

# Length variation in mtDNA control region in hatchery stocks of European sea bass subjected to acclimation experiments

Giuliana Allegrucci\*, Donatella Cesaroni, Federica Venanzetti,  
Stefano Cataudella, Valerio Sbordoni

Department of Biology, Tor Vergata University,  
via della Ricerca Scientifica, 00133 Rome, Italy

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**Abstract** – Length polymorphism of the mtDNA control region was analysed in samples from the sea bass *Dicentrarchus labrax* before and after acclimation to freshwater. Acclimation trials were repeated twice for two samples originating from the same broodstock. DNA amplification of 221 individuals made it possible to detect 40 different D-loop length variants and a mean level of gene diversity ( $k_c$ ) of 0.850. Out of 52 scored distinct genotypes, 18 were homoplasmic and the remaining ones were heteroplasmic for up to four variants. Shifts in frequencies between starting and acclimated samples in the same direction of the same genotypes in both years were not observed. Patterns of mtDNA control region variation were compared to previous allozymic and RAPD studies carried out on the same samples. While variation found in the allozyme alleles and the RAPD markers in replicate experiments revealed differential survival of the genotypes, stochastic processes, such as genetic drift, explained the observed variation of mtDNA control region. Very high levels of genotype diversity were observed within each sample, ranging from 0.77 to 0.97. This high diversity associated with the maternal inheritance of mtDNA makes this molecular marker especially suitable for studies in aquaculture genetics, when the allozyme studies fail to reveal genetic variation or when the identification of progeny based on the mother's contribution is relevant. © Inra/Elsevier, Paris

**fish mtDNA / D-loop region / European sea bass / hatchery stock / acclimation trials**

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Correspondence and reprints  
E-mail: allegrucci@uniroma2.it

**Résumé – Polymorphisme de la longueur de la région de contrôle de l'ADN mitochondrial dans des souches de bar européen soumis à des expériences d'acclimatation à l'eau douce.** Le polymorphisme de longueur de la région de contrôle de l'ADN mitochondrial (ADNmt) a été analysé dans des échantillons de bar (*D. labrax*) avant et après des expériences d'acclimatation à l'eau douce. Les expériences d'acclimatation ont été répétées sur deux échantillons de même origine. L'amplification de l'ADNmt de 221 individus a permis de détecter 40 variantes de longueur de la boucle D et une diversité génique ( $K_c$ ) de 0,850 en moyenne. Sur 52 génotypes analysés, 18 ont été homoplasmiques et le reste hétéroplasmiques, avec jusqu'à quatre variants chez le même individu. La variation de la région de contrôle de l'ADNmt a été comparée à celle observée lors d'études précédentes sur les mêmes échantillons pour les allozymes et aux marqueurs RAPD. Alors que la variation liée aux allozymes et aux marqueurs RAPD trouvés a indiqué des différences de survie des génotypes après acclimatation, des processus stochastiques, comme la dérive génétique, peuvent expliquer la variation observée. À l'intérieur de chaque échantillon, on a observé de hauts niveaux de diversité génétique avec une variation comprise entre l'indice 0,77 et l'indice 0,97. Cette diversité associée à l'hérédité maternelle de l'ADNmt semble rendre ce marqueur moléculaire très utile pour les études génétiques appliquées à la pisciculture. © Inra/Elsevier, Paris

poisson / bar / ADN mitochondrial / D loop / acclimatation

## 1. INTRODUCTION

The European sea bass, *Dicentrarchus labrax*, is an Euryhaline species found in the northeastern Atlantic Ocean and in the Mediterranean sea. It occurs in coastal waters, lagoons and estuaries. This species is very important in the fishery industry and is also widely used in aquaculture [7, 26]. The genetic identification and discrimination of aquaculture stocks is a fundamental requirement in culture programmes especially for those directed at using genetically characterized breeders. The knowledge of genetic heterogeneity within and among stocks makes it possible to minimize the deleterious effects of inbreeding and/or specific selective agents by planning appropriate crossing schedules.

For the past few years we have been analysing the distribution of genetic variability in wild and cultivated populations of sea bass *D. labrax*, using different genetic markers, such as allozymes [1, 3], RAPDs [2, 13] and mitochondrial DNA (mtDNA; [14–16, 29]).

Over two subsequent years (1989 and 1990) we followed the changes in genetic variation in reared samples, before and after acclimation to freshwater. Both allozyme and RAPD markers showed that survival to acclimation to freshwater was not random. Variability estimates based on 28 allozyme loci decreased between the starting and acclimated samples by about 50 %. Survival rates and relative fitnesses per genotype per locus were estimated and in both years the same genotype at certain loci (i.e. *Ck-4*, *Est-2*, *G6pd*, *Me*, *Np*) showed the highest probability of survival [1]. Similar shifts were also observed in RAPD markers. Even though only one band out of the 126 markers scored showed concordant shifts in both years, which were statistically significant, several other markers also varied concordantly [2]. These results suggested the hypothesis that regimes associated with acclimation trials, which are selected

within an heterogeneous array of genotypes, probably reflect the different geographic origins of broodstock [1, 2].

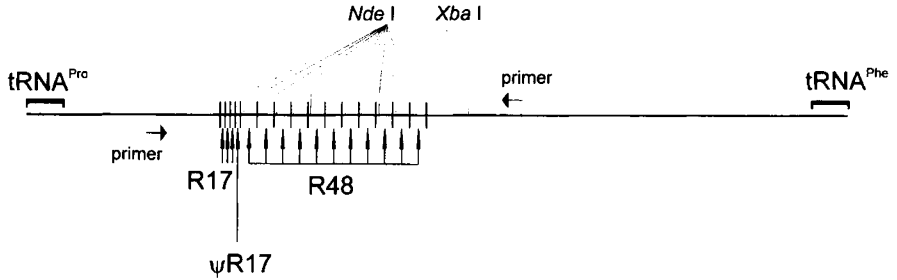
We followed the behaviour of both allozymes and RAPD markers in wild populations from the Mediterranean sea, sampled either in open sea or in coastal lagoons. Interestingly, the allozyme genotypes and RAPD bands, which increased in frequency in the samples acclimated to freshwater, were also more frequently found in wild populations from brackish water rather than from the open sea [3, 13]. Wild populations were also investigated using a third marker, the D-loop or control region of mtDNA, which revealed the existence of length polymorphism [16].

In this paper we analyse the same cultivated samples previously studied by allozymes and RAPDs, studying the length polymorphism of the mtDNA control region, before and after acclimation to freshwater. This region has proven to be particularly suitable for population genetic studies [9], because it has the highest nucleotide substitution rate of all the mitochondrial genome, with an evolutionary rate up to five times higher than the coding portion [20, 30]. The control region is primarily responsible for the observed variation in the total length of the vertebrate mitochondrial genome (see review in Meyer [21, 22]). In vertebrates, the D-loop region often varies in length because of tandem duplications [23, 28]. In fish, length variation is not a rare phenomenon and in most cases it is due to variation in the copy number of tandem repeats in the control region [5, 8, 11, 12, 22, 24, 31]. In *D. labrax*, the control region has proven to be only responsible for the observed individual variation in total length of the mtDNA molecule [29]. The sequencing of length variants from three distinct *D. labrax* individuals revealed the existence of a) a variable number of copies of two strictly related 17-bp tandemly repeated sequences; b) a variable number of copies of two strictly related 48-bp tandemly repeated sequences; and c) an 'imperfect' 17-bp repeat occurring only once in the D-loop region, which separates the array of 17-bp repeats from the array of the 48-bp repeats (*figure 1*; [15]). A survey of 209 individuals collected in eight Mediterranean localities confirmed that mtDNA length variation in *D. labrax* is the outcome of the contemporary variation in repeat copy number of these two repeat arrays. Moreover, it revealed high levels of heteroplasmy (33.3–70%), and genetic diversity, especially between individuals within populations [16].

The aim of the present study was to characterize and quantify length variation in a large sample of reared individuals, in order to evaluate differences after acclimation to freshwater. Comparisons with allozyme and RAPD variation were also carried out, in order to examine the usefulness of the three molecular markers as a source of genetic markers in sea bass aquaculture.

## 2. MATERIAL AND METHODS

Samples were taken from the ENEL fish farms, 'Torrevaldaliga' (Civitavecchia, Latium) and 'La Casella' (Piacenza, Lombardy). Samples from 'Torrevaldaliga' were obtained from artificial reproduction in 1989 and 1990. In both years, the same broodstock, made up of 190 individuals from different localities along the Italian coasts (i.e. 50% from Tyrrhenian sea and 50% from northern Adriatic sea), contributed to artificial reproduction, the sex ratio being two males to one female. Fingerlings (ca. 60 000 per year) were reared in



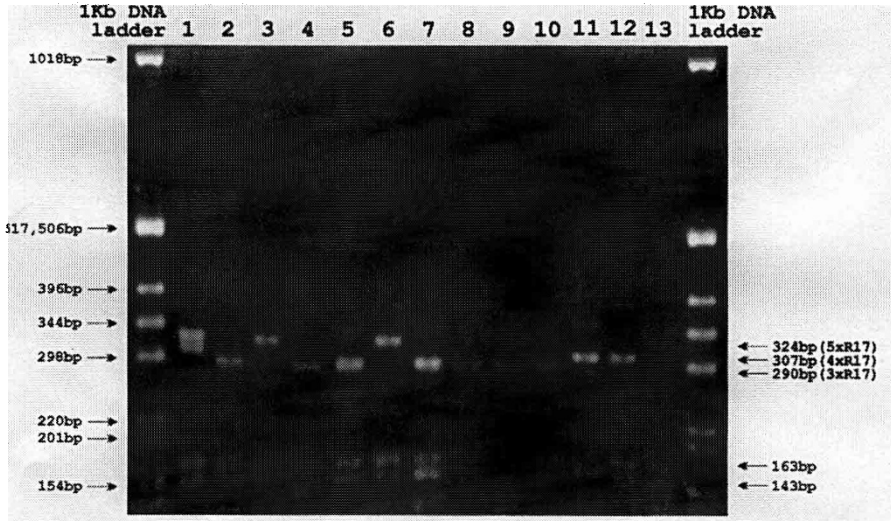
**Figure 1.** Schematic representation of the *D. labrax* mitochondrial control region, enclosed between two tRNA genes, showing the R17 and R48 repeat arrays and the unique sequence R17 between them. The position of PCR primers and of *Nde I* and *Xba I* restriction sites are also indicated.

seawater conditions up to a size of 70 g in weight. Average age was 10 months (1989 sample) and 1 year (1990 sample). Yearly samples (ca. 7 000 sea basses in 1989 and 2 600 in 1990) were taken from this stock and transferred to the ENEL fish farm 'La Casella', where they were acclimated to freshwater (for further details see Allegrucci et al. [1]). At the sampling time (i.e. after 15 months for 1989 sample and 7 months for 1990 one) mortality rates were 94.4 % for 1989 sample and 74.7 % for 1990 one. Starting samples from Torrevadali collected before freshwater acclimation were named S89 for 1989 and S90 for 1990. Samples from La Casella collected after acclimation trials were named A89 for 1989 and A90 for 1990.

DNA was extracted from individual fishes, following a standard phenol-chloroform procedure detailed in Allegrucci et al. [2]. We amplified the variable portion of the D-loop region by PCR. Primer sequences were derived from Cecconi et al. [15]. They are complementary to unique sequences flanking the tandem arrays located at positions 284–302 (CCCAGTGCCACAAACGCG) and positions 1509–1490 (TTGTCCCTGAACTAACAGCC) of the *D. labrax* D-loop sequence (EMBL Data Library accession no. X81472).

Amplifications were performed with a Perkin-Elmer Cetus thermal cycler in 50  $\mu$ L of a solution containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, each dNTP at 2.5 mM, about 20 ng of template DNA, 1U of Amplitaq, 15 ng for each of the two primers used. Amplification conditions included a total of 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min and 30 s at 72 °C, using the fastest available transition between each temperature. Individual PCR amplification products were separated by electrophoresis through ethidium-bromide stained 1–1.5 % agarose gels in Tris-borate buffer and their size was scored in comparison to appropriate molecular weight standards.

Each individual fragment was double digested with the restriction endonucleases *Nde I*, recognizing a site within all the sequences of R48 repeats, and *Xba I*, which cleaves the amplified region at a unique site 143 bp away from 3' end (figure 1). After digestion, the products were visualized on 2 % TBE agarose gels and compared to molecular weight standards. As shown in figure 2, from double digestions of each amplified D-loop portion, we always obtained fragments of known length (143 and 163 bp), *n* 48-bp-long fragments (not visible



**Figure 2.** Example of pattern obtained after mtDNA fragments are double digested with the restriction endonucleases *Nde I* and *Xba I*. See text for details.

in the figure) and a residual fragment whose variable length depends on the number of R17 repeats. The 143-bp fragment derives from the cleavage site of *Xba I* away from 3' end of the amplified region; the 163-bp is comprised on the D-loop map between the *Xba I* restriction site and the first *Nde I* site away from 3' end. *Nde I*, cleaving once in each R48 repeat, produces  $n$  48-bp fragments. In figure 2, the three fragments of 290, 307 and 324 bp (belonging to different individuals) illustrate the length variability of the D-loop fragment including the R17 repeats. Each one of these variable fragments includes the primer (at the 5' end), 26 bp of the first R48 element and 194 bp (non-repeated sequence between the primer and the first R17 element) for a total of 239 bp plus  $n$  R17. The number of R48 repeats was inferred by comparing the fragment sizes before and after digestion. In fact, the difference in molecular weight among *D. labrax* D-loops is due only to the different composition in number of R17 and/or R48. Once we calculated the repeat combination for each amplified fragment, the molecular weight of each D-loop examined was also indirectly inferred.

The statistical data analysis, i.e. frequency distribution and multivariate analysis were performed by using standard procedures included in the STATISTICA package (ver. 5.1, StatSOFT, Inc. [27]).

Estimates of genetic diversity were calculated within each sample for both length variants and genotypes, according to the general formula  $K = 1 - \sum x_i^2$ , where  $x_i$  is the frequency of the  $i$ th size length variant or composite genotype within the sample [10]. Estimates of gene diversity ( $K_c$ ) were obtained when frequencies of length variants were used and estimates of genotype diversity ( $G$ ) were obtained when frequencies of composite genotypes were used. Standard errors were estimated using a jackknife procedure within each sample. Each length variant or each genotype in turn was omitted and  $K_c$  or  $G$  was re-estimated. For  $n$  length variants or  $n$  genotypes, one obtains  $n$  different

estimates. The jackknife mean is the average of the  $n$  estimates and the jackknife variance is their variance.

Likelihood ratio test analyses were used to determine if the genotype frequencies were significantly different among samples. The exact test was used because the sparseness of data sets, using SPSS package (vers. 6.0, Metha and Patel/SPSS Inc., 1993).

Multivariate ordination of individual fish based on the D-loop genotypes was studied by means of correspondence analysis, using individual profiles. Each individual was characterized for each variable (length variant) as 1 in the presence of the length variant and as 0 in its absence. This analysis was carried out separately on the two yearly samples (S89-A89 and S90-A90, respectively).

### 3. RESULTS

Each individual amplification produced a maximum of four fragments of different lengths, which were interpreted as heteroplasmic variants if they were present in the same individual. In *table I*, the total number of fragments, number of length variants and number of distinct genotypes for each sample (S89, A89, S90 and A90) are reported. Estimates of heteroplasmy within each sample are also reported. The percentage of heteroplasmy was about 30 % in S89, S90 and A90 and reached 70 % in A89.

**Table I.** *Dicentrarchus labrax*. Basic data and heteroplasmy in the two yearly samples of sea bass before (S89, S90) and after acclimation (A89, A90) to freshwater.

	S89	A89	S90	A90
Sample size	75	50	47	49
Total fragments	103	90	66	67
No. length variants	21	13	18	22
No. distinct genotypes	25	16	20	20
% Heteroplasmy	30.7	70.0	31.9	28.6

Among the 221 specimens of *D. labrax* analysed, 40 different D-loop length variants were detected. For each variant, the overall size of the amplified product, the number of tandemly repeated R17 and R48 sequences, and the relative frequency in each sample are reported (*table II*). The minimum and maximum length of the amplified portions were 884 and 1 463 bp, respectively. The total length variation was due to the combined variation of the number R17 and R48 repeats, as described in Cesaroni et al. [16]. More than 50 % of the scored D-loop length variants (22 out of 40) were singletons, and occurred only in one of the four samples. Between the two yearly samples only 15 out of 40 length variants (35 %) were in common. Starting and acclimated samples within each year shared 21 and 44 % (in 1989 and 1990, respectively) of the D-loop length variants (*table II*). Estimates of gene diversity,  $K_c$ , for this mtDNA region ranged from 0.750 to 0.899 and are reported in *table III*.

Out of 221 assayed specimens in the two yearly samples, a total of 52 distinct genotypes, named LP1–LP52, were found. Genotypes from LP1 to LP17 were

**Table II.** *Dicentrarchus labrax*. Base pair, R17 and R48 repeat copy number and frequency of length variants (numbered from 1 to 40) in the two yearly samples of sea bass before (S89, S90) and after acclimation (A89, A90) to freshwater.

	Size (bp)	Number R17	Number R48	S89	A89	S90	A90
1)	1 463	6	18	0.019	0.000	0.000	0.000
2)	1 370	9	15	0.000	0.000	0.000	0.015
3)	1 364	3	17	0.000	0.011	0.000	0.000
4)	1 316	3	16	0.019	0.000	0.000	0.015
5)	1 288	7	14	0.000	0.000	0.000	0.015
6)	1 280	15	11	0.010	0.000	0.000	0.000
7)	1 271	6	14	0.029	0.000	0.000	0.075
8)	1 268	3	15	0.000	0.011	0.000	0.000
9)	1 263	14	11	0.010	0.000	0.000	0.000
10)	1 257	8	13	0.000	0.000	0.000	0.015
11)	1 254	5	14	0.000	0.000	0.015	0.000
12)	1 226	9	12	0.000	0.000	0.000	0.015
13)	1 223	6	13	0.019	0.000	0.000	0.015
14)	1 220	3	14	0.010	0.000	0.000	0.000
15)	1 192	7	12	0.000	0.000	0.015	0.000
16)	1 189	4	13	0.000	0.011	0.000	0.000
17)	1 181	12	10	0.010	0.000	0.000	0.000
18)	1 178	9	11	0.000	0.000	0.000	0.015
19)	1 175	6	12	0.000	0.000	0.045	0.000
20)	1 172	3	13	0.029	0.067	0.030	0.015
21)	1 141	4	12	0.019	0.044	0.030	0.075
22)	1 127	6	11	0.000	0.000	0.015	0.000
23)	1 124	3	12	0.194	0.344	0.167	0.224
24)	1 110	5	11	0.010	0.000	0.076	0.030
25)	1 093	4	11	0.049	0.111	0.167	0.119
26)	1 076	3	11	0.058	0.333	0.076	0.104
27)	1 062	5	10	0.000	0.000	0.227	0.045
28)	1 045	4	10	0.019	0.000	0.015	0.000
29)	1 028	3	10	0.039	0.011	0.015	0.045
30)	1 017	8	8	0.000	0.011	0.000	0.000
31)	1 014	5	9	0.000	0.000	0.015	0.000
32)	1 000	7	8	0.000	0.011	0.000	0.000
33)	997	4	9	0.000	0.000	0.015	0.015
34)	980	3	9	0.078	0.000	0.045	0.015
35)	969	8	7	0.000	0.011	0.000	0.000
36)	952	7	7	0.000	0.022	0.000	0.000
37)	949	4	8	0.049	0.000	0.000	0.045
38)	932	3	8	0.078	0.000	0.015	0.015
39)	901	4	7	0.243	0.000	0.015	0.075
40)	884	3	7	0.010	0.000	0.000	0.000

homoplasmic. Heteroplasmic genotypes with two bands were named LP18–LP39. Eleven genotypes were made up of three fragments (LP40–LP50) and only two genotypes (LP51 and LP52) were heteroplasmic for four fragments. Heteroplasmic individuals with three or four bands were rare, and they were very often singletons (*figure 2*).

Mean genotype diversity estimates,  $G$ , calculated for each sample, indicated a reduction between starting and acclimated samples in 1989 ( $G_{S89} = 0.865 \pm 0.0004$ ,  $G_{A89} = 0.772 \pm 0.001$ ) and a slight increase in the 1990 sample ( $G_{S90} = 0.876 \pm 0.0006$ ,  $G_{A90} = 0.911 \pm 0.0004$ ). Although genotype diversity estimates were rather similar in both years, the pattern of distribution of the D-loop genotypes was remarkably different (*figure 3*). In 1989, only six out of 35 genotypes were shared by the two samples, S89 and A89, before and after acclimation to freshwater. The other genotypes either in the starting sample or in the acclimated one were mostly singletons. In 1990, 9 out of 30 genotypes were in common between the S90 and A90 samples, before and after acclimation to freshwater. The other genotypes occurred in one or at least two individuals either in the starting sample or in the acclimated one. Between the two yearly samples only 13 out of 52 genotypes were in common, although diversity estimates were similarly high (*table III*). In both years, only a few genotypes had a high frequency of occurrence ( $\geq 10\%$ ). In 1989, four genotypes (LP1, LP8, LP10 and LP19) were the most common. In 1990, three genotypes (LP6, LP8 and LP10) had frequencies higher than 10%. Only the LP8 and LP10 genotypes were frequent in both years (*figure 3*).

Likelihood ratio tests were carried out to evaluate differences in genotype frequencies between starting and acclimated samples and between the two yearly samples. Statistically significant differences (exact probability,  $P \ll 0.01$ ) were observed between all samples, except for the S90/A90 comparison which showed an exact probability equal to 0.087.

Results from the multivariate correspondence analysis, carried out on individual D-loop genotype profiles, did not reveal any shift in genotype frequencies between the starting and acclimated samples in both years.

## 4. DISCUSSION

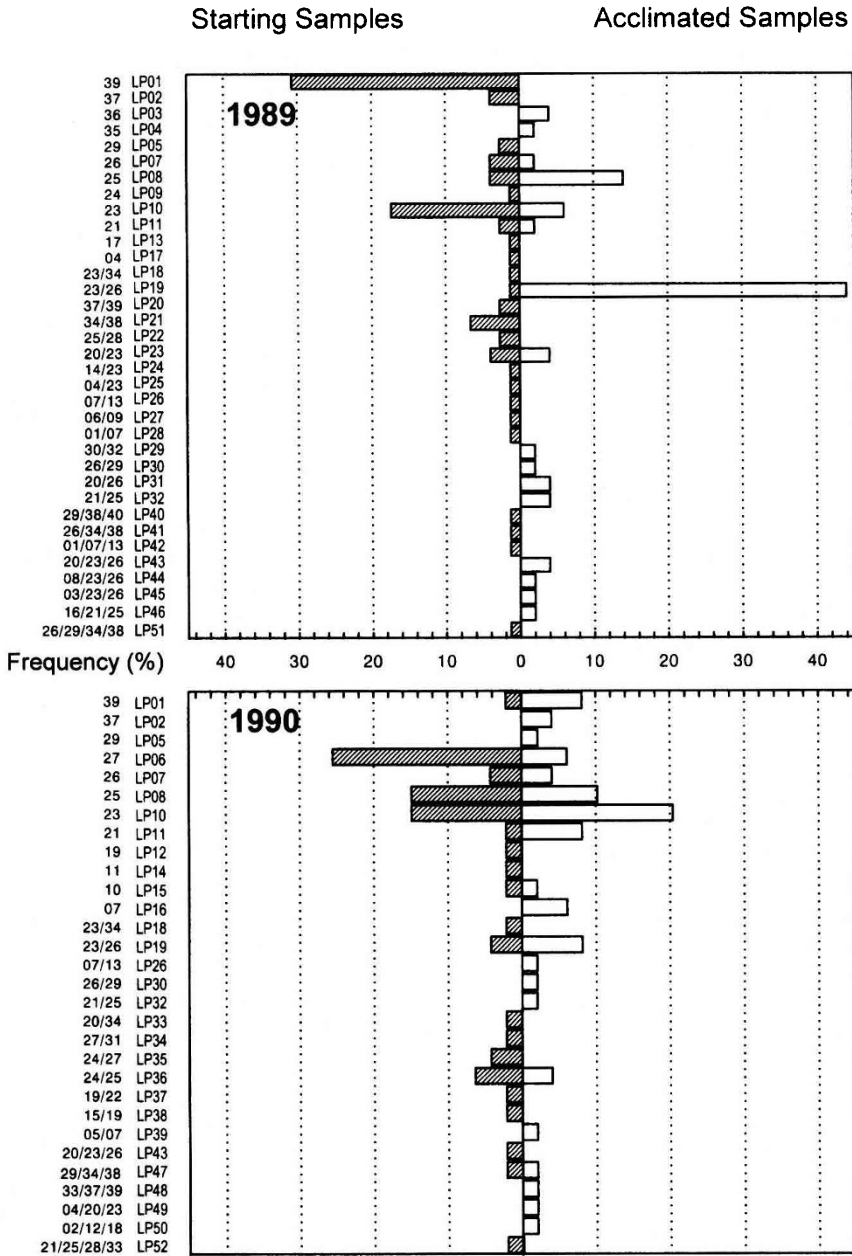
### 4.1. Levels of genetic variation

The results of this study confirmed the occurrence of high levels of length polymorphism in the mtDNA D-loop region of *D. labrax*, as already observed in wild populations [16]. The high variation in copy number of both the R17 and R48 repeats, associated with the high degree of heteroplasmy found in *D. labrax* populations, were probably responsible for the high number of distinct genotypes found either in the wild populations or in the cultivated samples.

Statistically significant differences were found between the genotype frequency distribution of the two yearly samples (exact probability  $\ll 0.01$ , *figure 3*). This suggested that these samples, since mtDNA is maternally inherited, originated from genetically different maternal lines within the same broodstock. This result is in agreement with those obtained from allozyme and RAPD markers analyses, carried out on the same individuals [1, 2]. As with allozymes and RAPD markers, the D-loop length variation suggested that the two starting populations were genetically heterogeneous. Indeed, the original broodstock was heterogeneous, as it was composed of individuals from different geographic populations from Italian coastal waters.

When the levels of diversity of the hatchery stock samples were compared with those from wild populations, a reduction in the amounts of D-loop





**Figure 3.** Observed frequencies of D-loop genotypes in the two yearly samples of *D. labrax* before and after acclimation to freshwater. Genotypes are named LP1-LP52 and next to each genotype symbol the number and type of length variants is shown (see table II for correspondance).

**Table III.** *Dicentrarchus labrax*. Estimates of gene ( $K_c$ ) and genotype ( $G$ ) diversities in the two yearly samples of sea bass before (S89, S90) and after acclimation (A89, A90) to freshwater. Standard errors, obtained by jackknife procedure, are also indicated.

	$K_c \pm \text{Std error}$	$G \pm \text{Std error}$
S89	$0.878 \pm 0.0002$	$0.865 \pm 0.0004$
A89	$0.750 \pm 0.0003$	$0.771 \pm 0.0011$
S90	$0.873 \pm 0.0003$	$0.875 \pm 0.0006$
A90	$0.898 \pm 0.0003$	$0.911 \pm 0.0004$

length variation could be observed. The study of wild populations coming from eight Mediterranean localities, ranging from France (Gulf of Lyons) to Egypt (Bardawill lagoon), revealed a total of 104 D-loop length variants and an average level of gene diversity,  $K_c$ , equal to  $0.937 \pm 0.013$  [16]. As previously pointed out, the cultivated samples of sea bass originated from a broodstock composed of individuals belonging to different Italian populations both from Adriatic and Thyrrhenian seas. Unfortunately, we did not analyse the mtDNA length variation in any other Adriatic populations. We could only compare the genetic diversity between hatchery stock samples and wild populations, by using the wild populations coming from Thyrrhenian sea. The four Thyrrhenian populations showed 76 length variants across 121 assayed individuals and an average level of gene diversity,  $K_c$ , equal to  $0.930 \pm 0.022$ . The cultured stock showed a significant reduction in mtDNA length variation with respect to the wild populations, having only 40 D-loop length variants over 221 specimens, with a  $K_c$  of  $0.850 \pm 0.033$ .

The reduction in genetic variability in cultivated stocks of aquatic organisms is a known phenomenon. This reduction can be due to the limited number of founding parents relative to wild populations, where genetic drift mediated by reductions in effective population size can cause lower levels of observed genetic variation [4]. Studies carried out on wild and cultured stocks of salmonids by restriction fragment length polymorphisms (RFLPs) in mtDNA demonstrated the existence of lower variation in cultured stocks. For example, Ontario aquaculture stocks of rainbow trout (*Onchorhynchus mykiss*) showed an average nucleon diversity less than one-half of that of the wild populations [17]. Swedish stocks of brown trout have only 25 % of the mtDNA variability of the wild populations [19]. The loss of diversity in *D. labrax* is probably due to domestication. However, we do not know the level of variation in the Adriatic populations. If they have less variation than the Thyrrhenian ones, this could also explain the reduced polymorphism levels of the cultured stocks.

When analysing allozyme polymorphisms of wild and hatchery stocks of *D. labrax*, we did not observe a loss of genetic variation, in fact the average allozyme heterozygosity was of the same order of magnitude in both the hatchery and the wild stocks of sea bass (average heterozygosity was of about 10 %; [1, 3]). Ferguson et al. [17] reported similar results in *Onchorhynchus mykiss*, with aquaculture stocks not having reduced enzyme heterozygosity relative to the wild populations.

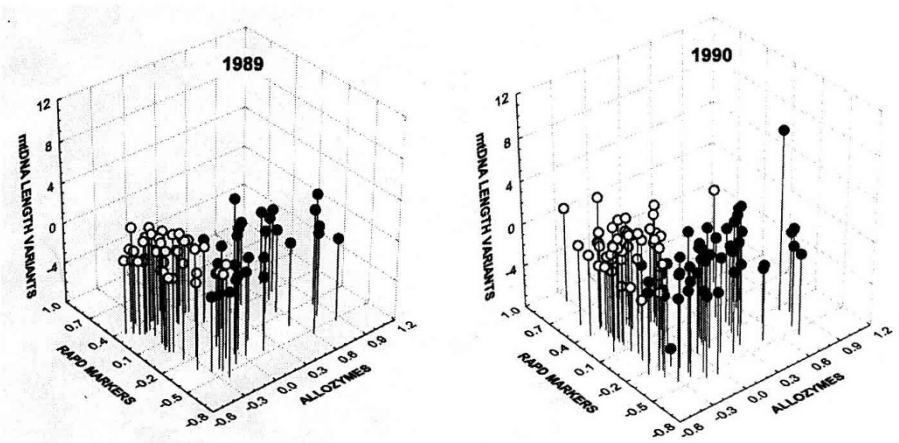
A possible explanation of these data can be found in the properties of the mtDNA. It is predominantly inherited maternally and no recombination between mitochondrial genomes is known [6]. These factors contribute to the reduction of the effective population size for mtDNA with respect to that for the nuclear genes of the same organisms (generally, in case of a 1:1 sex ratio, the effective population size is one-fourth of that for nuclear genes; [25]) and make it more susceptible to genetic drift and founder effect.

#### 4.2. D-loop length variation versus rapid and allozyme polymorphisms

As previously pointed out, the same individuals were studied for allozymic and RAPD variation [1, 2]. Multivariate ordination analysis (correspondence analysis) was carried out for each of the three data sets. Using the first principal axis score as a synthetic descriptor of variation expressed by each genetic marker, we were able to compare the results from the three different molecular techniques. Using the three synthetic descriptors as dimensional axes of a 3D space, we obtained a schematic representation of the cumulative genetic variation in sea bass (*figure 4*). Using the axis relative to D-loop genotype variation, it was not possible to distinguish between starting and acclimated samples in both years. This outcome contrasts with those from RAPDs and, especially, allozymes, where a clear separation can be observed in both years (*figure 4*). RAPD markers and allozyme loci revealed a differential survival rate for the different genotypes to acclimation to freshwater, because some of the RAPD bands and most of the allozyme alleles changed in the same direction in both years. On the basis of these results, we have suggested that selection was responsible for the observed shifts in frequencies in these markers [1, 2]. In the mtDNA control region, we also only observed statistically significant differences in genotype frequencies between starting and acclimated samples in 1989 (exact  $P \ll 0.01$ , *figure 3*). Differences between starting and acclimated samples were also found in 1990, although they were not significant (exact  $P = 0.08$ , *figure 3*). However, we did not observe shifts in frequencies in the same direction of the same genotypes in both years.

This result is not surprising if one considers that these three genetic markers are marking different parts of the genome. Allozymes are parts of the functional genome and therefore they are more probably subjected to selective pressures. RAPD markers are, by definition, randomly amplified and most of them may mark less functional parts of the genome and thus are not expected to trace selective effects. The control region of mtDNA (D-loop) is a non-coding region of the mitochondrial genome and is more likely to be neutral than allozymes. On the other hand, specific D-loop genotypes could in principle be linked to genes subjected to selection. However, selection would have to be very strong in order to overcome genetic drift because the effective population size of mtDNA is much lower than that of nuclear genes.

In conclusion, the mtDNA control region did not give any indication of selection acting on sea bass during the acclimation to freshwater. However, the high levels of genotype diversity observed in hatchery stock (*table III*) and in wild populations of sea bass [16] make this molecular marker very helpful in aquaculture genetics where the identification of progeny based on the



**Figure 4.** A three-dimensional plot of *D. labrax* specimens in the two yearly samples before (black circles) and after (white circles) acclimation to freshwater. The three coordinates are the first principal axes obtained from correspondence analyses of each set of genetic descriptors (allozymes, RAPD markers and mtDNA length variants, respectively). See text for details.

mother contribution may be relevant. Wild populations show genotype diversity ranging from 0.855 to 0.967, comparable to that obtained in the present paper for starting samples (*table III*). Such high diversity within populations ensures a probability of 73–93.5 % for identifying any individual by a given genotype. Moreover, this level of polymorphism is comparable to that observed in a single microsatellite locus [18]. When studying reared and wild stock of species of economic relevance such as *D. labrax*, the opportunity of individual characterization could be beneficial.

The combined study of highly variable markers, such as mtDNA control region and microsatellites would be highly desirable in aquaculture programmes, for three major reasons: a) both these molecular techniques use a PCR mediated approach which has the advantage of characterizing samples by taking tissue biopsies from reared specimens without sacrificing them; b) the analysis of nuclear loci, such as microsatellites, could be used as a complement to avoid the bias of using only one maternally inherited genetic marker; and c) it could provide information on both maternal and paternal genetic contribution in reared stocks. Such information could be useful for reconstructing pedigrees of cultured stocks, which are essential for identifying founder individuals, avoiding deleterious inbreeding and maintaining variability by maximizing founder contributions.

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