	2
T.m. 14	D	C	A	A	A	A			1			
T.m. 15	A	A	A	B	D	A			1			1
T.m. 16	A	A	A	C	A	A			4			2
T.m. 17	A	A	A	F	A	A			1			
T.m. 18	A	A	A	G	F	A			1			
T.m. 19	O	A	A	A	D	A					1	
T.m. 20	O	A	A	A	A	A					1	
T.m. 21	A	C	A	B	A	A				1		
T.m. 22	D	A	A	H	C	A				1		
T.m. 23	H	C	A	E	A	A				1		
T.m. 24	D	A	A	A	D	A						1
T.m. 25	D	A	A	A	A	A						1
Nucleotide diversity (%)							0.91	0.61	1.15	1.04	0.99	0.99
Haplotype diversity (%)							77.33	65.32	82.66	69.17	78.32	73.79
±SE (%)							6.63	8.14	5.24	12.8	5.15	8.02
Total sample size							37	32	32	16	35	30

Table 4. *Trachurus picturatus*. Composite haplotypes, haplotype frequency, nucleotide and haplotype diversity values (%) including standard error (SE), and sample sizes of the studied populations. Composite haplotypes are denoted by capital letters in the following order: *DdeI*, *HhaI*, *HinfI*, *NlaIII*, *HincII*, *TaqI*. Roman numerals refer to the following area: IXa (Portugal); see also Fig. 1. Capital letters of composite haplotypes refer to different patterns obtained for each restriction enzyme

Haplotype	<i>Trachurus picturatus</i> composite haplotype						Sample locality				
							N Aegean	S Aegean	Ionian	W Italy	IXa
T.p. 1	D	A	A	C	A	B	4	6	7	7	3
T.p. 2	D	A	A	C	B	B	6	4	6	3	5
T.p. 3	D	A	A	C	A	A	4	6	7	6	5
T.p. 4	D	A	A	C	B	A	1	1	1	1	
T.p. 5	D	A	A	C	D	B	2	1	3		3
T.p. 6	D	A	A	C	D	A	2	1	4		1
T.p. 7	E	A	A	C	A	A			1		
T.p. 8	D	A	A	A	D	B	1	1	1		4
T.p. 9	D	A	A	A	A	A	1	1	1		2
T.p. 10	E	A	B	C	A	B		1			
T.p. 11	D	A	A	C	E	B		1			1
T.p. 12	D	A	A	C	C	A		1			
T.p. 13	D	A	A	D	B	B	3	1		1	
T.p. 14	D	A	A	D	A	B	1				
T.p. 15	D	A	A	A	A	B	2	1		1	1
T.p. 16	D	C	A	A	A	B	1				
T.p. 17	D	A	A	F	D	B	1	1			
T.p. 18	D	A	A	F	A	B	1				
T.p. 19	F	A	A	C	A	B		1			
T.p. 20	D	A	A	E	A	A		1			
T.p. 21	D	A	A	D	D	B		1			
T.p. 22	D	A	A	F	B	B		1			
T.p. 23	D	A	A	A	D	A					2
T.p. 24	D	A	A	A	B	B					1
T.p. 25	D	C	A	A	B	B					1
Nucleotide diversity (%)							1.54	1.53	1.25	0.71	1.60
Haplotype diversity (%)							92.41	92.26	85.81	77.19	91.63
±SE (%)							2.59	3.02	2.98	6.22	2.45
Total sample size							30	31	31	19	29

### Phylogenetic relationships of haplotypes

Pairwise sequence divergence between haplotypes revealed after restriction analysis with the 6 enzymes that discovered polymorphism, ranged between 0.68 to 6.39% for *Trachurus trachurus* (when African samples of *T. trachurus* were not included in the analysis, the maximum value decreased to 4.10%), 0.62 to 6.39% for *T. mediterraneus* and 0.59 to 4.55% for *T. picturatus*. After the addition of the 6 mostly monomorphic enzymes, the pairwise sequence divergence values for the 12 enzymes were lower and ranged between 0.38 to 3.76% for *T. trachurus* (when African samples of *T. trachurus* were not included in the analysis, the maximum value decreased to 2.64%), 0.39 to 3.10% for *T. mediterraneus* and 0.35 to 2.87% for *T. picturatus*.

A UPGMA tree based on Nei's divergence values between 78 haplotypes, estimated after the analysis of all individuals of the 3 studied species is presented in Fig. 2. These haplotypes clustered in 2 main groups. One group included the haplotypes of *Trachurus*

*mediterraneus* and *T. picturatus*, whereas the second group included the lineage of *T. trachurus*. The *T. trachurus* group was divided in 2 subgroups: one including the haplotypes present in European populations and the other consisting of the haplotypes present in African populations. The topology of the tree was not supported by high bootstrap values. The maximum parsimony method gave the same grouping of haplotypes. The FITCH (distance) phenogram (not shown) resulted in the same topology with the exception that the *T. trecae* outgroup was placed between the 2 groups of *T. trachurus* haplotypes.

### Sample diversity

The number of observed haplotypes within samples varied from 6 to 12 for *Trachurus trachurus*, 7 to 17 for *T. mediterraneus* and 6 to 18 for *T. picturatus*. Three haplotypes (with total frequency 61.3%) were common for all European samples of *T. trachurus*, 2 haplotypes (with total frequency 57.6%) for *T. mediterraneus* and

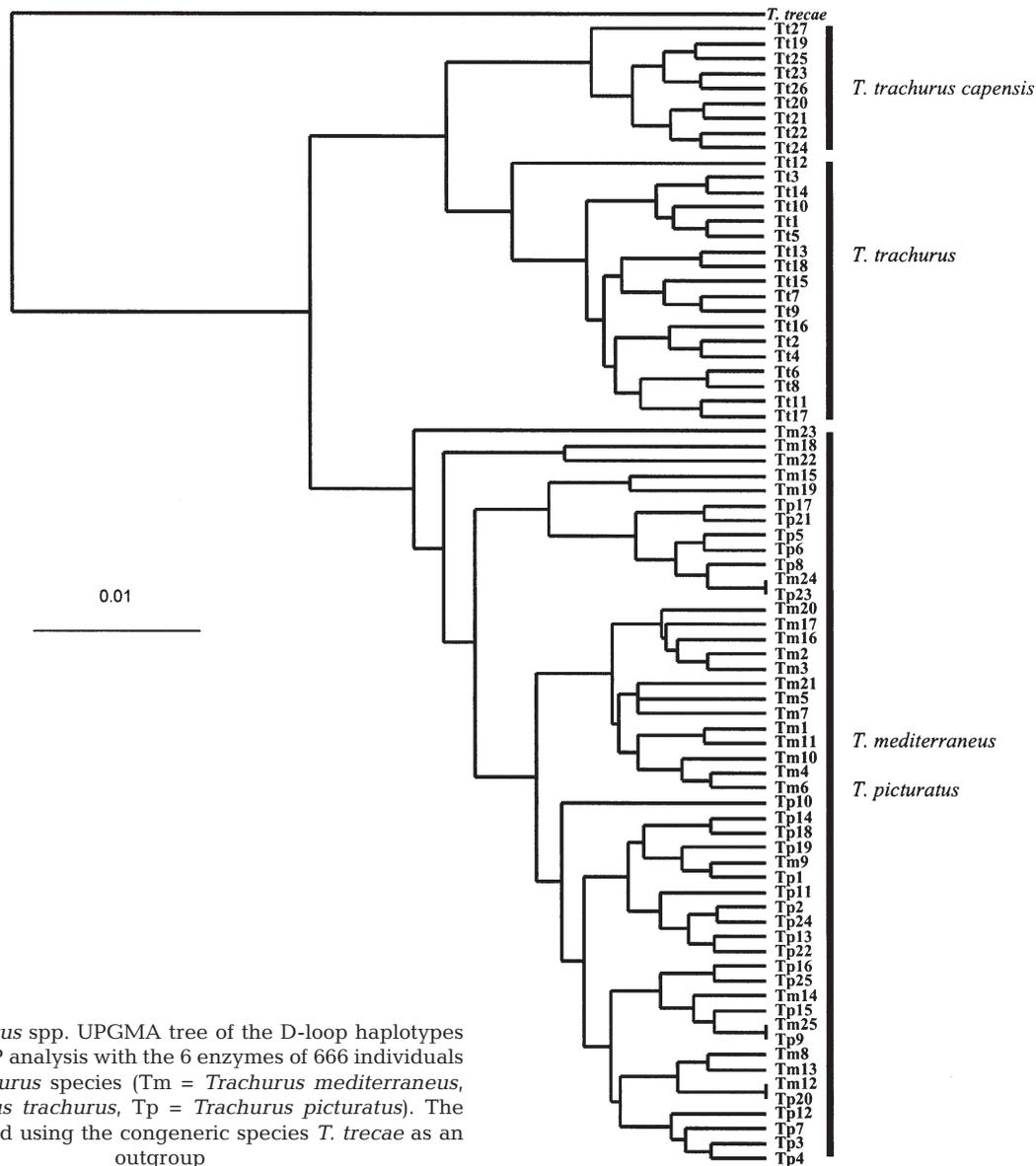


Fig 2. *Trachurus* spp. UPGMA tree of the D-loop haplotypes based on RFLP analysis with the 6 enzymes of 666 individuals of all 3 *Trachurus* species (Tm = *Trachurus mediterraneus*, Tt = *Trachurus trachurus*, Tp = *Trachurus picturatus*). The tree was rooted using the congeneric species *T. trecae* as an outgroup

3 for *T. picturatus* (with total frequency 55.4%). The majority of the recorded haplotypes were low frequency haplotypes, i.e. observed less than twice in different samples. Thus, 17 out of 27 haplotypes were unique (present in only 1 sample) for *T. trachurus*, 19 out of 25 for *T. mediterraneus* and 17 out of 25 for *T. picturatus*.

Haplotypic diversity values, estimated after the analysis of the 6 enzymes that discovered polymorphism (Tables 2, 3 & 4), were higher for *Trachurus picturatus* ( $87.86 \pm 0.09\%$ ) than for *T. trachurus* ( $79.60 \pm 0.04\%$ ) ( $81.17 \pm 0.03\%$  excluding African samples of *T. trachurus*) and *T. mediterraneus* ( $74.43 \pm 0.07\%$ ). Additionally, nucleotide diversity values estimated within *Trachurus* spp. populations were higher for *T. picturatus* ( $1.33 \pm 0.00027\%$ ) than

for *T. trachurus* ( $0.93 \pm 0.00002\%$ ) ( $0.94 \pm 0.00002\%$  excluding African samples of *T. trachurus*) or *T. mediterraneus* ( $0.94 \pm 0.00006\%$ ). When the 6 mostly monomorphic enzymes were included in the analysis, the mean values of nucleotide diversity were 1.14% for *T. picturatus*, 0.79% for *T. mediterraneus* and 0.83% for *T. trachurus*.

All global and pairwise tests of sample differentiation did not result in a significant outcome for the 6 samples of *Trachurus mediterraneus* ( $p = 0.662$ ) and the 5 samples of *T. picturatus* ( $p = 0.886$ ). On the other hand, the global test for the 12 samples of *T. trachurus* resulted in significant population differentiation ( $p < 0.0001$ ). This differentiation could be attributed to the inclusion of Namibian and South African populations since when these samples were omitted,

the global test was not significant. Within European *T. trachurus* samples, only in the case of the VIIIb region were 5 out of the 9 pairwise tests statistically significant, but none of these was significant after the Bonferroni correction.

Results of the hierarchical analysis of molecular variance (AMOVA) for European *Trachurus trachurus* indicated that most of the variation is within samples ( $F_{ST} = 0.008$ , Table 5). On the other hand, when African populations were added, a statistically significant amount of the variation ( $F_{ST} = 0.307$ ,  $p < 0.001$ ) was due to differences between samples. For *T. mediterraneus*, all genetic variation was distributed within samples, while for *T. picturatus* only 2.4% of the variation was due to differences between samples without this value being statistically significant ( $p = 0.051$ ). Additionally,  $F_{ST}$  values remained statistically non significant, even if samples were grouped according to Atlantic or Mediterranean origin (Table 5).

Simultaneously, the Mantel test showed that there was no correlation between the genetic and the geographical distance matrices for any species (*Trachurus mediterraneus*:  $p = 0.095$ , *T. picturatus*:  $p = 0.44$ , European *T. trachurus*:  $p = 0.22$ ). Only when African samples of *T. trachurus* were included in the analysis, was there a positive correlation between genetic and geographical distances ( $p = 0.005$ ).

Table 5. *Trachurus* spp. Results of the hierarchical analysis of molecular variance with or without grouping. The probability (p) estimated from permutation tests as well as the  $F$ -statistics are given at each hierarchical level (Excoffier et al. 1992). Med. = Mediterranean. The phrases 'no structure' or 'Atlantic/Med.' correspond to the *a priori* hypotheses/groupings that are tested

Species	Source of variation	Fixation index	p	
<i>T. trachurus</i> & <i>T. t. capensis</i>	No structure	$F_{ST} = 0.307$	0.000	
<i>T. trachurus</i>	No structure	$F_{ST} = 0.008$	0.167	
	Atlantic/ Med.	Among regions	$F_{CT} = 0.002$	0.220
		Among samples/ within regions	$F_{SC} = 0.004$	0.433
		Within samples	$F_{ST} = 0.006$	0.220
<i>T. mediterraneus</i>	No structure	$F_{ST} = -0.003$	0.566	
	Atlantic/ Med.	Among regions	$F_{CT} = -0.008$	0.830
		Among samples/ within regions	$F_{SC} = -0.006$	0.700
		Within samples	$F_{ST} = -0.008$	0.822
<i>T. picturatus</i>	No structure	$F_{ST} = 0.024$	0.051	
	Atlantic/ Med.	Among regions	$F_{CT} = 0.007$	0.214
		Among samples/ within regions	$F_{SC} = -0.008$	0.974
		Within samples	$F_{ST} = -0.007$	0.671

A UPGMA tree based on the distance matrix of nucleotide divergence (DA) was constructed for all the 3 studied species (Fig. 3). For the 5 samples of *Trachurus picturatus* and the 6 samples of *T. mediterraneus*, no specific clustering of samples was observed. For *T. trachurus* populations, the samples clustered in 2 distinct clades, the one including the African samples (Namibia and South Africa) and the other consisting of the European samples. No geographical structuring was found within this clade.

## DISCUSSION

### No levels of population differentiation within the 3 *Trachurus* species

Analysis of the genetic structure of the 3 *Trachurus* species was performed both within and between the Mediterranean and Atlantic waters. The heterogeneity exact tests, the  $F_{ST}$ -based analysis and the Mantel tests did not reveal significant variation among samples within *T. picturatus* and *T. mediterraneus* or within European samples of *T. trachurus*. This phenomenon could be commonly attributed to high levels of current gene flow as a result of the species high dispersal abilities in the marine environment and the fact that marine waters are often viewed as boundless habitats, and therefore, an ongoing exchange of migrants is taking place (e.g. Arnason & Palsson 1996, Vis et al. 1997). Only the pairwise comparison of Namibian and South African samples to European *T. trachurus* samples resulted in significant differentiation ( $p = 0.005$ ). This differentiation is consistent with the placement of African *T. trachurus* populations into a different subspecies known as *Trachurus trachurus capensis* expanding from the Gulf of Guinea to South Africa, indicating that enough time has elapsed for the African population to differentiate from the European populations. The lack of significant population structuring was also evident in the phylogenetic trees (Fig. 3) that did not reveal pronounced phylogeographic structuring for *T. mediterraneus* and *T. picturatus*. For *T. trachurus*, 2 major genetic divisions were defined: one including the European populations grouped without any evident geographical meaning and the other, the African ones.

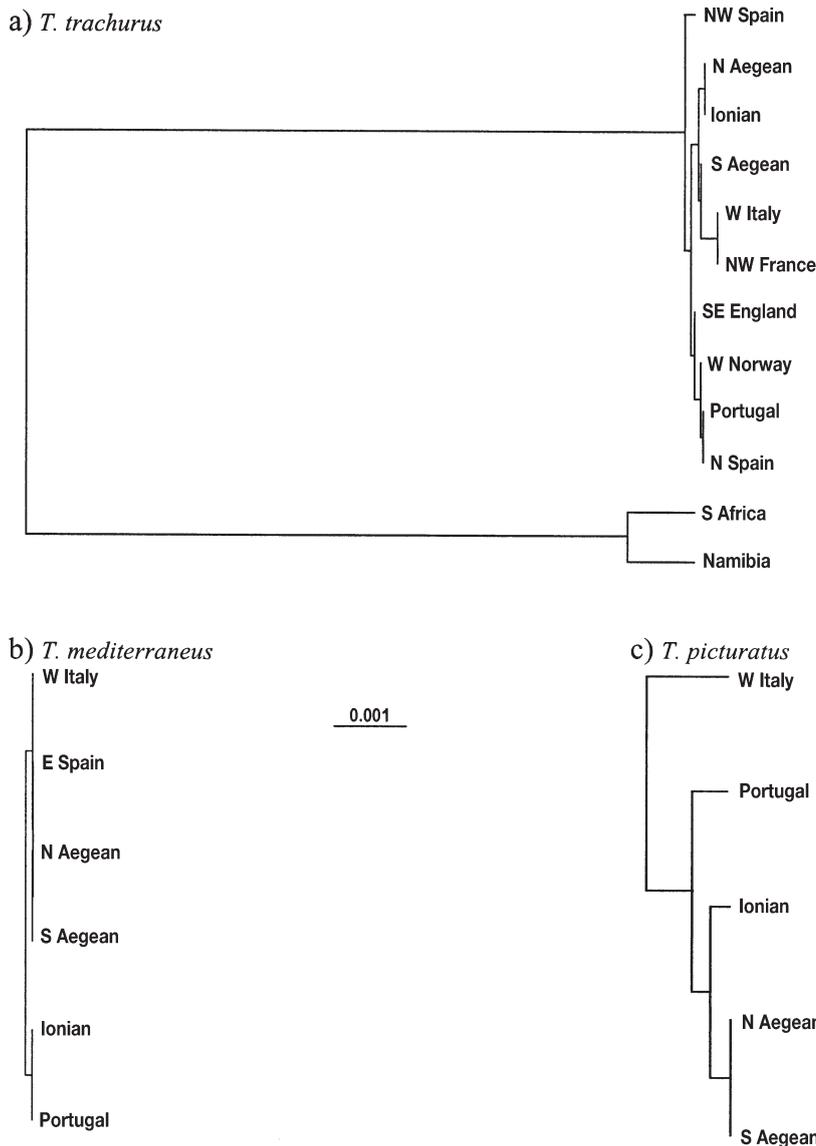


Fig 3. *Trachurus* spp. UPGMA phenograms clustering: (a) the 12 samples of *Trachurus trachurus*, (b) the 6 samples of *T. mediterraneus* and (c) the 5 samples of *T. picturatus* based on pairwise divergence values. ICES abbreviations are: Portugal (IXa), N Spain (VIIIc), NW Spain (VIIIb), NW France (VIIe), SE England (IVb–c), W Norway (IVa)

For a proportion of species presently occurring in both the Atlantic and the Mediterranean, the Strait of Gibraltar represents a corridor sufficient for gene flow; thus, an ongoing exchange of individuals between the 2 seas is taking place (see reviews in Borsa et al. 1997b). Species of the same family with very similar biological features may react differently to oceanographic conditions. A very characteristic example is the case of 5 sparidae species (Bargelloni et al. 2003). For 3 (*Dentex dentex*, *Lithognathus mormyrus* and *Spondyliosoma cantharus*) out of 5 species of the family, no free mixing between the Mediterranean and the Atlantic popula-

tions is taking place as hydrographic conditions lower the rate of gene flow between them at such levels that prevent their genetic homogenization and thus, act as a force for the maintenance of genetic differentiation. On the contrary, the other 2 congeneric sparidae species (*Pagellus bogaraveo* and *Pagrus pagrus*) display no differentiation at all. For all 3 *Trachurus* species, the Gibraltar Strait does not present a phylogeographic boundary, as there was no sign of Atlantic–Mediterranean division. Specific life history traits may provide *Trachurus* species with flexibility to oceanographical constraints and give them the ability to migrate across them.

Another (not necessarily exclusive) scenario for this lack of differentiation between the Atlantic and Mediterranean populations could be the effect of recent colonization without sufficient time for the Mediterranean populations to diverge. In order to test this scenario, Tajima’s *D* (Tajima 1989) values were estimated for all studied samples of the 3 *Trachurus* species to search for departures from mutation-drift equilibrium (Tajima’s *D* values should be significantly negative). None of Tajima’s *D* values was significantly negative for any of the samples under study. This means that the shallow population differentiation detected for the 3 species is not a consequence of a recent colonization but probably reflects populations in long and stable demographic equilibrium.

The fact that conclusions are often based on the evidence of 1 methodology and 1 single mtDNA marker is usually criticized. The present results are consistent with the results obtained by the sequence analysis of cytochrome b of mtDNA (Karaiskou et al. 2003). Global and pairwise tests obtained from the analysis of 9 European samples with a comparable although restricted sampling scheme (NW Spain and SE England were not included) of the 3 *Trachurus* species did not reveal marked differences within *T. mediterraneus* ( $p = 0.980$ ) and *T. trachurus* ( $p = 0.824$ ). The test for *T. picturatus* resulted in significant population differentiation ( $p = 0.026$ ) but it was attributed only to the pairwise comparison of Portugal to the S Aegean Sea samples, probably due to the

small sample size. Thus, the shallow genetic structure is supported by concordant evidence of sequence and RFLP data of 2 different mtDNA markers.

### Patterns of genetic diversity in *Trachurus* species

A typical phenomenon of many marine species, both vertebrate and invertebrate, is the high level of within population haplotypic diversity. This has been observed for many fish species (Camper et al. 1993, Zwanenburg et al. 1992, Grant & Waples 2000) and is also evident in the case of the 3 studied *Trachurus* species (Tables 2, 3 & 4). However, the majority of the haplotypes (63, 76 and 68% for *T. trachurus*, *T. mediterraneus* and *T. picturatus*, respectively) occur at low frequencies. Explanations for the occurrence of high diversity values and of large numbers of low frequency haplotypes may lie in the enormous population sizes of marine organisms, which may cause retention of numerous haplotypes and result in undersampling of the populations (Bucklin & Wiebe 1998). This is true especially in the case of *T. trachurus* which is a fairly long-lived species, reaching a maximum age of over 30 yr (Eltink & Kuitert 1989) and thus, occasional strong year classes (such as in 1982, Abaunza et al. 2003) lead to high abundance of the species. Additionally, levels of nucleotide diversity obtained after the analysis of the 6 (or even the 12) enzymes, were high for all 3 species and close to the values obtained after the sequence analysis of the D-loop region of the species *Scomber scombrus* (Perciformes), where values ranged from 1.6 to 3% (mean  $h = 0.76$  and  $\pi = 2.8\%$ : Nesbø et al. 2000). The observed values of the haplotype and nucleotide diversity are within the range of values reported in a review of mtDNA variation in 32 marine teleost species (range  $h = 0.70$ –1 and  $\pi = 0.80$  to 3.20%) by Grant & Bowen (1998). According to these authors, high haplotype and nucleotide diversity values are typical for large stable populations with a long evolutionary history or are the result of secondary contact between previously extended geographic isolated populations, which, however, is unlikely for middle pelagic *Trachurus* species because of the high dispersal capabilities of the species (Bas 1995). Additionally, all populations shared some common haplotypes with high frequencies and therefore, there was no evidence of different past-history refugia. Thus, the high diversity values probably reflect large and stable populations through time and further supports previous conclusions based on Tajima's  $D$  values.

The values of haplotype diversity (87.86%) as well as nucleotide diversity found in the samples of *Trachurus picturatus* (average 1.33%) were higher than those found in the other 2 species. This result is in agreement

with the results obtained from the sequence analysis of cytochrome b of the 3 species (Karaiskou et al. 2003). This might be a reflection of the fact that *T. picturatus* is subjected to lower levels of fishing mortality as presumed from the reported annual catch statistics (Food and Agriculture Organization, www.fao.org), which are an order of magnitude less than those of *T. trachurus* and *T. mediterraneus*. Thus, it is quite likely that *T. picturatus* maintains a larger population size resulting in higher levels of genetic diversity. However, this hypothesis cannot be tested with the data at hand because *T. picturatus* is not subjected to annual assessments in the NE Atlantic. Thus, no fish mortality estimates are available. One should, however, also not exclude as a possible explanation that higher haplotypic and nucleotide values might reflect an intrinsic characteristic of *T. picturatus* (e.g. lower levels of natural mortality).

The amount of haplotypic diversity revealed after the sequence analysis of cytochrome b (Karaiskou et al. 2003) for *Trachurus picturatus*, *T. trachurus* and *T. mediterraneus* (64.40, 26.66 and 24.10%, respectively) was considerably lower compared to the D-loop, especially for *T. trachurus* and *T. mediterraneus*. The same status existed for nucleotide diversity values of cytochrome b, within populations of *T. picturatus* (average 0.47%), *T. trachurus* (0.13%) or *T. mediterraneus* (0.11%). This is a well-documented fact because of the higher mutation rate in the D-loop, which leads to higher polymorphism and thus, to a larger number of haplotypes. Additionally, it could be due to lower sample sizes in the cytochrome b analysis.

### Lack of congruence between genetic and ICES stock data

It is assumed that there are 3 distinct stocks of *Trachurus trachurus* in the NE Atlantic (ICES 1999): (1) the southern horse mackerel around the Iberian peninsula (ICES Divisions: VIIIc and IXa); (2) the western horse mackerel in the Norwegian Sea, northern North Sea, west and south off the British Isles, western Channel and west off France (ICES Divisions: IIa, IIIa, Vb, IVa, VIa, b, VIIa to c, e to k and VIIIa, b, d, e); and (3) the North Sea horse mackerel, mainly restricted to the central and southern North Sea, and eastern English Channel (ICES Divisions: IVb, c and VIIId). This separation of stocks was based on circumstantial evidence, such as egg distribution and temporo-spatial distribution of fishery or morphological differences (Kerstan 1991). Therefore, the need for stronger biological justification has been recognized.

An attempt was made to evaluate the existence of 3 different stocks according to ICES, by using genetic analysis of samples from different geographical areas

within each of the 3 determined units. Thus, the assumed southern stock was represented (Fig. 1) by samples obtained from areas VIIIc and IXa, the Northern stock by a sample from the area between IVb and IVc, and the western stock by samples from areas VIIIb, VIIe and IVa. The test for differences between the 3 assumed Atlantic stocks (samples within stocks were pooled) resulted in significant population differentiation ( $p = 0.03$ ). However, this could be attributed only to the pairwise tests of the VIIIb area to the other areas, as when this region was omitted, no global and/or pairwise test was statistically significant. This could probably be due to sampling error as can be concluded in the case of the S Aegean sample. In our initial experiments, the pairwise comparison of the S Aegean to the other studied populations was also statistically significant but when the sample size was increased (63 individuals), no significant population differentiation was detected for European *Trachurus trachurus*.

Thus, the data obtained by the global test of differentiation did not support the initial hypothesis of 3 different stocks for *Trachurus trachurus* as these 6 sampling areas in Atlantic waters could not be considered as reproductively isolated. These results probably suggest a revision of ICES stock boundaries. This was also evident in the case of European *Merluccius merluccius* (Lundy et al. 1999), where microsatellite data supported a different structure than the one determined by ICES.

According to Waples (1998), patterns of differentiation that are consistent across time will unlikely be due to sampling artifacts. Lundy et al. (2000) have demonstrated that the spatial patterns obtained for *Merluccius merluccius* are not temporally stable since very high levels of interannual differentiation are detected. Thus, it could not yet be supported that the European populations of *Trachurus trachurus* could be managed as a single stock and not as 3 different stocks. It is necessary to ensure that patterns seen here of unlimited migration are temporally stable, both annually and during spawning seasons, when mature adults migrate to common spawning sites which are far from feeding grounds in the case of *T. trachurus* (Abaunza et al. 2003). From a management perspective, it is also important to stress that whether the hypothesis of recent colonization of the Mediterranean from the Atlantic was true, then the Mediterranean and the Atlantic stocks should be managed separately.

### Phylogenetic relationships

The phylogenetic analyses, based on distance and maximum parsimony methods, revealed the existence of 2 clusters: one with haplotypes of *Trachurus trachu-*

*rus*, and the other including both *T. mediterraneus* and *T. picturatus* haplotypes. This result is not in absolute accordance with the results obtained by the sequence data of cytochrome b where the 22 haplotypes determined for the 3 species clustered in 3 distinct lineages (Karaiskou et al. 2003). A plausible explanation for this discrepancy is that nucleotide saturation in the D-loop region obscures the usefulness of this segment for closely related sequences. This phenomenon might have also decreased the number of species-specific sites overall, resulting in low bootstrap values in the UPGMA phylogenetic tree of haplotypes. However, one should not forget the inherent limitations of the RFLP methodology; a phylogenetic analysis based on the sequence of the D-loop region might increase the bootstrap values. The same discrepancy was found for rainbow fishes by Zhu et al. (1994). These authors found that the most probable cause of the discrepancy is rapid saturation of transitions in the D-loop, presumably as a result of a more biased base composition (e.g. higher A + T content) compared to cytochrome b.

The clear discrimination of *Trachurus trachurus* was, however, also evident by the existence of species-specific enzymes, such as *DdeI* and *HhaI*, that can be used to discriminate *T. trachurus* from the other 2 species, while no possible enzyme was found to discriminate between *T. mediterraneus* and *T. picturatus*. Additionally, the shared haplotypes between *T. mediterraneus* and *T. picturatus* further reinforced the closer relationship of these 2 species compared to *T. trachurus*, which may have differentiated earlier from the common ancestor than the other 2 species (Karaiskou et al. 2003).

### Conclusions and implications for management

As discussed in detail by Waples (1998), the genetic signal indicating population structure is relatively weak for species displaying a high dispersal capacity. It has been assumed that the usually high level of genetic variation observed at microsatellite loci would provide enough resolution power to increase the genetic signal and hence, the likelihood of correctly describing the population structure of marine fishes. Thus, a further analysis using microsatellite data may maximize the chances of detecting genetic differences among *Trachurus* spp. populations, since even such a highly polymorphic region as the D-loop region appears to reveal population differentiation less often than microsatellites with similar levels of variability (Hauser & Ward 1998). Hauser et al. (2001) demonstrated that mtDNA and microsatellite data did not support the same results in the Atlantic herring *Clupea harengus*, most likely due to differences in mutation

rate and demographic dynamics of the 2 marker systems, and thus underlying the need for simultaneous application of the 2 markers to the same sample sets. Additionally, both biological and ecological features (e.g. growth and mortality) should be taken into consideration when addressing management issues.

The results based on the RFLP analysis of the D-loop region showed no clear genetic structuring between the studied samples of the 3 *Trachurus* species (*T. trachurus*, *T. mediterraneus* and *T. picturatus*). Additionally, no Atlantic–Mediterranean population differentiation was observed. However, based on the fact that every methodology and genetic system selected to uncover the population structure may not lead to detectable genetic differences, extreme caution is suggested, when policy decisions need to be taken. Thus, despite the lack of differentiation, it would be wise to apply precautionary management approaches until the present information is also compared with a number of genetic systems and possible techniques.

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