Tuğba ŞENEL

M.S. Thesis in Biology

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DETECTION OF SOMATIC and GONADAL MOSAICISM IN *Sparus auratus* and *Dicentrarchus labrax* USING POLYMERASE CHAIN REACTION WITH RANDOM PRIMERS

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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January 2011

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ABSTRACT

Maricultured fish species have a very important contribution for the food demand wordwide. Among the aquacultured species, sea bream (*Sparus auratus L.*) and sea bass (*Dicentrarchus labrax L.*) are among the most widely maricultured ones worldwide and in Turkey. Natural water sources in Turkey enable aquaculture, but still it is not at its climax, because of high number of parameters to be controlled, like the quality parameters of culture area, quality parameters for the feed of fish and the quality parameters of the cultured fish itself as the food.

Environmental risks of aquaculture and environment's impact on aquaculture both exist as a generalized term pollutant stress within a biological system and can be monitored at various levels starting from molecular level which is the earliest of biomarker signals. In this thesis our aim is to follow the various DNA and RNA level variations with modifications of RAPD-PCR and RT-RAPD-PCR profiles.

DNA and RNA mutations can be innate, spontaneous and/or induced according to the causes and somatic and gonadal depending on the cell types they originate or occur. In this thesis the aim is to show only the extend of total genomic variation either innate or induced on various somatic tissues and gonads of cultured *Sparus auratus L.* and *Dicentrarchus labrax L.* samples collected from the same culture area at the same times of the year. DNA and RNA of organs and tissues from cultured fish: *Sparus auratus* and *Dicentrarchus labrax* have been analyzed by RAPD-PCR amplifications with 12 random primers. Among the 8 random 10-mer nucleotide primers tested, four that yield stable, well reproducible and differentiating profiles of amplification products were chosen for further genome-wide mosaicism analysis. With these primers, the differences in the RAPD and RT-RAPD profiles of some tissues were detected. These differences

were associated with the modification of mobility or with the gain/loss of the fragment in the RAPD profile and could be caused by either genomic rearrangements or mutations involving the regions of DNA-primer pairing. Different epigenetic factors may also contribute to this process.

Keywords: Fish aquaculture, Sea bream (*Sparus auratus* L.), Sea bass (*Dicentrarchus labrax* L.), DNA variation, genotoxicity, target organ toxicity, RAPD-PCR, RT-PCR, somatic mosaicism, gonadal mosaicism

Sparus auratus ve _Dicentrarchus labrax_'da RASTGELE SEÇİLMİŞ PRİMERLERLE POLİMERAZ ZİNCİR REAKSİYONU KULLANARAK SOMATİK ve GONADAL MOZAİKLEŞMENİN TESBİTİ

Tuğba ŞENEL

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ÖZ

Ülkemizde ve dünyada yaygın olarak özel hazırlanmış havuzlarda bilimsel yöntemlerle balık üretme işi olan kültür balıkçılığında yetiştirilen balıkların çevresel ve kimyasal etkilere ne ölçüde maruz kaldığı ve bu etkiler sonucu balıkların genetik yapısında oluşan olumsuz değişiklik ve hasarların gösterilmesini amaçlayan çalışmamız, dünyada da, ülkemizde de bu düzeyde yapılan bilimsel temele dayalı bilinçlendirme amaçlı nadir çalışmalardandır.

Kültür balıkçılığıyla elde edilen balıklar tüm dünyada ve ülkemizde tüketilen balıkların % 30'unu oluşturmaktadır. Özellikle ülkemizde yeni gelişmekte olan bu sektörde var olan balık çiftliklerinin neredeyse tamamı hiçbir kritere dayandırılmadan gelişigüzel kurulmuştur ve çevreye çok büyük zarar vermektedir. Ayrıca bu çiftliklerde balık yetiştirmek üzere belirlenmiş olan deniz suyu temizliği ve ortam koşullarının sürdürülebilir olması gibi çeşitli kriterlerin olmadığı bilinmektedir. Bu tip eksikler yanında yetiştirilme ortamına karışabilen bir takım kimyasallar tüm yetiştirilen balık türlerini olumsuz yönde etkilemektedir.

Yapılan çalışmamızla öncelikli olarak belirlenen Çipura ve Levrek balıklarının bu etkenlerden kalıtım materyalimiz olan Deoksiribonüklikasit (DNA) ve yardımcısı Ribonükleikasit (RNA) seviyesinde etkilenme şekilleri, özellikle de hangi organ ve dokularının DNA ve RNA'larında daha fazla bozulma olduğu gösterilmektedir.

Ülkemizde en çok tüketimi yapılan kültür balığı tipleri Çipura ve Levrek olduğu için çalışmamıza model organizma olarak bu balıklar seçilmiştir. Ayrıca bu balıkların diğer kültürü yapılan deniz canlılarına nazaran tüm mevsimlerde ulaşılabilir olması da diğer bir tercih nedenidir.

Yapılan tüm denemeler ve optimizasyonlar sonucu iki balık türünde de DNA ve RNA düzeyinde ciddi bozulma olarak yorumlayabileceğimiz değişiklikler ve

kararsızlıklar görülmüştür. En çok etkilenen dokunun ise tükettiğimiz kısmı olan kas dokusu olduğu görülmüştür. Ayrıca diğer bir etkilenen dokunun da gonad, üreme hücrelerini meydana getiren üreme organı olduğu görülmüştür. Bu dokudaki DNA ve RNA bozulmaları gelecek nesillere aktarılabilir, kalıtsal bozukluklar olabilecektir. DNA/RNA bazında en az bozulma gösteren dokuların ise iki balık türü için de beyin ve karaciğer olduğu görülmüştür. Bu dokuların diğer dokulara nispeten DNA hasarlarına karşı daha korunmuş olmalarının sebebi, beyin dokusunda bulunan kan-beyin bariyeri ve rejeneratif özelliği olan karaciğer dokusunun detoksifikasyondan sorumlu organ olması ve birçok metabolizma enzimi ile toksik maddelerle mücadele eden bir mekanizmaya sahip olmasıyla açıklanabilir.

Teorik olarak aynı canlının DNA'sının bütün dokularda aynı olması beklenirken, RAPD-PZR sonuçlarında böyle olmadığı hatta her bir dokunun da çeşitli olası maruziyetler yüzünden mozaikleştiği ve tıpatıp aynı bantlaşmayı vermediği gözlemlenmektedir.

Anahtar Kelimeler: Kültür balıkçılığı, Çipura (*Sparus auratus* L.), Levrek (*Dicentrarchus labrax* L.), DNA varyasyonu, genotoksisite, hedef organ toksisitesi, RAPD-PZR, RT-PCR, somatik mozaikleşme, gonadal mozaikleşme

Dedicated to my parents Köksal ŞENEL, Songül ŞENEL, my sister Büşra ŞENEL and my best friend Hale YEDİYILDIZ.

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My heartfelt thanks to my father, my mother and my sister for their love, care, pray and sacrifice. Their support has been unconditional all these years; they have cherished with me every great moment and always been there for me whenever I needed.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Abbreviation
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
cDNA	Complementary DNA
mtDNA	Mitochondrial DNA
RAPD	Random amplification of polymorphic DNA
RAMD	Random amplified mosaic DNA
RFLP	Restriction fragment length polymorphism
AFLP	Amplified fragment length polymorphism
SNP	Single nucleotide polymorphism
eSNP	Expressed single nucleotide polymorphism
cSNP	Coding region single nucleotide polymorphism
EST	Expressed sequence tag
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNA	Small interference RNA
piRNA	Piwi-interacting RNA
miRNA	microRNA
RNAi	RNA interference

CHAPTER 1

INTRODUCTION

1.1 Aquaculture

Aquaculture, also known as aquafarming, is the farming of aquatic organisms such as fish, molluscs, crustaceans and aquatic plants. Aquaculture involves cultivating saltwater and freshwater populations under controlled conditions. Farming implies individual or corporate ownership of cultivated stock and some forms of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators.

Particular kinds of aquaculture include algaculture (the production of kelp/seaweed and other algae), shrimp farming, fish farming, oyster farming, and the cultivation of ornamental fish. Particular methods include aquaponics, which integrates fish farming and plant farming. Fish culture, also named as pisciculture, refers to the husbandry of finfish. The most popular aquaculture species are finfish that are grown in fresh waters, accounting for over 40 % of total aquaculture production (1).

Aquaculture is considered an agricultural activity, mainly producing protein crops with the disadvantage of accumulation of pollutants in the culture environment. Consequently, aquaculturists must manage their production units to ensure that water quality not to deteriorate environment and become stressful to the culture organisms (2).

Fish farming is an industry that accounts for increasing share of world fisheries production. In previous years, it is a business for small farms, but now pursued by large

agribusinesses. By the early 2000s it had become almost as significant a source of fish as the as wild fisheries and other varieties (3, 4).

Mariculture is a specialized branch of aquaculture involving the cultivation of marine organisms for food and other products in the open ocean, an enclosed section of the ocean, or in tanks, ponds or raceways which are filled with seawater. Side products of mariculture include fish meal, nutrient agar, jewelries (e.g. cultured pearls), and cosmetics (5, 6).

1.2 Aquaculture in Turkey and in the World

Aquaculture in Turkey started with carp and trout farming in 1970s and gained momentum with commencement of gilthead sea bream / sea bass farming in the Aegean Sea and Mediterranean Sea beginning from the midst of 1980s; cage culture of trout in the Black Sea during 1990s; and tuna rearing in the Aegean Sea and the Mediterranean Sea in early 2000s. In 1990s, the attempts for salmon culture in the Black Sea and shrimp culture in the Mediterranean Sea (Manavgat) started but wasn't become successful (7).

The inland resources are also very rich in Turkey with 175 km rivers, 1 million ha natural lakes, 170.000 ha reservoirs, 70.000 ha lagoons, 700 small reservoirs (8).

Turkey import raw fish and export mollusks and crustacean; the need for raw fish may be met through aquaculture. 70%-80% of export is to European Union countries (Belgium, Germany and France), followed by Japan. Also in recent years exportation to China is also increasing continously. Necessary precautions to prevent pollution and decrease in fish stocks should be taken, evaluated and the present potentials of seas and inland waters of Turkey. It is necessary that new aquaculture methods in inland waters and dams to be introduced and encouraged, and additionally fish varieties with high economic value should be adapted without harming the domestic fauna (9).

Table 1.1 Number of fish farms, their capacity and amount of production in Turkey (7).

Species	Number of fish farms	Capacity (tons/year)	Product (tons/year)
Rainbow trout	1215	29998	39674
Rainbow trout (sea)	11	1139	1194
Sea bream and sea bass	345	51211	37773
Mussel	2	320	815
Carp	86	2613	543
Total	1659	85281	79943

98.5% of the production is from the carnivorous fish species (trout, sea bass, gilthead sea bream and tuna). This is one of the typical characteristics of aquaculture in Turkey that aquaculture is mostly based on the intensive production of carnivorous fish species (Table 1.1). Trout ranks have the first place (56.4%) amongst the species cultured, followed by sea bass (23.4%) and gilthead sea bream (19.1%) (7).

1.3 Sparus auratus (Gilthead sea bream)

Sparus auratus (Linn., 1758) is a fish of the bream family Sparidae found in the Mediterranean Sea and the eastern coastal regions of the North Atlantic Ocean. It grows to about 60 cm length. The gold spot just behind the gill is the main distinguishing feature of the gilthead bream.

The gilthead sea bream has been cultured for many years in Mediterranean coastal lagoons and brackish/salt water ponds and especially in the northern Adriatic valleys in Italy. In ancient times, the extensive fish rearing systems acted like natural fish traps, taking advantage of the natural trophic migration of juveniles from the sea. Restocking was usually performed with wild fry and juveniles, collected by fishermen. By the late

1970s, the reduced availability of wild fry and the increasing demand from intensive farms enhanced the development of induced spawning techniques (10).

1.3.1 Global Aquaculture Statistics of *Sparus auratus*

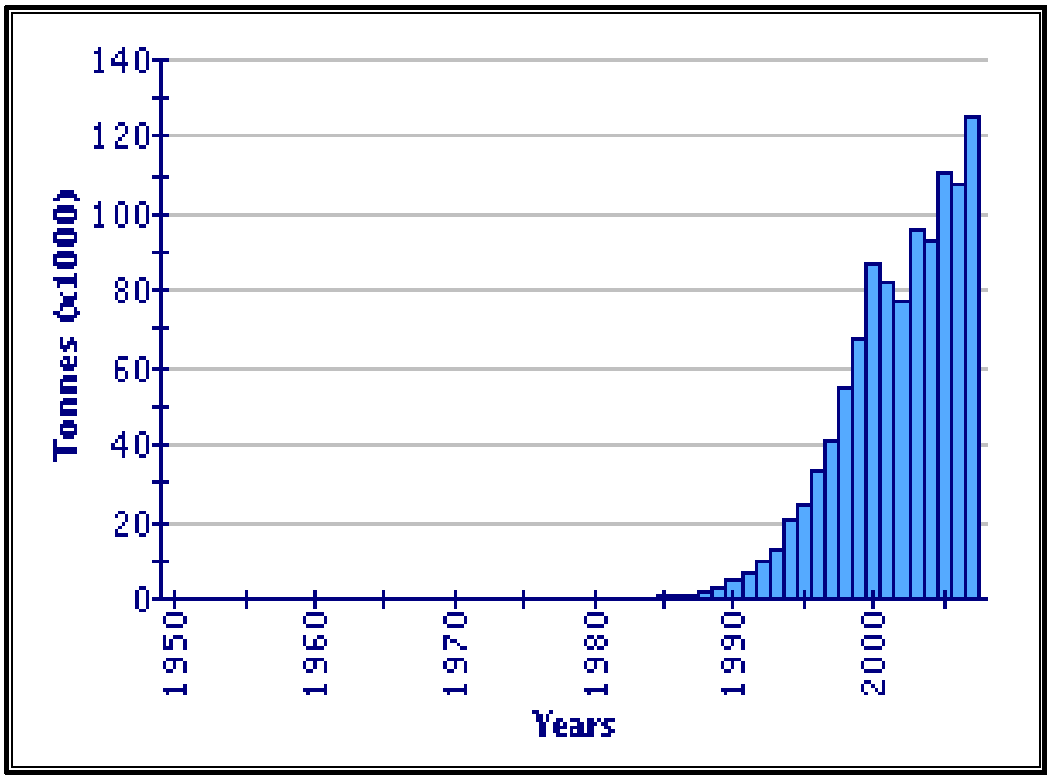

Figure 1.1 *Sparus auratus* Global Aquaculture Statistics (11).

World production occurs mostly in Mediterranean and Greece (49 %) being by far the largest producer in 2002. Besides Turkey (15 %), Spain (14 %) and Italy (6 %) are also the major Mediterranean producers. Additionally considerable production occurs in Cyprus, Malta, Egypt, Croatia, France, Morocco, Portugal and Tunisia. Also in Red Sea, the Persian Gulf and the Arabian Sea, gilthead sea bream production is being made. The main producer is Israel and Oman and Kuwait are minor producers (11).

1.3.2 Taxonomy

Taxonomy of the *Sparus auratus* is given bellow (12).

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Infraclass	Teleostei
Superorder	Acanthopterygii
Order	Perciformes
Suborder	Percoidei
Family	Sparidae
Genus	Sparus
Species	*Sparus auratus* (*Sparus aurata*)

1.3.3 Biology

The gilthead sea bream inhabits sandy bottoms and seagrass beds as well as the surf zone. It is reported as a sedentary fish; even so migrations are likely to occur on the Eastern Atlantic coast, from Spain to British Isles. It occurs either in small aggregations or solitary. It is a euryhaline species and moves in spring towards protected coastal waters in search for abundant food and milder temperatures that means trophic migration. In late autumn it returns to the open sea for breeding purposes, being very sensitive to low temperatures (lower lethal limit is 2ºC). Sea bream is mainly carnivorous (shellfish, including mussels and oysters) and sometimes herbivorous (13).

Sea bream is a proterandrous hermaphrodite: it is a functional male in the first two years and over 30 cm in length becomes female. During the male phase, the bisexual gonad has functional testicles with asynchronous spermatogenesis and non-functional ovarian areas (14, 15).

Ovarian development is also asynchronous, and females are batch spawners that can lay 20.000-80.000 eggs per day for a period of up to 3 months. In the Mediterranean, they reproduce between October and December. The eggs are pelagic and spherical with a diameter lower than 1 mm and with a single large oil droplet. The planktonic larval stage lasts about 50 days at 17 ºC -18 ºC (16).

Microalgae are used to feed the rotifer or to indirectly feed the larvae of *Sparus auratus* at first step. Algae help holding the quality of various water parameters at optimum ranges in algae tanks, minimises bacterial contamination and nitrogen concentration and stimulates larvae immune systems. *Isocrysis galbana*, *Nannochloropsis gadinota*, *Nannochloropsis oculata* and *Tetracelmis suecica* are the most common species for feeding the larvaes. These species are chosen for their rich protein potentials, even though some of these algae species are not enough nutritious especially on ώ3 fatty acids concentrations. These species are selected for their sizes, production aptitudes and absence of toxic effects. For adult feeding, two different types of feeding is applied; for startup from 3-4 months to ovogenesis period a protection diet is being applied that daily feeding rate is about to 1-1.5% of total weight. Enriched diet is serving as main feed for gametogenesis and during hatching period (10).

Before harvesting the fish some days of starvation are needed. The length of this period varies according to feeding rate and temperature (for example, at 25 °C, 24 hours may be enough). 48-72 hours are necessary at lower temperatures. The fish are ready to be harvested after correct starvation. The presence of dying or dead fish needs to be checked before starting this process.

1.3.4 Life Cycle of *Sparus auratus*

There are two life cycles of *Sparus auratus* known as extensive and intensive system. (Figures 1.2 and 1.3)

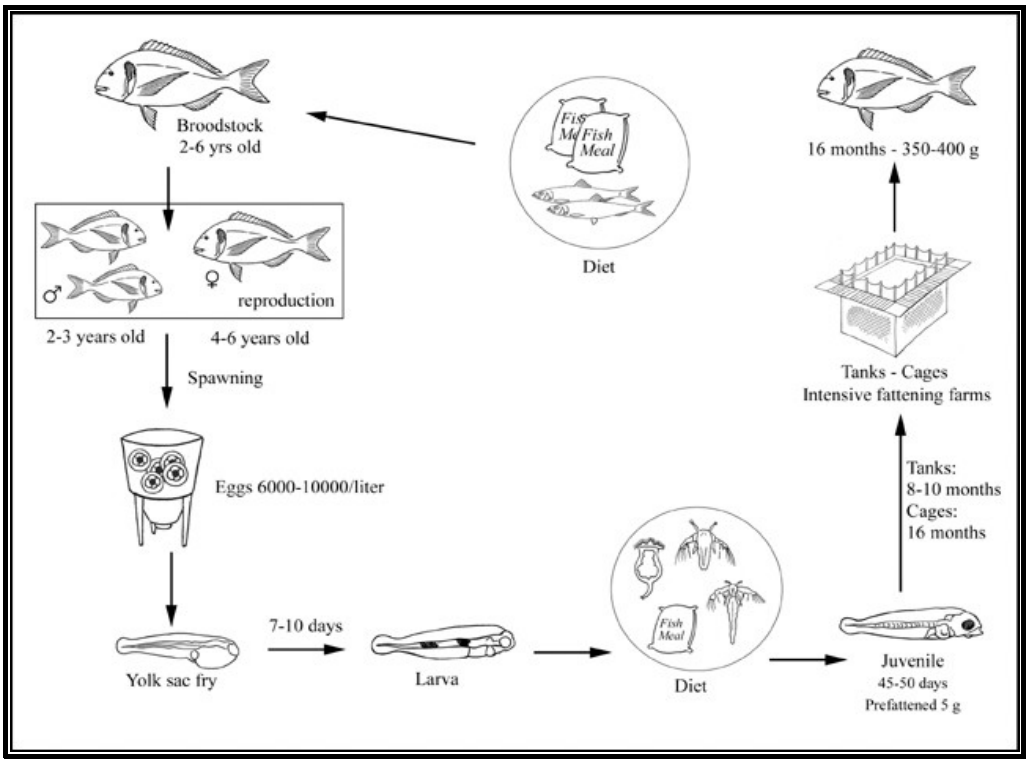

Figure 1.2 Life Cycle of *Sparus auratus* (Intensive System) (17).

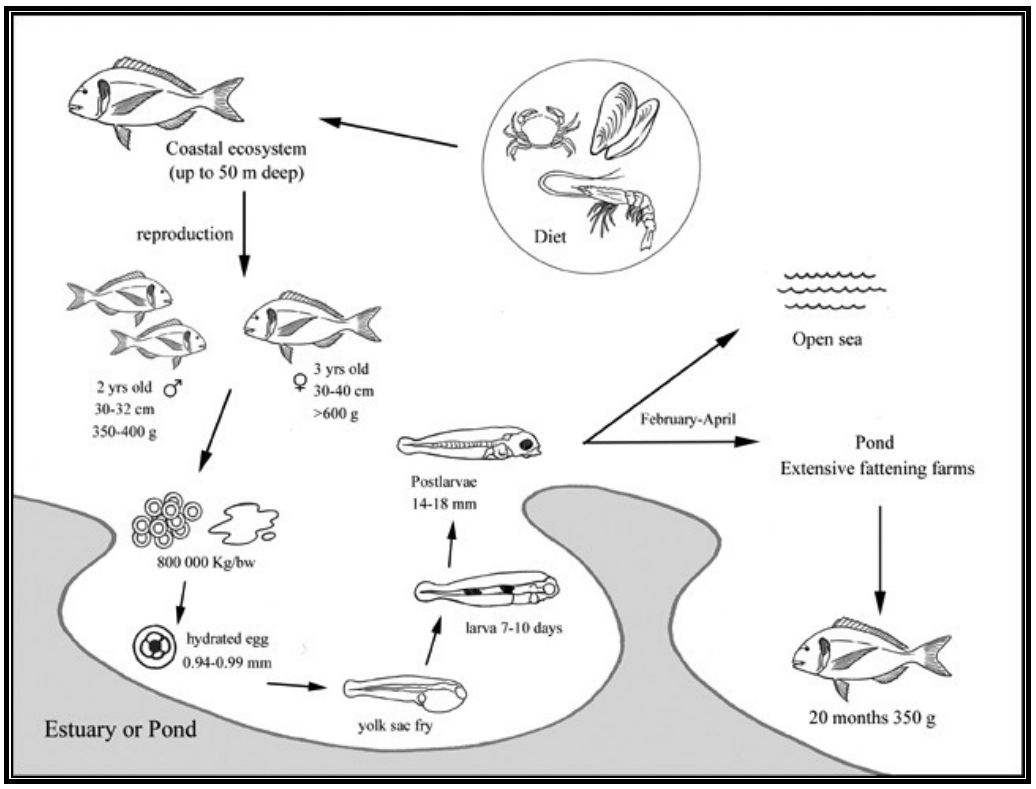

Figure 1.3 Life Cycle of *Sparus auratus* (Extensive System) (17).

Intensive grow-out follows other intensive farming phases, named reproduction, pre-fattening and larval rearing, as described above. Gilthead sea bream intensive pre-fattening and grow-out phases may be carried out in land-based installations with rectangular concrete tanks that vary in size (200-3000 m^3) according to fish size and the demands of production. Also grow-out may occur in sea cages, either in sheltered or semi-exposed sites named floating cages or totally exposed sites (semi-submersible or submersible cages).

Extensive system is based on the natural migration of euryhaline fish, when the fish may be caught, generally in typical fishing traps. Since this practice provides a very limited and unpredictable source of natural juveniles, many modern commercial extensive production units rely on both wild-caught and hatchery-reared juveniles.

1.4 *Dicentrarchus labrax* (SEA BASS)

The European sea bass, *Dicentrarchus labrax* (Linn., 1758) is found in coastal waters of the Atlantic Ocean from South of Norway (60°N) to Western Sahara (30ºN) and almost found everywhere in the Mediterranean Sea and the Black Sea. It has been introduced for culture purposes in Israel, and recently in Oman and the United Arab Emirates (18).

Cultivation of sea bass was started in coastal lagoons in the late 1960s. Fish culture was initially associated with salt production in coastal evaporation pans and marshes in ancient times. The salt was harvested during the high evaporation season of autumn and summer; fish were cultured during winter and spring. This culture came from trapping of fish that lived in these estuarine areas.

One of the largest success stories in European aquaculture has been the sea bass industry, which in less than 15 years grew from a few thousand tonnes to 57.000 tonnes today (19, 20).

1.4.1 Global Aquaculture Statistics of *Dicentrarchus labrax*

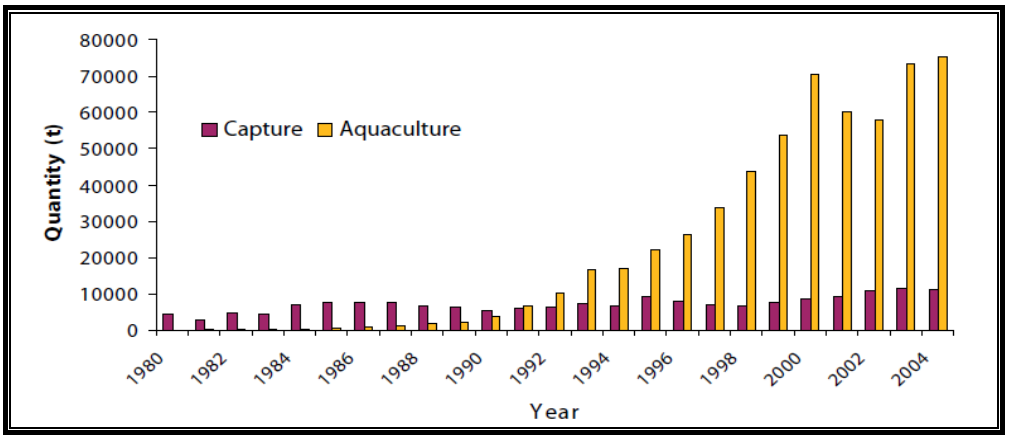

Figure 1.4 Capture fisheries and aquaculture production for European sea bass in the Mediterranean and in Europe (18).

In 2004, the global sea bass capture fisheries production was of 11.481 tonnes (19) and aquacultured sea bass capture is about to 75.000 tonnes.

1.4.2 Taxonomy

Taxonomy of the *Dicentrarchus labrax* is given bellow (21).

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Infraclass	Teleostei
Superorder	Acanthopterygii
Order	Perciformes
Suborder	Percoidei
Family	Moronidae
Genus	Dicentrarchus
Species	*Dicentrarchus labrax*

1.4.3 Biology

The European bass is a member of the Moronidae family and the name *Dicentrarchus* comes from the presence of two dorsal fins. It has silver sides and a white belly and juveniles maintain black spots on the back and sides. This fish's operculum is spined and serrated. *D.labrax* may grow to a total length of over 1 m and 15 kg of weight (21).

D.labrax has a body rather elongated. These fish have opercle with 2 flat spines; preopercle with large, forward-directed spines on its lower margin and a diffuse spot been on the edge of opercle. The color of the fish is silvery grey to bluish on the back, silvery on the sides, belly sometimes tinged with yellow and young ones have some dark spots on upper part of body but adults never spotted (19).

Sea bass is a gonochoristic species. Females spawn in winter in the Mediterranean Sea (December to March) and spawn in the Atlantic Ocean up to June. They present a high fecundity (on average 200.000 eggs / kg of female). These fish start to reproduce over 2 kg and can reach 6 to 7 years in the wild (22, 23).

1.4.4 Life Cycle of *Dicentrarchus labrax*

There are two life cycles of *Dicentrarchus labrax* known as extensive and intensive system that given in the Figures 1.5 and 1.6.

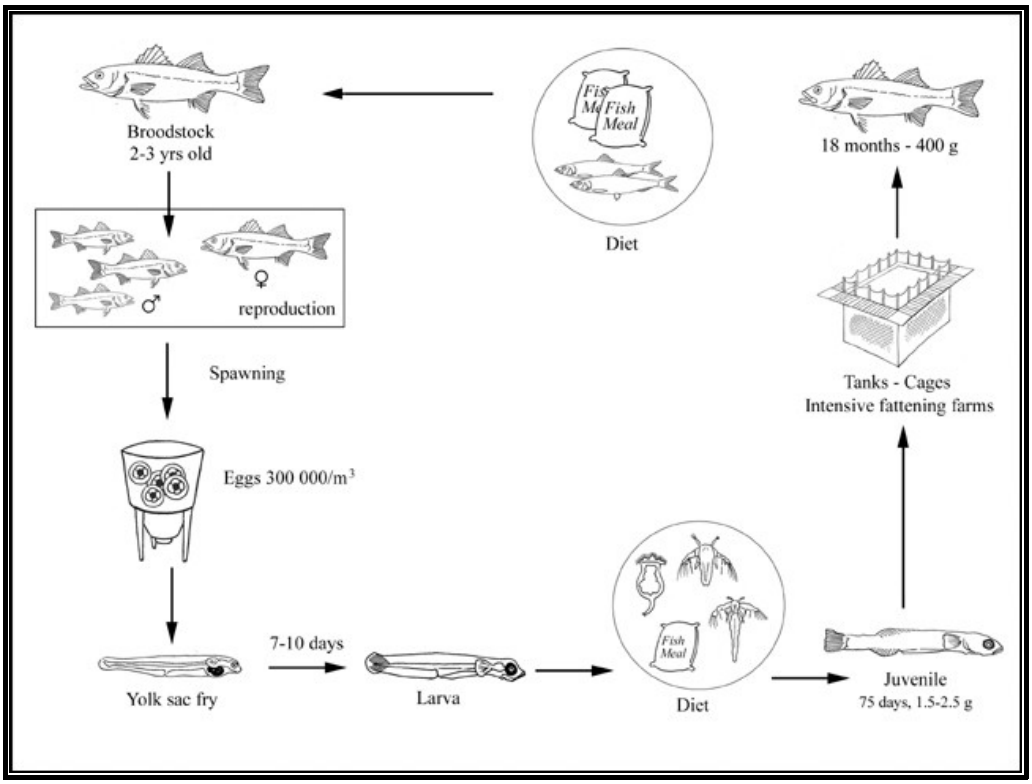

Figure 1.5 Life Cycle of *Dicentarchus labrax* (Intensive System) (19).

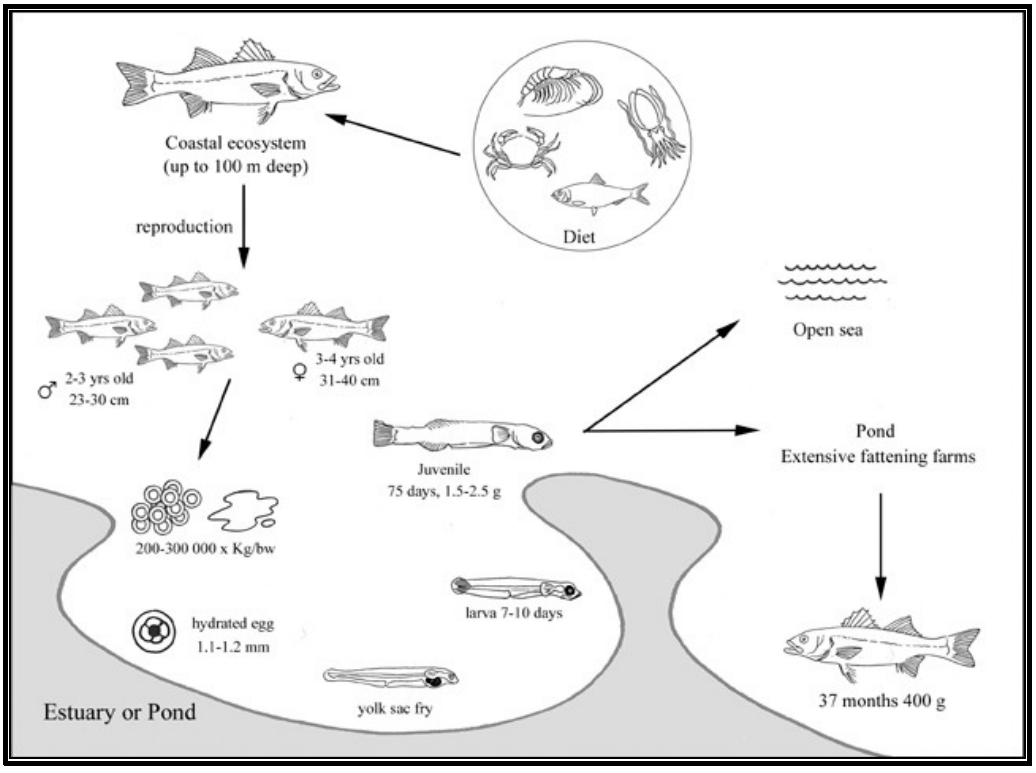

Figure 1.6 Life Cycle of *Dicentrarchus labrax* (Extensive System) (19).

In intensive production, ongrowing units are supplied with fry from hatcheries and controlled diet is provided. Juveniles are sold to farmers at a size of 1.5-2.5 g. The ongrowing juveniles reach 400-450 g in 18-24 months. Feeds are distributed by automatic feeders every 10-15 minutes for small fish (2-15 g), or by hand for larger fish. Grading is necessary at least two or three times per cycle, in order to avoid growth differentiation and cannibalism. Fattening can occur in tanks or in cages system.

The traditional extensive method of lagoon management places special barriers in appropriate lagoon sites to capture fish during their autumn migration to the open sea. In this system sea bass is usually cultured in polyculture with sea bream, mullets and eels. Sea bass reach a commercial size of 400-500 g in 37 months, with a total lagoon production of 50-150 kg. The limiting factor is in the natural feeding behaviour of the sea bass that, as predators, may drastically reduce the natural resources of the lagoon ecosystem (19).

1.5 Molecular Researches on Aquaculture

The development of DNA-based genetic markers has a revolutionary impact on animal genetics. It is theoretically possible to observe and exploit genetic variation in the entire genome with DNA markers. Popular genetic markers in the aquaculture community include allozymes, mitochondrial DNA, RAPD, RFLP, AFLP, SNP, microsatellite and EST markers. The application of DNA markers has allowed rapid progress in aquaculture investigations of genetic variability and inbreeding, species and strain identification, parentage assignments and the construction of high-resolution genetic linkage maps for aquaculture species (24).

Several marker types are highly popular in aquaculture genetics. In the past, allozyme and mtDNA markers have been popular in aquaculture genetics research but more recent marker types that are finding service in this field include restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers (25). Table 1.2 summarizes the basic properties of mentioned marker types.

Table 1.2 Types of DNA markers, their characteristic and potential applications (24).

Marker type	Acronym or alias	Requires prior molecular information	Mode of inheritance	Locus under investigation	Likely allele numbers	Major applications
Allozyme		Yes	Mendelian codominant	Single	2-6	Linkage mapping
Mitochondrial DNA	mtDNA	No	Metarnal İnheritance		Multiple haplotypes	Maternal lineage
Restriction fragment length polymorphism	RLFP	Yes	Mendelian codominant	Single	2	Linkage mapping
Random amplified DNA	RAPD AP-PCR	No	Mendelian dominant	Multiple	2	Fingerprinting for population studies Hybrid identification
Amplified fragment length polymorphism	AFLP	No	Mendelian dominant	Multiple	2	Linkage mapping Population studies
Microsatellites	SSR	Yes	Mendelian codominant	Single	Multiple	Linkage mapping Population studies

Molecular markers are classified into two categories: type I are markers associated with genes of known function, while type II markers are associated with anonymous genomic segments (Table 1.2). Under this classification, most RFLP markers are type I markers because they were identified during analysis of known genes. Likewise, allozymes markers are type I markers because the protein they encode has known function. RAPD markers are type II markers because RAPD bands are amplified from anonymous genomic regions via the polymerase chain reaction (PCR). AFLP markers are type II because they are also amplified from anonymous genomic regions.

Microsatellite markers are type II markers unless they are associated with genes of known function. EST markers are type I markers because they represent transcripts of genes. SNP markers are mostly type II markers unless they are developed from expressed sequences (eSNP or cSNP). Indels are becoming more widely used as markers since they often are discovered during genomic or transcriptomic sequencing projects; they can be either type I or type II markers depending on whether they are located in genes (24).

1.5.1 Applications of DNA Markers in Aquaculture Genetics

One of the questions at the beginning of any genome research is what type of marker is most suitable for the species of interest. There is no simple answer to this question, and much depends on the specific objectives of the study (Table 1.3). However, with a good understanding of the DNA marker technologies, appropriate decisions can be reached.

Table 1.3 Applications of DNA Markers in Aquaculture Genetics (24).

Task	Recommended Marker System	Other Useful Marker Types
Species identification	RAPD	AFLP, microsatellites, isozymes
Strain identification	AFLP, microsatellites	RAPD
Hybrid identification	RAPD	AFLP, microsatellites, mitochondria
Paternity determination	Microsatellites	
Genetic resource	AFLP, microsatellites	RAPD
Genetic mapping	Type I markers, Microsatellites SNP	AFLP, RFLP
Comparative mapping	Type I markers	ESTs, conserved microsatellites

1.6 Genetic Diversity

Genetic diversity is a level of biodiversity that refers to the total number of genetic characteristics in the genetic makeup of a species. It is distinguished from genetic variability, which describes the tendency of genetic characteristics to vary.

Genetic diversity plays a very important role in survival and adaptability of a species because when the environment of a species changes, slight gene variations are necessary for it to adapt and survive. A species that has a large degree of genetic diversity among its population will have more variations from which to choose the fittest alleles. Species that have very little genetic variation are at a great risk. With very little gene variation within the species, healthy reproduction becomes increasingly difficult, and offspring often deal with similar problems to those of inbreeding (26).

There are many different ways to measure genetic diversity. Many works for the loss of animal genetic diversity have been done (27, 28). A 2007 study conducted by the National Science Foundation found that genetic diversity and biodiversity are dependent upon each other, that diversity within a species is necessary to maintain diversity among species, and vice versa. According to the lead researcher in the study, Dr. Richard Lankau, "If any one type is removed from the system, the cycle can break down, and the community becomes dominated by a single species" (29).

Genetic diversity refers to the variation at the level of individual genes (polymorphism), and provides a mechanism for populations to adapt to their ever-changing environment. The more variation, the better the chance that at least some of the individual genetic diversity refers to any variation in the nucleotides, genes, chromosomes, or whole genomes of organisms (the genome is the entire complement of DNA within the cells or organelles of the organism). Genetic diversity at its most elementary level is represented by differences in the sequences of nucleotides (adenine, cytosine, guanine, and thymine) that form the DNA (deoxyribonucleic acid) within the cells of the organism. The DNA is contained in the chromosomes present within the cell; some chromosomes are contained within specific organelles in the cell (for example, the chromosomes of mitochondria and chloroplast). Nucleotide variation is measured for discrete sections of the chromosomes, called genes. Thus, each gene

compromises a hereditary section of DNA that occupies a specific place of the chromosome, and controls a particular characteristic of an organism. Individuals will have an allelic variant that is suited for the new environment, and will produce offspring with the variant that will in turn reproduce and continue the population into subsequent generations (30).

The rapid advances in molecular biology have provided a range of techniques for direct examination of variation in DNA. To date most populations studies have used restriction fragment length polymorphisms of the mitochondrial genome. The mitochondrial (mt) DNA is small and relatively easy to purify, and the fragments generated with restriction enzyme digests are easy to interpret. Variations in fragment numbers are generated by additions and deletions of restriction sites, and in fragment lengths by insertions or deletions of blocks of bases. Similar techniques can be applied to nuclear (n) DNA, but the considerably larger size of DNA means that small pieces of the genome have to be analyzed with specific probes. Several regions of the nuclear genome contain multiple repeats of short minisatellite sequences which are resolved as DNA fingerprints. The hypervariable nature of these variable number tandem repeats has lead to widespread use of DNA fingerprinting in forensic studies, but the technique has had limited application in marine population studies (31, 32).

Morphological characters, the tools of traditional taxonomy, have been used to describe variation among individuals and populations. The characters used are meristic (countable) such as number of fin rays or vertebrae and morphometric (measurable) expressed as ratios of standard length or fork length. Morphological characters have limitations for describing intraspecific genetic diversity as they are polygenic and expression can be modified by the environment. Their use in population-stock identification studies has been superseded for the most part by the development of direct genetic methods.

Invertebrates generally have higher levels of genetic diversity than vertebrates as measured by protein electrophoresis (33). Within the vertebrates amphibia have the highest and teleosts have the lowest levels of genetic diversity (34). Marine invertebrates show wide variation in levels of genetic diversity. In 26 species of

molluscs, heterozygosities range from 2 to 32 %. Crustacea have lower levels of genetic diversity ranging from 0.4 to 10.9 % in 44 species of decapod, from 0.8 to 6.4 % (35).

1.7 Genetic Variation

Genetic variability is a measure of the tendency of genotypes within a population to differentiate. Individuals of the same species are not identical. Although they are recognizable as belonging to the same species, there are many differences in form, function and behavior. For every characteristic of an organism, variations will exist within the species.

The most obvious cases of genetic variability are to be found in the domesticated species, where humans use the variability to create breeds and varieties of maize, beans, apples, pumpkins, horses, cattle, sheep, dogs and cats (36).

There are many sources of genetic variability in a population:

Homologous recombination is a significant source of variability. During meiosis in sexual organisms, two homologous chromosomes from the male and female parents cross over one another and exchange genetic material. The chromosomes then split apart and are ready to form an offspring. Chromosomal crossover is random and is governed by its own set of genes that code for where crossovers can occur (in cis) and for the mechanism behind the exchange of DNA (in trans). Being controlled by genes means that recombination is also variable in frequency, location, thus it can be selected to increase fitness by nature, because the more recombination the more variability and the more variability the easier it is for the population to handle changes (37).

DNA errors can take two forms: mutations and DNA damages. DNA damage tends to interfere with gene expression by preventing transcription of RNA from DNA, whereas mutation usually results in transcription that usually produces proteins with diminished or altered functionality. Mutations that are not lethal to a cell are more likely to be perpetuated in dividing cells. DNA damage rather than DNA mutation is posited as a cause of aging and cancer (38).

It is important to distinguish between DNA damage and mutation. Damages are physical abnormalities in the DNA, such as single and double strand breaks, 8-hydroxydeoxyguanosine residues and polycyclic aromatic hydrocarbon adducts (39). DNA damages can be recognized by enzymes, and thus they can be correctly repaired if redundant information, such as the undamaged sequence in the complementary DNA strand or in a homologous chromosome, is available for copying.

If a cell retains DNA damage, transcription of a gene can be prevented and thus translation into a protein will also be blocked. Replication may also be blocked and/or the cell may die. In contrast to DNA damage, a mutation is a change in the base sequence of the DNA (40).

Mutations can occur spontaneously or can be induced. Spontaneous mutations can result from abnormalities in cellular or biological processes, for example errors in DNA replication. Induced mutations are caused by environmental agents. These agents can be chemical and physical agents that are known to alter DNA structure and termed as mutagens (41).

The great majority of mutations that are not neutral in their effect are harmful to a cell's survival. Thus, in a population of cells comprising a tissue with replicating cells, mutant cells will tend to be lost. However infrequent mutations that provide a survival advantage will tend to clonally expand at the expense of neighboring cells in the tissue. This advantage to the cell is disadvantageous to the whole organism, because such mutant cells can give rise to cancers (38).

Genetic mutations may contribute to the genetic variability within a population and can have positive, negative or neutral effects on fitness. This variability can be easily propagated throughout a population by natural selection if the mutation increases the affected individual's fitness and its effects will be minimized if the mutation is deleterious. However, the smaller a population and its genetic variability are, the more likely recessive or hidden deleterious mutations will show up causing genetic drift (42).

Sources of new genetic variation that occur in populations showed in Table 1.7.

Table 1.4 Sources of new genetic variation (41).

Type	Description
Independent assortment	The independent segregation of different homologous chromosomes may give rise to new combinations of alleles in offspring.
Crossing over	Recombination (crossing over) between homologous chromosomes can also produce new combinations of alleles that are located on the same chromosome.
Interspecies crosses	On occasion, members of different species mat breed with each other to produce hybrid offspring.
Prokaryotic gene transfer	Prokaryotic species possess mechanisms of genetic transfers such as conjugation, transduction, and transformation.
New alleles	Point mutations can occur within a gene to create single nucleotide polymorphisms (SNPs). IN addition, genes can be altered by small deletions and additions.
Gene duplications	Events, such as misaligned crossovers, can add additional copies of a gene into a genome and lead to the formation of gene families.
Chromosome structure and number	Chromosome structure may be changed by deletions, duplications, inversions, and translocations. Changes in chromosome number result in aneuploid, polyploid, and alloploid offspring.
Exon shuffling	New genes can be created when exons of preexisting genes are rearranged to make a gene that encodes a protein with a new combination of protein domains.
Horizontal gene transfer	Genes from one species can be introduced into another species and become incorporated into that species' genome.
Changes in repetitive sequences	Short repetitive sequences are common in genomes due to the occurrence of transposable elements and due to tandem arrays. The number and lengths of repetitive sequences tend to show considerable variation in natural populations.

In a population of cells, mutant cells will increase or decrease in frequency according to the effects of the mutation on the ability of the cell to survive and reproduce. Although distinctly different from each other, DNA damages and mutations are related because DNA damages often cause errors of DNA synthesis during replication or repair and these errors are a major source of mutation (41).

1.7.1 Epigenetic Inheritance and its Molecular Basis

Epigenetic inheritance refers to a pattern in which a modification occurs to a nuclear gene or chromosome that alters gene expression. However, the expression is not permanently changed over the course of many generations. Epigenetic changes are caused on DNA by chromosomal modifications. These changes can occur during oogenesis, spermatogenesis or early embryonic development.

Specific epigenetic processes include: paramutation, Piwi-interacting RNA (piRNA), microRNA (miRNA), short interfering RNAs (siRNAs), bookmarking, imprinting, gene silencing, X chromosome inactivation, position effect, reprogramming, transvection, maternal effects, the progress of carcinogenesis, many effects of teratogens, regulation of histone modifications, heterochromatin and methylation.

DNA methylation is a change in chromatin structure that silences gene expression and carried out by the enzyme DNA methyltransferase. It is common in some eukaryotic species, but not all. For example, yeast and *Drosophila* have little DNA methylation, vertebrates and plants have abundant DNA methylation.

DNA methylation usually inhibits the transcription of eukaryotic genes especially when it occurs in the vicinity of the promoter. In vertebrates and plants, many genes contain CpG islands near their promoters. These CpG islands are 1.000 to 2.000 nucleotides long and they contain high number of CpG sites. In housekeeping genes, the CpG islands are unmethylated genes tend to be expressed in most cell types. In tissue-specific genes, the expression of these genes may be silenced by the methylation of CpG islands. Methylation may change with binding of the transcription factors. Methyl-CpG-binding proteins may recruit factors that lead to compaction of the chromatin.

There are two main kinds of methylation; hypermethylation, lots of genes that have vital function in the genom have been silenced by hypermethylation, for example, DNA repair genes, cell cycle regulators, apoptosis and detoxification related genes. Only the methylation that occurs in/by promoter region will be resulted as gene silencing. The second one is hypomethylation, this is a mechanism of drug, toxin and viral effects in cancer. For example, cervical cancer latency is caused by

hypermethylation of HPV16 genome and cadmium inhibits DNA methyltransferase activity and leads to acute hypomethylation, which is followed by hypermethylation of DNA after chronic exposure to this “epigenetic’ carcinogen.

DNA methylation is heritable. Methylated DNA sequences are inherited during cell division and this mechanism can be explained by genomic imprinting. Specific genes are methylated in gametes from mother or father. Pattern of one copy of the gene being methylated and the other not is maintained in the resulting offspring (41-43).

1.7.2 Regulation of RNA Processing and Translation

In eukaryotic species, it is common for gene expression to be regulated at the RNA level. These gene expression mechanisms are; alternative splicing, RNA editing, RNA stability, RNA interference, general regulation of translation and translational regulation of specific mRNAs.

RNA modification is analysis of bacterial genes in the 1960s and 1970 revealed the following: The sequence of DNA in the coding strand corresponds to the sequence of nucleotides in the mRNA. This in turn corresponds to the sequence of amino acid in the polypeptide. This is termed the colinearity of gene expression. Analysis of eukaryotic structural genes in the late 1970s revealed that they are not always colinear with their functional mRNAs. Instead, coding sequences, called exons, are interrupted by intervening sequences or introns. Transcription produces the entire gene product; introns are later removed or excised and exons are connected together or spliced. This phenomenon is termed RNA splicing. It is a common genetic phenomenon in eukaryotes.

One very important biological advantage of introns in eukaryotes is the phenomenon of alternative splicing. Alternative splicing refers to the phenomenon that pre-mRNA can be spliced in more than one way. In most cases, large sections of the coding regions are the same resulting in two alternative versions of a protein that have similar functions. Nevertheless, there will be enough differences in amino acid sequences to provide each protein with its own unique characteristics.

The term RNA editing refers to a change in the nucleotide sequence of an RNA molecule. It involves additions or deletion of particular bases or a conversion of one type of base to another. RNA editing can have various effects on mRNAs like generating start or stop codons and changing the coding sequence of a polypeptide.

Stability of RNAs varies considerably. The stability of mRNA can be regulated so that its half-life is shortened or lengthened. This will greatly influence the mRNA concentration and consequently gene expression. Factors that can affect mRNA stability include length of the polyA tail and destabilizing elements. Length of the polyA tail can be find name as most newly made mRNA. A polyA tail is about 200 nucleotides long. It is recognized by polyA-binding protein which binds to the polyA tail and enhances stability. As an mRNA ages, its polyA tail is shortened by the action of cellular nucleases. The polyA-binding protein can no longer bind if the polyA tail which is less than 10 to 30 adenosines long. The mRNA will then be rapidly degraded by exo- and endonucleases. Destabilizing elements are found especially in mRNAs that have short half-lives. These elements can be found anywhere on the mRNA. However, they are most common at the 3’ end between the stop codon and the polyA tail.

RNA transcripts have different functions. Once they are made, RNA transcripts play different functional roles. Well over 90% of all genes are structural genes producing mRNA and translated into a protein product. The RNA transcripts from nonstructural genes are not translated. They do have various important cellular functions. They can still confer traits. In some cases, the RNA transcript becomes part of a complex that contains protein subunits, for example ribosomes, spliceosomes and signal recognition particles (41-47).

1.8 Target Organ Toxicity and Mosaicism

Toxicology is the study of the adverse effects of chemicals or physical agents on living organisms. Toxicity is complex with many influencing factors; dosage is one of the most important. Xenobiotics, or chemicals foreign to the body, may cause many types of toxicity by a variety of mechanisms. Some chemicals are themselves toxic and are sometimes referred to as "parent" compounds. Others must be metabolized

(chemically changed within the body) before they cause toxicity. Toxicity can result from adverse cellular, biochemical, or macromolecular changes. Examples are cell replacement, such as fibrosis, damage to an enzyme system, disruption of protein synthesis, production of reactive chemicals in cells and DNA damage (48, 49).

Occupational and environmental exposure to chemicals frequently occurs, and various organs in the body have been identified as targets for toxicity resulting from such exposures. Some compounds can induce adaptive processes in their target cells. Changes in gene expression at the transcriptional, translational or post-translational levels can result from the induction or inhibition of enzyme systems, or by up-regulation or down-regulation of receptor-ligand interactions, thereby changing the functions of intracellular signal cascades. For instance, the increase of metallothionein expression following exposure to low doses of metals permits a higher toxic dose to be tolerated (50).

All the tissues are susceptible in varying degrees to the toxic chemicals' effects, but many chemicals exhibit a marked tendency to damage specific organs, these are known as the target organs of toxicity**.**

There are many kinds of specific toxicity areas like, blood and cardiovascular toxicity, dermal toxicity, eye toxicity, hepatotoxicity (toxicity in liver), immunotoxicity (toxicity in immune system), nephrotoxicity (toxicity in kidney), neurotoxicity (toxicity in neural system), reproductive toxicity (toxicity in male and female reproductive system) and respiratory toxicity (Toxicity relates to effects on the upper respiratory system, nose, pharynx, larynx, and trachea).

The term "mosaicism" describes a situation in which different cells in the same individual have different numbers or arrangements of chromosomes. It is called "mosaicism" because, in a way, the cells of the body are similar to the tiles of a mosaic. In a mosaic piece of art, each tile is different. They have different shapes and colors. The tiles are fitted together to make a whole picture.

Mosaicism can be diagnosed in different ways. Sometimes, different cells in the blood have different chromosome make-ups. For these individuals, a blood test may be

able to detect mosaicism. In other individuals, it is more difficult to diagnose mosaicism. For example, a blood test would not be able to diagnose mosaicism if the chromosome change is only located in the skin cells. In cases like this, additional testing may be needed to detect mosaicism. Usually, this would involve examination of other tissues of the body, such as the skin. A geneticist can help determine whether this type of additional testing is appropriate (51).

Genetic mosaics can be extraordinarily useful in the study of biological systems, and can be created intentionally in many model organisms in a variety of ways. They often allow for the study of genes that are important for very early events in development, making it otherwise difficult to obtain adult organisms in which later effects would be apparent. Furthermore they can be used to determine the tissue or cell type in which a given gene is required and to determine whether a gene is cell autonomous. That is, whether or not the gene acts solely within the cell of that genotype, or if it affects neighboring cells which do not themselves contain that genotype, but take on that phenotype due to environmental differentiation.

1.8.1 Somatic Mosaicism

The phenomenon of somatic mosaicism implies the presence of genetically different cell lines in a single organism. The differences between the lines may be caused by mutations, chromosomal rearrangements, modifications in mini- and microsatellite loci at DNA replications, the loss of heterozygosity through mitotic recombination and different epigenetic factors (52).

Somatic mosaicism, the presence of genetically distinct populations of somatic cells in a given organism is frequently masked, but it can also result in major phenotypic changes and reveal the expression of otherwise lethal genetic mutations. Mosaicism can be caused by DNA mutations, epigenetic alterations of DNA, chromosomal abnormalities and the spontaneous reversion of inherited mutations (53).

Many somatic mutations are deleterious and kill the cells in which they occur. Others may be selected against in rapidly dividing or differentiating populations of

cells. When either of these effects of somatic mutation occurs early enough during development to disrupt embryonic structures, birth defects may be produced. Studies in experimental animals exposed to mutagenic agents confirm these mechanisms, but extent to which they are involved in human birth defects is not much known. Chromosomal mosaicism, however, has been well documented in some children with birth defects (54).

1.8.2 Gonadal Mosaicim

The phenotypic effects of somatic mutations depend on when the mutations occur; the earlier in development, the more prevalent will be their contributions. There is mounting evidence that mitotic missegregation of chromosomes in very early gestation frequently leads to mosaicism that is confined to the placenta.

Most importantly for genetic counseling, early embryonic somatic mutations and chromosomal missegregation can introduce genetic disorders into the germ-line by producing gonadal mosaicism. Several rounds of cell division occur between fertilization and differentiation of germ cells in the developing embryo (54). Gonadal mosaicism, also known as germline mosaicism, is the condition in which an individual's germline cells developed mutations during embryonic growth, while the individual's somatic cells did not. Particularly, the germline mutation will result in disease in the individual's offspring, while the individual will not be affected.

The term “gonadal mosaicism” refers to a particular type of mosaicism that only affects one group of cells. The germline cells include eggs and sperm. In some individuals that have had a child with a chromosome change, there is mosaicism in these cells. This means that some eggs or sperm have a normal chromosomal make-up while others carry a chromosome change. If one of the eggs or sperm with a chromosome change is used in fertilization, the child may have a chromosome abnormality.

1.9 RAPD-PCR

RAPD, developed by Williams et al. (1990) and Welsh and McClelland (1990), is a PCR-based technique that amplifies DNA fragments of genomic DNA with single short primers of arbitrary nucleotide sequence under low annealing conditions. This technique is used extensively for species classification, genetic mapping and phylogeny etc. In addition, their use in surveying genomic DNA for evidence of various types of DNA damage and mutation shows that RAPD may potentially form the basis of novel biomarker assays for the detection of DNA damage and mutational events (e.g. rearrangements, point mutation, small insert or deletions of DNA and ploidy changes) in cells of bacteria, plants, invertebrate and vertebrate animals (55, 56).

Recent advances in molecular biology enabled the development of an assessment technique that has the potential of identifying both acute and chronic effects of pollution earlier than other presently used methods. This method uses RAPD-PCR to generate genetic profiles of individuals within populations. The combined analysis of the RAPD profiles can provide useful information regarding species endemic to sites impacted by pollution. RAPD profiles have proven effective in determining the overall genetic diversity levels harbored within populations of both aquatic and terrestrial species. By analyzing the genetic health of endemic populations, this protocol is capable of identifying populations at risk prior to their local disappearance. RAPD profiling is continuing to gain acceptance by the scientific community. However, continued refinement of the method is required to establish RAPD profiling as a standard bioindicator of environmental contamination.

There is a great concern about the effect of environmental contaminants on the genetic make-up of populations. One class of genetic effects includes alterations to the structure and function of DNA including DNA adducts DNA breakage, and mutations as a result of chemical exposure (genotoxic effects). However, indirect genetic effects can also arise as a consequence of the interactions of genotoxic agents with DNA. The RAPD and similar technologies have been used to detect not only DNA damage and mutations but also changes in genetic diversity and gene frequencies. However, most of the studies indicate that the observed changes in RAPD profiles which occurred among

the exposed population were the result of either DNA damage and mutations or population genetic effects (57, 58).

The RAPD method was used to detect genetic diversity among populations and genetic variabilities which had been exposed to environmental contaminants, including well-known genotoxins. It was reported that fish populations in the contaminated sites were consistently less genetically distant from each other than they were from each of the reference sites (59).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments and General Reagents

Laboratory equipments, chemicals, consumables and company communications are listed in Table 2.1 - 2.7.

Table 2.1 Laboratory Kits.

Kits	Brand Name	Catalog No	Distributor in TURKEY	Packsize
EZ-10 Spin Column PCR Products Purification Kit	BioBasic	BS364	BioGen	100 Preps
EZ-10 Spin Column DNA Gel Extraction Kit	BioBasic		BioGen	100 Preps
Allprep DNA&RNA Isolation Kit	QIAGEN	80204	GenOva	50 Preps
Allprep DNA&RNA Protein Mini Kit	QIAGEN	80004	GenOva	50 Preps
QUANT-IT DsDNA BR ASSAY KIT	INVITROGEN	Q32850	Medsantek	50 Preps
DNeasy Blood&Tissue Isolation Kit	QIAGEN	69506	GenOva	250 Preps
dNTP Set	BioBasic	D0046	BioGen	100 mM, 4x0.25 ml
dNTP Set	BioBasic	D0056	BioGen	10 mM, 1 ml

Table 2.2 Laboratory Machines.

Lab Machines	Brand Name	Catalog No	Distributor in TURKEY	Packsize & Information
Thermal Cycler		FTC41H2D	Elips	
Spectrafuge Mini Complete	LABNET	C1301-230V-EU	Elips	1,5/2,0ml rotor,strip tube rotor, 0.5 and 0.4 ml adapters, 230V
Gradient Thermal Cycler	TECHNE	FTC51H2D	Elips	96x0.2ml microtubes or 96well plate,120V/230V
Qubit Fluorometer	INVITROGEN	Q32857	Medsantek	
Autoclave	CERTO CLAW			
Electrophoresis Equipment	Mupid One			Up to 4 x 26 samples (max)
Transilluminator	Bio-Rad GelDoc 2000			25 x 26 cm UV transilluminator
Homogenizer	IKA Labortechnik	T25 Basic		
Refrigerator	Philips			+4ºC, -20ºC
Freezer	BEKO			-20ºC
Water Purification System	Millipore			
Microbalance	Sartorius			

Table 2.3 DNA Ladder.

DNA Ladder	Brand Name	Catalog No	Distributor in TURKEY	Packsize
DNA Ladder FastRuler	Fermantas	SM1103	Elips	(2x500µl)

Table 2.4 Micropipets and Related Consumables.

Filter Tips& Micropipets	Brand Name	Catalog No	Distributor in TURKEY	Packsize
0,1-10µl filter tips sterile	BioBasic	FT-215RNS	BioGen	DNAse RNAse free 96/rack
5-30µl filter tips sterile	BioBasic	S2532-RS	BioGen	DNAse RNAse free 96/rack
5-200µl filter tips sterile	BioBasic	S2533-RS	BioGen	DNAse RNAse free 96/rack
100-1000µl filter tips sterile	BioBasic	FT-222RS-1	BioGen	DNAse RNAse free 100/rack
Yellow Tips	Axygen		Maksigen	DNAse RNAse free (1000pack)
Yellow Tips	Axygen	T-200-Y	Elips	200µl 1000Tips
Filter Pipet Tips	CLP	BT1000	Metis	1000µl
Filter Pipet Tips	CLP	BT20	Metis	20µl
Filter Pipet Tips	CLP	BT200	Metis	200µl
DV200 Discovery Micropipet	HTL	4305	GenOva	20-200µl
DV1000 Discovery Micropipet	HTL	4306	GenOva	100-1000µl
DV8-10 Discovery Multichannel Micropipet	HTL	5221	GenOva	1-10µl
Micropipet Set	GLP Digital Kit-1	4700410	Elips	0,5-10 µl, 1-100 µl, 100-1000µl

Table 2.5 Polymerase Enzymes**.**

Taq Polymerase Enzyme	Brand Name	Catalog No	Distributor in TURKEY	Concentration
Taq DNA Polymerase	Fermentas	EP0402	Elips	500 units
Dream Taq	Fermentas		Elips	200 units

Table 2.6 Agarose Gel and Chemicals.

Other Materials	Brand Name	Catalog No	Distributor in TURKEY	Information
Agarose	Sigma	A5093	Maksigen	100 g
Agarose	OnBio		Metis	100 g
TBE 10x Buffer	CLP	5111	Metis	
Safeview Gel Stain				
Ethidium Bromide				

Table 2.7 Distributor companies in Turkey and their communication information.

Distributors in Turkey	E-mail	Telephone Number	Fax Number
BioGen	biogen@biogen.com	212 588 23 55	212 633 46 77
Elips	info@elipsltd.com	212 222 95 08	212 222 96 02
GenOva	info@genovamedikal.com	212 320 51 90	212 320 23 10
Metis	info@metismedikal.com.tr	312 397 64 99	312 397 55 42
Medsantek	info@medsantek.com	212 635 85 46	212 63583 50

2.1.2 Fish Samples

All fish samples were collected from the same fish farm (Kılıç Balıkçılık, Bodrum, Muğla, Turkey, 37°12'29.09"K, 27°38'59.51"E) and same location. Fish samples sizes and sites of collection are listed in Table 2.8.

Table 2.8 Fish samples sizes and sites of collection.

	Sparus auratus		***Dicentrarchus labrax***		***Sparus auratus* & *Dicentrarchus labrax***	
	Sample Name	Size	Sample Name	Size	Location	Collection Time
Sample 1	1SA	28 cm	1DL	32 cm	Bodrum	October,2010
Sample 2	2SA	31 cm	2DL	34 cm	Bodrum	January,2010
Sample 3	3SA	33 cm	3DL	40 cm	Bodrum	March,2010

Tables 2.9-2.11 refer to the names of DNA and RNA samples for both species, *Sparus auratus* and *Dicentrarchus labrax*.

Table 2.9 DNA names of *Sparus auratus* samples extracted separately for each organ and tissue type.

Tissue Type	Sample 1	Sample 2	Sample 3
Kidney	1-SA1	2-SA1	-
Spleen	1-SA2	2-SA2	-
Muscle	1-SA3	2-SA3	3-SA3
Brain	1-SA4	2-SA4	3-SA1
Heart	1-SA5	2-SA5	-
Gill	1-SA6	2-SA6	-
Liver	1-SA7	2-SA7	3-SA2
Stomach	1-SA8	-	-
Lipid	1-SA9	-	-
Blood	1-SA10	-	-
Gonad	-	2-SA8	3-SA4

Table 2.10 DNA names of *Dicentrarchus labrax* samples extracted separately for each organ and tissue type.

Tissue Type	Sample 1	Sample 2	Sample 3
Kidney	1-DL1	-	-
Spleen	1-DL2	-	-
Muscle	1-DL3	2-DL3	3-DL3
Brain	1-DL4	2-DL1	3-DL1
Heart	1-DL5	-	-
Gill	1-DL6	-	-
Liver	1-DL7	2-DL2	3-DL2
Stomach	1-DL8	-	-
Lipid	1-DL9	-	-
Blood	1-DL10	-	-
Gonad	-	2-DL4	3-DL4

Table 2.11 RNA names of *Sparus auratus* and *Dicentrarchus labrax* extracted separately for each organ and tissue type.

	Sparus auratus			***Dicentrarchus labrax***		
Tissue Type	**Sample1**	**Sample 2**	**Sample 3**	**Sample 1**	**Sample 2**	**Sample 3**
Brain	-	2-SA1	3-SA1	-	2-DL1	3-DL1
Liver	-	2-SA2	3-SA2	-	2-DL2	3-DL2
Muscle	-	2-SA3	3-SA3	-	2-DL3	3-DL3
Gonad	-	2-SA4	3-SA4	-	2-DL4	3-DL4

2.1.3 RAPD-PCR Reagents

Polymerase chain reaction reagents were taken from Fermentas and QIAGEN. Eight different 10-mer primers were assessed at first (OPA8, OPA9, OPB1, OPB5, OPB6, OPB7, OPB10 and OPB18) and four best resulting ones (OPB5, OPB6, OPB7 and OPB18) were chosen. The primers were synthesized by IONTEK. Sequences of the primers used are given in Table 2.12.

Table 2.12 Primers and sequences used in the thesis.

Primer	Sequence 5´to 3’
OPB 05	TGCGCCCTTC
OPB 06	TGCTCTGCCC
OPB 07	GGTGACGCAG
OPB 18	CCACAGCAGT
OPA8	GTGACGTAGG
OPA9	GGGTAACGCC
OPB1	GTTTCGCTCC
OPB10	CTGCTGGGAC

2.2 Methods

2.2.1 Fish Dissection

Dissection of the fish was done by research assistant Ergün Şakalar using dissection kit (Figure 2.1). All dissected tissues were weighed for DNA and RNA extraction and the rest were frozen at -80°C for further usages.

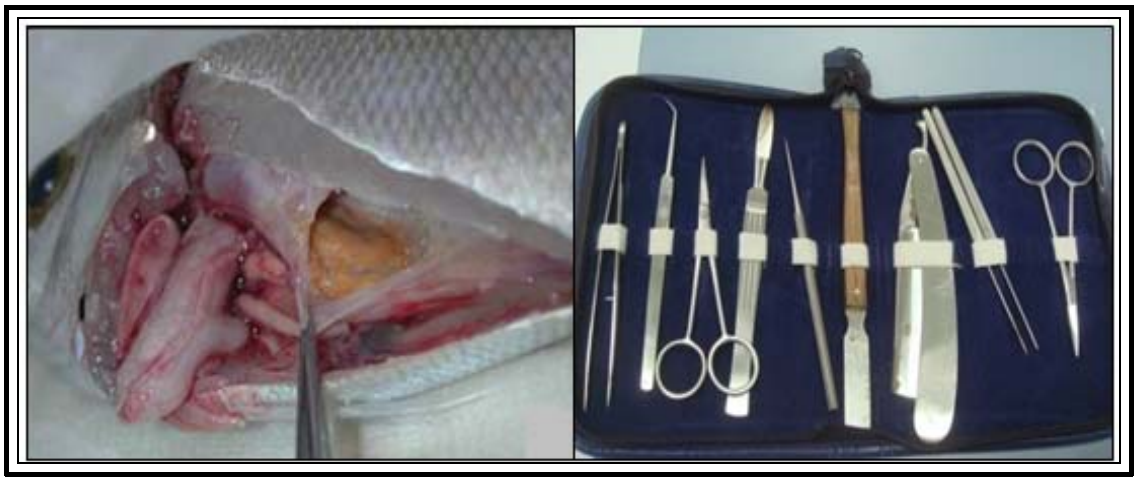

Figure 2.1 Dissection of tissues with the dissection kit.

2.2.2 DNA Isolation

DNA isolation was performed according to QIAGEN Blood DNA Isolation Kit protocol and QIAGEN All Prep DNA-RNA Isolation Kit protocol.

QIAGEN Blood DNA Isolation Kit protocol was performed step by step as described below:

- 25 mg (10 mg spleen) of tissues was weighted in a 1.5 ml microcentrifuge tube. 100 µl Buffer ATL was added for each sample and homogenization was done by IKA Labortechnik homogenizator.

- 20 µl proteinase K was added, mixed by vortexing and incubated at 56°C until the tissue completely lysed. The tubes were vortexed occasionally during incubation.

- The tubes were briefly centrifuged to remove drops from the inside of the tube lid.

- 200 µl Buffer AL was added to the samples. Samples were mixed by pulse vortexing and incubated at 70°C for 10 min. After incubation, a brief centrifugation was done.

- 200 µl ethanol (96-100%) was added to the samples and mixed by pulse vortexing for 15 s. After vortexing, the tubes were briefly centrifuged.

- The mixture was loaded to QIAmp Mini spin columns without touching the rim. The caps were closed and centrifugation was done at 8000 rpm for 1 min. The spin column was placed in a clean 2 ml collection tube and the flow-through was discarded after centrifugation .

- 500 µl Buffer AW1 was added to the columns and centrifuged for 1 min at 8000 rpm. Flow-through was discarded.

- 500 µl Buffer AW2 was added to the columns and centrifuged at 14000 rpm for 3 min. Collection tube with flow-through was discarded.

- Spin column was placed in a new 2 ml collection tube. 200 µl Buffer AE was added and incubated 1 min at room temperature, then centrifugation was done at 8000 rpm for 1 min.

- Pure DNA was collected at the end of procedure and eluted DNA concentration and purity was calculated using Qubit Fluorometer.

- Pure DNA was stored at -20°C.

QIAGEN All Prep DNA Isolation Kit was used for both DNA and RNA isolation from the same sample (Figure 2.2).

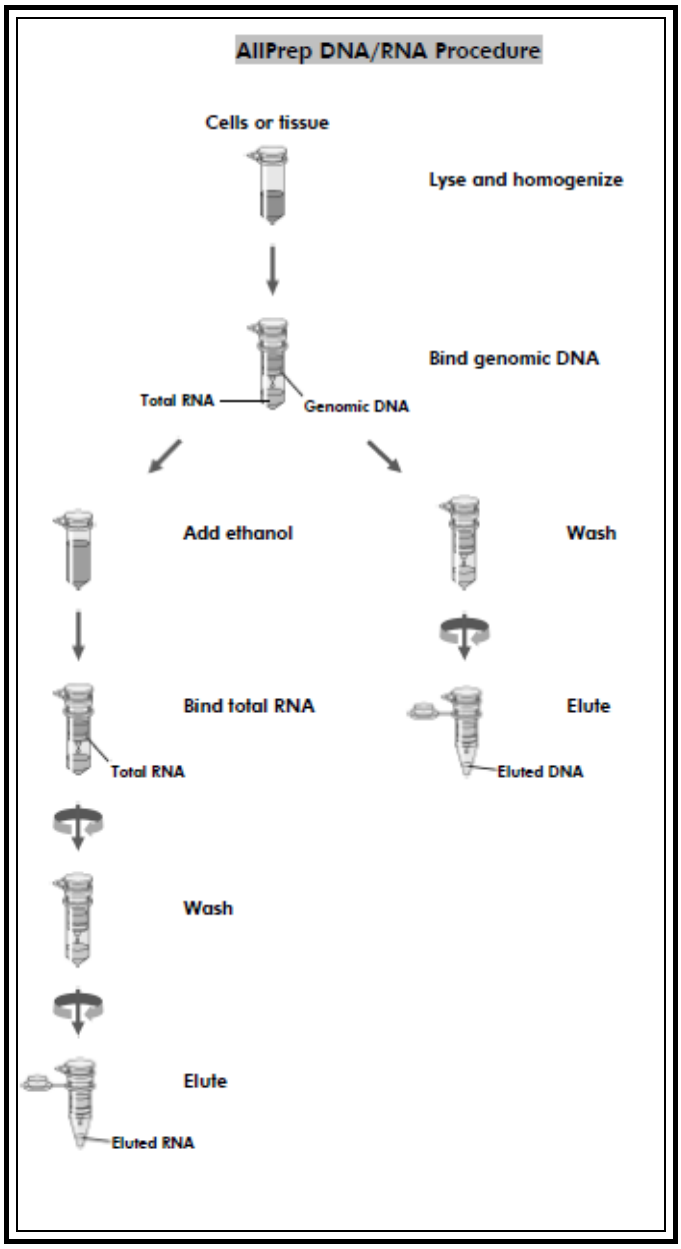

Figure 2.2 AllPrep DNA/RNA Isolation Procedure (60).

DNA extraction part of the kit protocol was performed step by step as described below:

- Tissue samples from the fish were weighed 30 mg. The tissues were disrupted and homogenized with homogenizator and then 350 µl Buffer was added.
- The lysate was centrifuged for 3 min at max speed. Supernatant was pipetted and transferred to the AllPrep DNA spin column placed in a 2 ml

collection tube. The spin column was centrifuged for 30 s at 10000 rpm.

- AllPrep DNA spin column was placed in a new 2 ml collection tube. 500 µl Buffer AW1 was added and centrifuged for 15 s at 10000 rpm. Flow-through was discarded.

- Buffer AW2 was added to the spin column and centrifuged for 2 min at max speed to wash the spin column membrane.

- The spin Column was placed in a new 1.5 ml collection tube. 100 µl Buffer EB was added and the column was incubated at room temperature for 1 min then centrifuged at 10000 rpm to elute the DNA.

- Highly pure DNA was extracted at the end of procedure and eluted DNA concentration and purity is calculated using Qubit Fluorometer.

- Extracted DNAs are stored at -20ºC.

2.2.3 RNA Isolation

RNA extraction was performed according to AllPrep DNA RNA Isolation Kit protocol as described below:

- Tissue samples were taken 30 mg. The tissues were disrupted and homogenized with homogenizator and then 350 µl Buffer was added.

- The lysate was centrifuged for 3 min at max speed. Supernatant was pipetted and transferred to the AllPrep DNA spin column placed in a 2 ml collection tube. The spin cloumn was centrifuged for 30 s at 10.000 rpm.

- 350µl %70 ethanol was added to the flow-through and mixed by pipetting.

- 700µl sample was transferred to the RNA spin column placed in a 2ml collection tube and centrifuged 15s at 10.000rpm. Flow-through was discarded.

- 700µl Buffer RW1 was added to the column and centrifuged 15s at 10.000rpm. Flow-through was discarded.

- 500µl Buffer RPE was added to the column and centrifuged 15s at 10.000rpm. Flow-through was discarded and 500µl Buffer RPE was added again and centrifuged 2 min at 10.000rpm.

- The column was placed in a new 1.5ml collection tube and 50µl RNAse free water was added. The collection tube was centrifuged for 1 min at 10.000rpm.

- Highly pure RNA was extracted at the end of procedure and eluted RNA concentrations and were measured using Qubit Fluorometer.

- Pure RNA was stored at -80°C.

2.2.4 DNA and RNA Concentration Measurement Assay

Quant-iT ™ dsDNA BR Assay Kit and Quant-iT ™ RNA Assay Kit were used for the concentration measurements of extracted DNAs and RNAs.

- Two tubes were used for the standards and one tube for each of our samples.

- Quant-iT Working Solution was prepared by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. 200 µl of Working Solution were required for each sample and standard.

- Assay Tubes were prepared according to the Table 2.13.

Table 2.13 DNA&RNA Concentration Assay.

	Standard Assay Tubes	**Sample Assay Tubes**
Volume of Working Solution to add	190 µL	180-199 µL
Volume of Standard to add	10 µL	—
Volume of Sample to add	—	1–20 µL
Total Volume in each Assay Tube	200 µL	200 µL

- And then all tubes were vortexed for 2–3 seconds.
- The tubes were incubated for 2 minutes at room temperature
- Readings of the tube were done by Qubit fluorometer.
- Concentrations of the samples are calculated according to the formula by multiplying the same concentration with the dilution factor (see Figure 2.4).

$$\text{Concentration of your sample} = \text{QF value} \times \left(\frac{200}{x}\right)$$

where:

QF value = the value given by the Qubit™ fluorometer

x = the number of microliters of sample you added to the assay tube

Figure 2.3 DNA concentration calculation formula.

2.2.5 RAPD-PCR & Modified RAPD Random Amplified Mosaic DNA-PCR Protocol (RAMD)

The conditions of RAPD amplifications were optimized with small modifications. PCRs were performed in reaction mixture of 25 µl containing the ingredients given in Table 2.14.

Table 2.14 RAPD-PCR Ingredients.

Reagent	Initial Concentration	Final Concentration	Final Volume
Taq Buffer	10X	1X	2.5 µl
dNTP	2 mM	0.2uM	1.0 µl
$MgCl_2$	25 mM	2 mM	3 µl
Primers	25 pmol/µl	25 pmol	4 µl
ddH_2O	-	-	x µl
Taq DNA Polymerase	5 U/µl	1 U	0.1 µl
Template DNA			y µl
Total Reaction Volume			25 µl

The RAPD-PCR protocol consisted of an initial denaturing step of 5 min at 94ºC, followed by 45 cycles at 94 ºC for 30 s (denaturation), 50ºC (instead of 37 ºC, 50ºC was used to increase the stringency and confirm the repeatability of the bands) for 60 s (annealing) and 72 ºC for 60 s (extension), with an additional extension period of 10 min at 72 ºC (61).

- **94 ºC 5 minutes (initial denaturation)**
- **94 ºC 30 seconds (denaturation)**
- **50 ºC 60 seconds (annealing)**
- **72 ºC 60 seconds (extension)**

} **45 Cycles**

- **72 ºC 10 minutes (final extension)**

The RAMD-PCR is a modification of the RAPD-PCR; Figure 2.5 is a schematic representation of the applied method. RAMD is random amplified mosaic DNA, here we assume that the tissues of the organism are highly exposed to many endogenous and exogenous exposures. DNA is becoming a mosaic of DNA changes that are either DNA damages or mutations because of these many attacks. After DNA extraction, we use this mosaic DNA in the RAMD-PCR by preparing a mastermix containing the same mosaic DNA and aliquot; distribute it to n tubes which are amplified in totally same conditions having the same DNA. The amplification products are run on the same gel and the profiles theoretically should be the same because the conditions and the DNA are completely the same, but usually we obtain some different patterns to be discussed with other related information that may be the causes of this variation in the profiles (62).

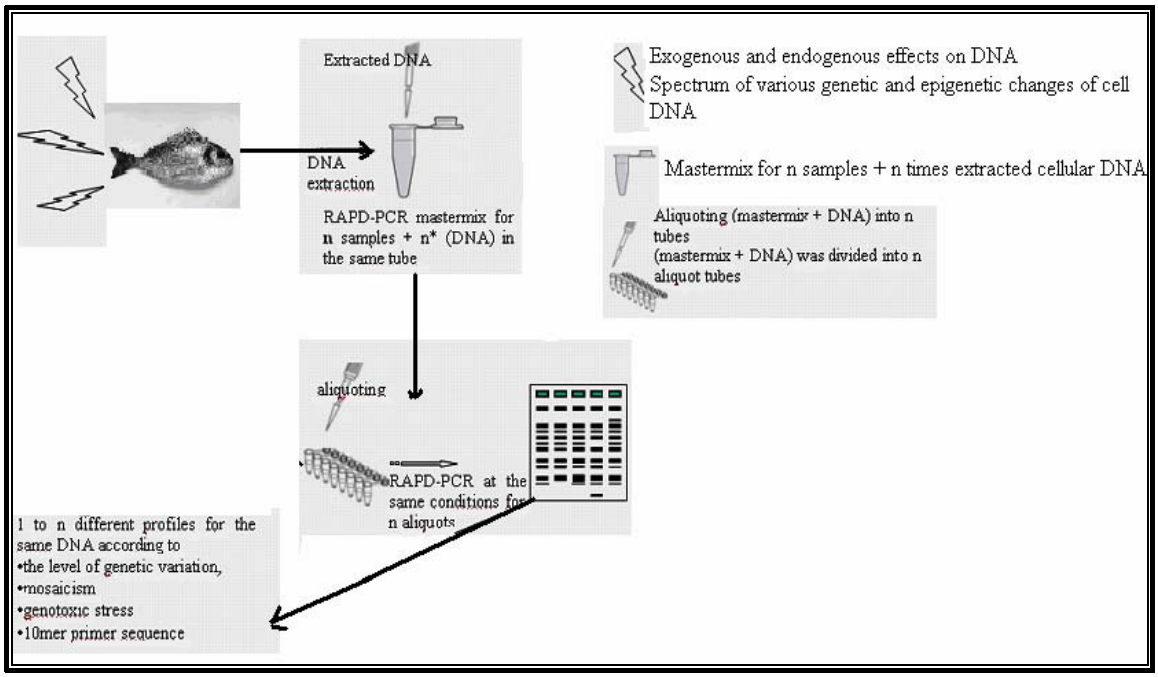

Figure 2.4 Representation of RAMD-PCR approach.

2.2.6 RT- PCR with RAPD Primers

2.2.6.1 cDNA Synthesis

QuantiTect Reverse Transcription Kit was used for cDNA synthesis. Template RNA was thawed on ice. gDNA wipeout buffer, quantiscript reverse transcriptase,

Quantiscript RT buffer, RT primer mix, and RNase-free water were thawed at RT (15–25°C). The genomic DNA elimination reaction components were used according to the kit protocol. Figure 2.5 shows a brief summary of the procedure of Quantitect Reverse Transcription Kit.

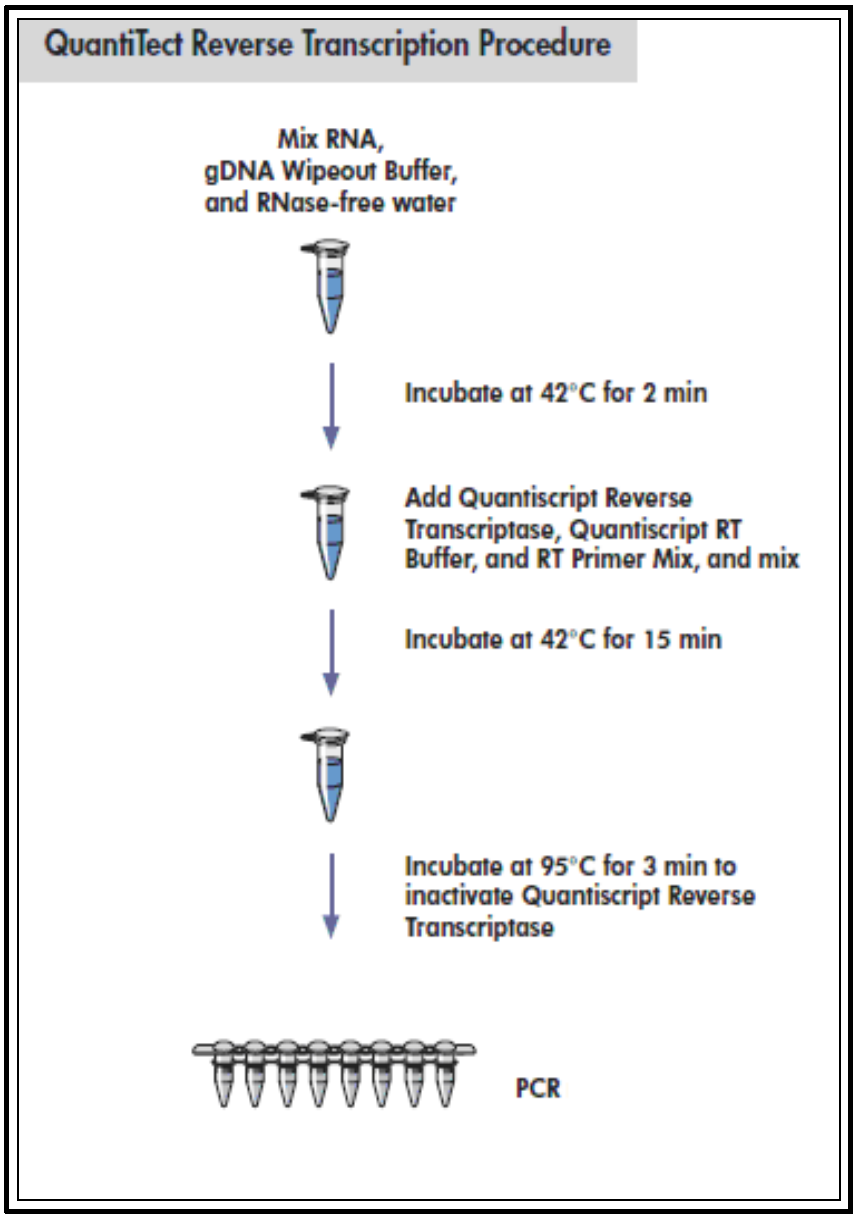

Figure 2.5 QuantiTect Reverse Transcription Procedure (63).

Template RNA was added to each tube containing reverse-transcription master mix. It was mixed and then stored on ice. Mastermix was incubated for 15min at 42°C and incubated for 3min at 95°C to inactivate Quantiscript reverse transcriptase. Then obtained cDNA was stored at -80°C.

RT-RAPD-PCR utilizes randomly selected RAPD 10-mer primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers were extended by cDNA polymerase and a copy of the strand was made after each cycle, leading to logarithmic amplification (see Figure 2.6).

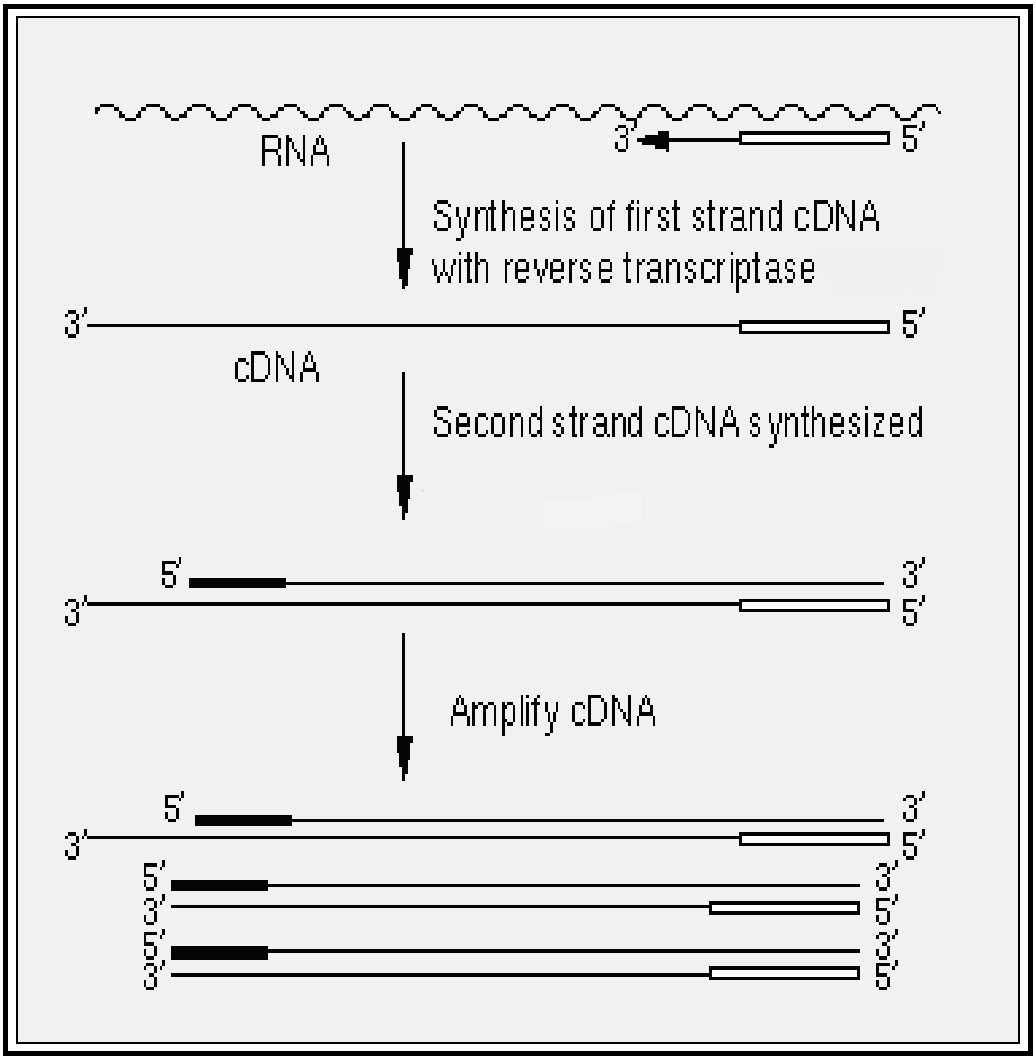

Figure 2.6 RT-PCR Representation.

PCR reactions were performed in reaction mixture of 25µl containing the components in Table 2.15.

Table 2.15 PCR solutions and their initial and final concentrations with final volumes.

Reagent	**Initial Concentration**	**Final Concentration**	**Final Volume**
Taq Buffer	10X	1X	2.5 µl
dNTP	2 mM	0.2 µM	1.0 µl
$MgCl_2$	25 mM	2 mM	3.0 µl
Primers	12,5 pmol/µl	12,5 pmol	4 µl
ddH2O	-	-	0.4µl
Taq DNA Polymerase	5 U/µl	1 U	0.1 µl
cDNA			14 µl
Total Reaction Volume			25 µl

The RT-RAPD protocol consisted of an initial denaturing step of 5 min at 94 ºC, followed by 45 cycles at 94 ºC for 30 s (deneturation), 37ºC for 60 s (annealing) and 72 ºC for 60 s (extension), with an additional extension period of 10 min at 72 ºC.

- **94 ºC 5 min (initial denaturation)**
- **94 ºC 30 s (denaturation)**
- **37 ºC 60 s (annealing)**
- **72 ºC 60 s (extension)**

45 Cycles

- **72 ºC 10 min (final extension)**

2.2.7 Agarose Gel Electrophoresis and Documentation

2 % agarose gel was used to visualize DNA and RNA PCR products Safe view stain was added to the gel after it boiled and cooled to visualize the double stranded DNA bands under UV light.

Loading of the Gel;

1. 15 µl of PCR products mixed with 2 µl loading dye, bromophenol blue (6x), were loaded in each slot mixed with loading buffer.

2. 1 µl of a 100 bp DNA Ladder (MBI Fermentas, Hanover, MD, USA) was mixed with 1 µl deionized water and 1 µl bromophenol blue. Then 2 µl of this mix was put into one of the slots as a molecular marker.

3. The gel was run at 50 V in 0.5 X TBE buffer for 50 min for maximum separation and resolution.

4. The gel was placed in Gel Doc 2000 (Biorad, Milan, Italy) apparatus and the bands were detected under UV using a transilluminator and documented with Quantity one program.

CHAPTER 3

RESULTS AND DISCUSSION

RAPD-PCR based approaches were used in this thesis to obtain the below results related to somatic and gonadal mosaicism detection on the basis of genetic and epigenetic changes followed by RAPD and RT-RAPD PCR.

We grouped our results under three major parts with subtitles; the first part is about optimizations of RAPD-PCR method for suitable primer selection and annealing temperature determination in discriminating the diagnostic patterns for our intensions with sample DNA and RNA quality evaluations. The two other parts are related with genome wide screening of DNA changes using different approaches of RAPD-PCR and the third part is epigenetic screening strategy with RT-RAPD PCR approaches using the RNA of the same tissues in second part that the DNAs have been screened for various level of variation.

3.1 Observations of Samples and Sampling Sites

All samples for this study are from the same sampling site; Bodrum, Muğla, Turkey. The name of the facility is named as ‘Kılıç Balıkçılık’. This facility is one of the biggest aquaculture and mariculture facilities in Turkey. It has the highest production capacity of aquacultured and maricultured fish in Europe (64).

Figure 3.1 Kılıç Balıkçılık mariculture fish tanks (64).

Figure 3.2 Harvesting maricultured fish from sampling site (64).

3.2 Dissection of Fish Organs and Tissues with the Determination of DNA&RNA Concentrations

In this thesis 2 different maricultured fish species *Sparus auratus* and *Dicentrarchus labrax* have been used. 3 different individuals for each species have been collected from same aquaculture facility (Kılıç Balıkçılık) at various times (October, January and March respectively).

Dissection of the organs and tissues were made carefully with high quality laboratory dissection kit. Some photographs of dissected organs and tissues are given in Figure 3.3.

Dorsal Muscle Gill Lipid

Figure 3.3 Dissected tissues of the fish samples, dorsal muscle, gill and lipid tissues.

DNA and RNA quantification should be done prior to all PCR applications which is especially important in mosaicism profiling with 10-mer primers. The Qubit fluorometer measurements of DNA and RNA are given for each dissected tissue in Tables 3.1, 3.2 and 3.3.

Table 3.1 DNA Concentrations of 1.*Sparus auratus* (SA) and *Dicentrarchus labrax* (DL).

Tissues	**Sample Name SA**	**Concentration** (µg/ml)	**Sample Name DL**	**Concentration** (µg/ml)
Kidney	1-SA1	54.1	1-DL1	54.0
Spleen	1-SA2	19.6	1-DL2	54.6
Muscle	1-SA3	0.652	1-DL3	1.21
Brain	1-SA4	61.5	1-DL4	59.0
Heart	1-SA5	12.5	1-DL5	5.02
Gill	1-SA6	14.4	1-DL6	1.86
Liver	1-SA7	27.6	1-DL7	12.2
Stomach	1-SA8	5.29	1-DL8	2.13
Lipid	1-SA9	0.359	1-DL9	0.546
Blood	1-SA10	140.0	1-DL10	1.68

Table 3.2 DNA and RNA Concentrations of 2. and 3. *Sparus auratus* (SA).

Tissues	**Sample name**	**DNA Conc.** (µg/ml)	**RNA Conc.** (µg/ml)	**Sample name**	**DNA Conc.** (µg/ml)	**RNA Conc.** (µg/ml)
Kidney	2-SA1	220.0	56.9	-	-	-
Spleen	2-SA2	19.7	89.0	-	-	-
Muscle	2-SA3	-	-	3-SA3	13.3	-
Brain	2-SA4	19.3	9.15	3-SA1	38.7	-
Heart	2-SA5	23.7	6.27	-	-	-
Gill	2-SA6	13.6	35.9	-	-	-
Liver	2-SA7	18.0	-	3-SA2	32.9	-
Stomach	2-SA8	41.5	37.0	-	-	-
Gonad	2-SA9	46.9	65.5	3-SA4	91.6	180.0

(-) indicates out of range concentrations.

Table 3.3 DNA and RNA Concentrations of 2. and 3.*Dicentrarchus labrax* (DL).

Tissues	Sample Name	DNA Conc. (µg/ml)	RNA Conc. (µg/ml)	Sample Name	DNA Conc. (µg/ml)	RNA Conc. (µg/ml)
Brain	2-DL1	120.0	32.5	3-DL1	35.5	48.6
Muscle	2-DL2	3.98	-	3-DL2	160.0	58.1
Liver	2-DL3	48.5	24.4	3-DL3	-	-
Gonad	2-DL4	11.9	97.9	3-DL4	27.7	33.2

(-) indicates out of range concentrations.

3.3 RAPD Profiling and Primer Optimizations

Suitable primer selection and annealing temperature optimizations are done for the study. Low annealing temperatures (34–36º C) are used in RAPD to ensure a maximal number of primer binding events and consequent generation of large number of amplified DNA fragments for analytical purposes. However, the low stringency of the accompanying DNA hybridisation can result in the formation of spurious amplifications (65, 66, 67). With selected primers, reproducible DNA profiles, consisting of a large number of amplified fragments with a heterogeneous size range could be produced at an annealing temperature of 50º C under optimised PCR conditions. The main advantage of high stringency conditions is that non-specific reactions are significantly reduced. Thus, protocols that use a high annealing temperature (48-52 º C) should always be preferred for mosaicism reproducibility and also this keeps the production of non-specific reactions to a very low level. In this context, we used RAPD assay are really advised to use high annealing temperature such as 50º C for 10-mer primers (55, 56).

After annealing temperature was optimized, primer trials were done to choose the most discriminating ones for the study. Eight 10-mer primers (see Table 2.12) were adopted from Atienzar's article (55) and four of them were chosen as they gave most consistent results in itself; they gave the same results in low and high DNA

concentrations (5 ng and 20 ng) and reproducibility was confirmed by repeating the experiments twice (Figure 3.4).

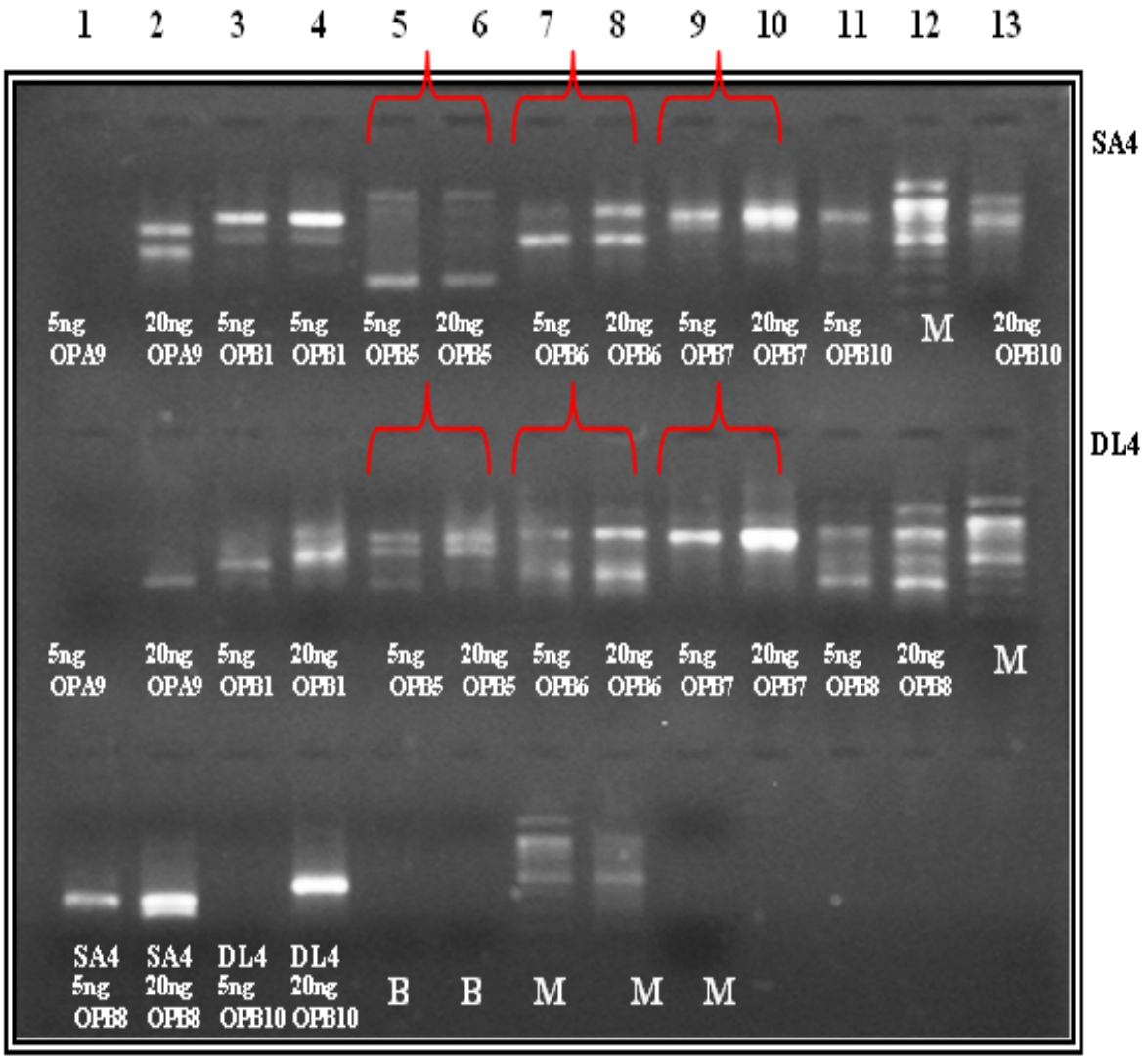

Figure 3.4 2% Agarose gel photograph of 10-mer primer selection for different DNA concentrations using 1-SA and 1-DL brain tissue. **M:** DNA Size Marker 100-1000 kb. Selected primers for down stream applications are indicated by brackets.

As shown in the Figure 3.4, OPB-5, OPB-6 and OPB-7 with both 5 ng and 20 ng DNA concentrations have given the same band profiles for *Sparus auratus* and *Dicentrarchus labrax*. These primers were chosen and 5 ng DNA concentration has been used throughout the study.

3.4 Genome-Wide RAPD Screening Results

For each selected primer (OPA-8, OPB-5, OPB-6, OPB-7 and OPB-18) all organs and tissues have been screened to detect genetic variation levels of the tissues of both fish (Figure 3.5).

3.4.1 Organ and Tissue RAPD Profiling

To determine comparative tissue specific of RAPD profiles two different random 10-mer primers OPA-8 and OPB-7 are used. The results are shown in Figure 3.5 for *Sparus auratus* and *Dicentrarchus labrax* respectively. OPB-7 has been used for *Sparus auratus* and OPA-8 has been used for *Dicentrarchus labrax* to show high genetic variation for these two fish.

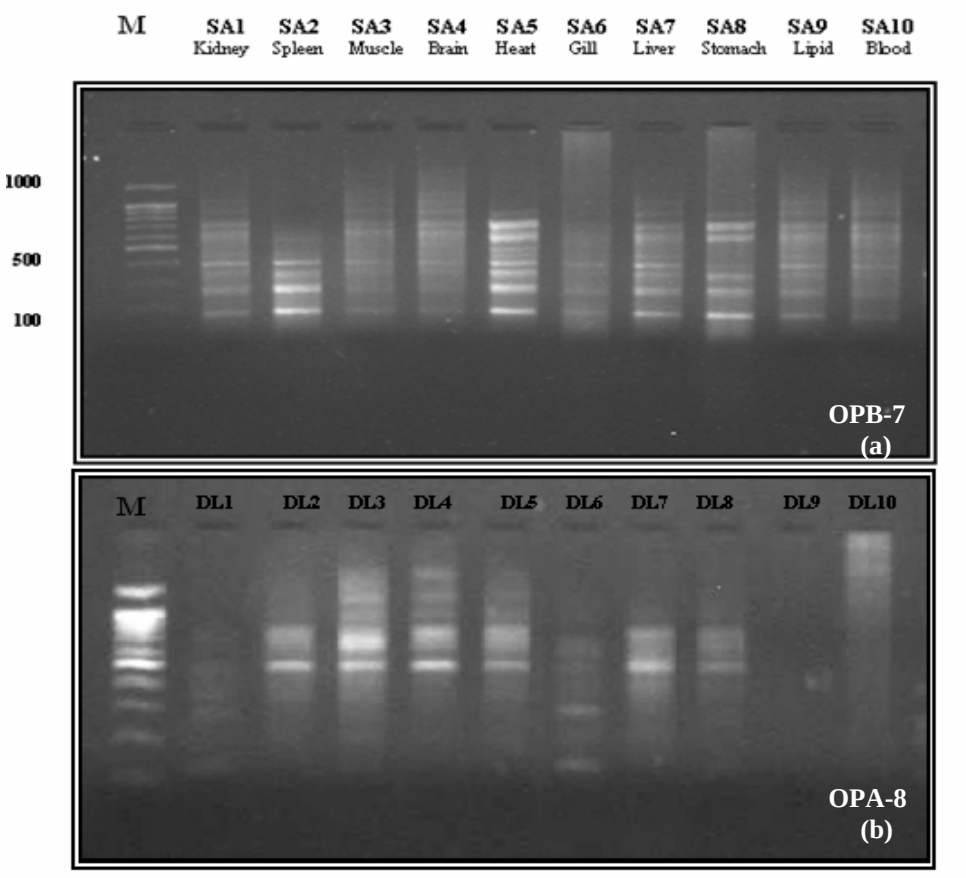

Figure 3.5 Comparative tissue-specific RAPD profiles of *Sparus auratus* (a) and *Dicentrarchus labrax* (b) various organs and tissues with OPB-7 and OPA-8 primers. **M:** DNA size marker.

All of the organs show significantly different RAPD profiles showing a high genetic heterogenity. For these two different species environment is the same (water quality; physical and chemical breeding conditions and also exposures). The only difference is the genetic difference of two species.

Genetic diversity which we have mentioned before makes whole genome complexity for the members of the same species. OPB-5 and OPB-18 primers were used for 3 samples, collected from the same sampling site (Muscle and liver tissues respectively). The experiment has been repeated two times and the samples are loaded tandemly. For both *Sparus auratus* and *Dicentrarchus labrax* three fish samples even the genetic diversity is very high.

This figure is important in two respects show the high diversity for both fish species. This figure clearly shows that there is a need for determination of tissue specific DNA mosaicism.

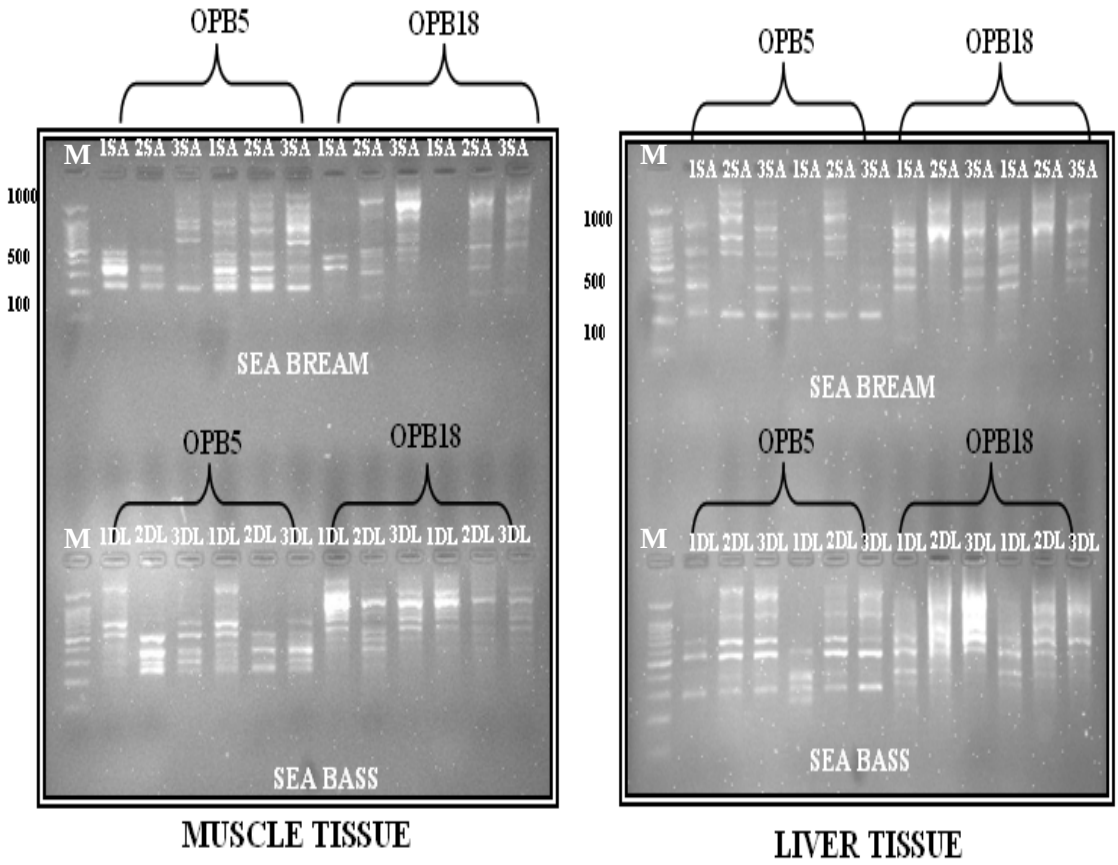

Figure 3.6 Determination of genetic diversity, genetic variation, allellic variation and genetic variabilty using RAPD-PCR using OPB-5 and OPB-18. **M:** DNA size marker.

3.4.2 Organ and Tissue Random Amplified Mosaic DNA (RAMD) Profiling

In this part of the work we have tried to assess the mosaicism level of each organ and tissue of the fish samples by using RAMD (Random Amplified Mosaic DNA) Analysis. Here we have prepared triplicates of each DNA sample that should have the same conditions for every component, but still showed different RAPD profiles.

In 3.7, spleen, muscle and lipid tissues show the most prominent different profiles, whereas kidney, brain, heart gill, liver, stomach and blood tissues show more conserved, stable and less mosaic pattern.

In figure 3.8, brain, gill, liver and blood tissues show the most prominent different profiles, whereas kidney, spleen, muscle, heart, stomach and lipid tissues show more conserved, stable and less mosaic pattern. This shows us different primers (OPB-5 and OPB-18) can show different levels of variations that can further be analyzed to show various conserved regions and variable regions of the genome.

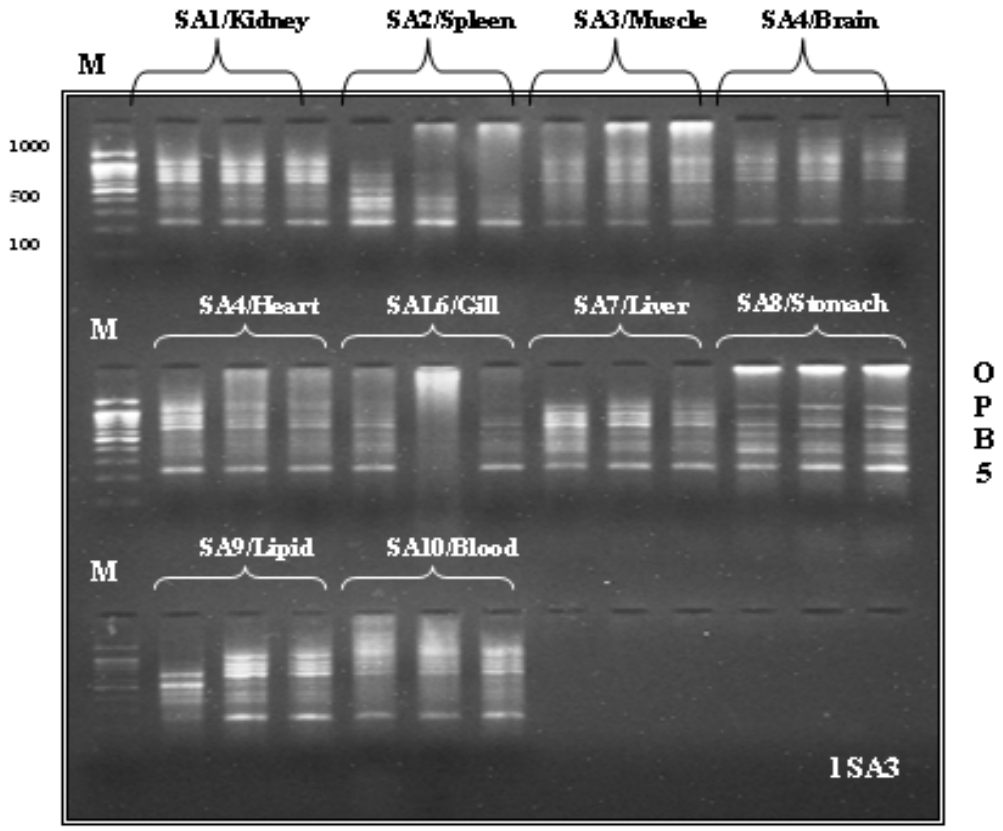

Figure 3.7 Triplicate RAMD Profiling of dissected tissues of 1.*Sparus auratus* using OPB-5 primer. **M:** DNA size marker.

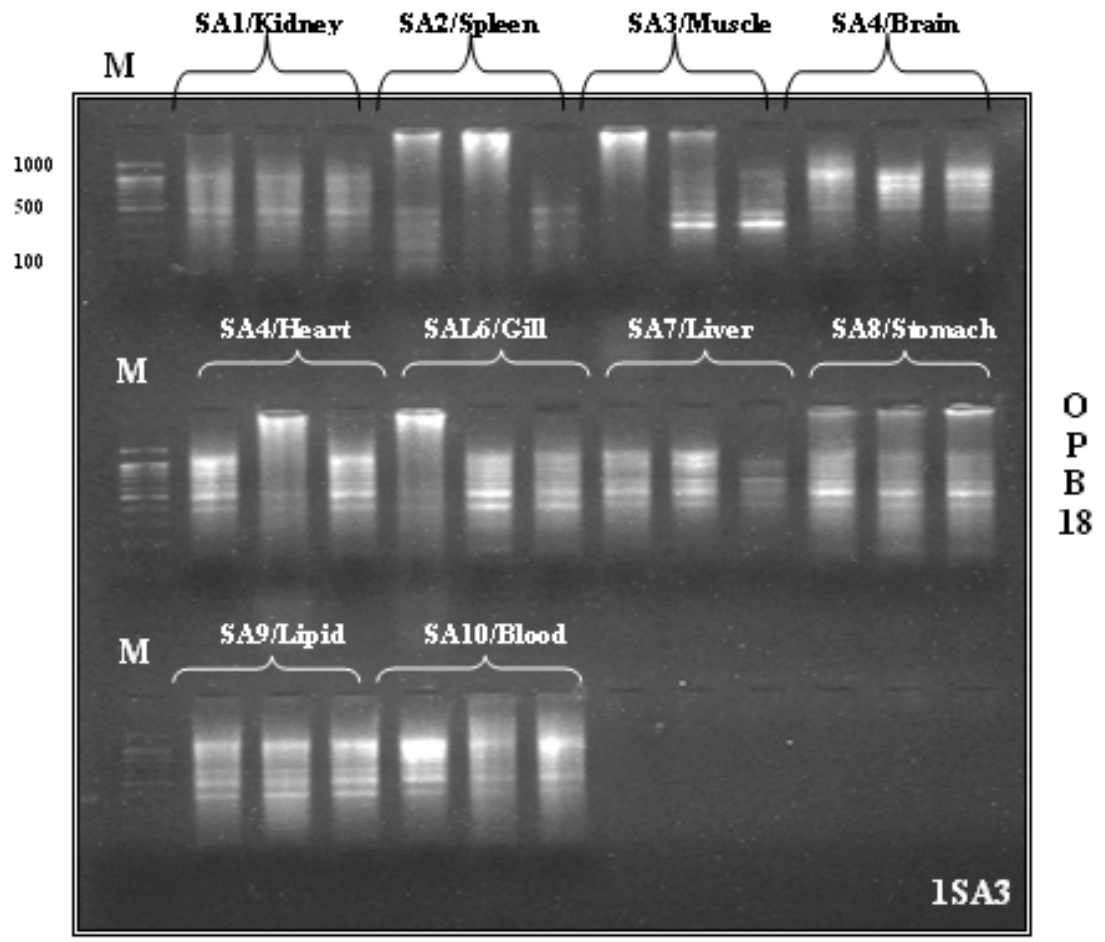

Figure 3.8 Triplicate RAMD Profiling of dissected tissues of 1.*Sparus auratus* using OPB-18 primer. **M:** DNA size marker.

In figure 3.9, kidney, spleen, muscle, liver and lipid tissues show the most prominent different profiles, whereas brain, heart, gill, stomach and blood tissues show more conserved, stable and less mosaic pattern.

In figure 3.10, kidney, muscle, brain, gill, liver, stomach and lipid tissues show more heterogeneous profiles whereas spleen, heart and blood show more conserved and stable patterns.

In this work OPB-5 primer is shown to be a good candidate primer to show the different variations at interspecies, intraspecies and even intraindiviual levels.

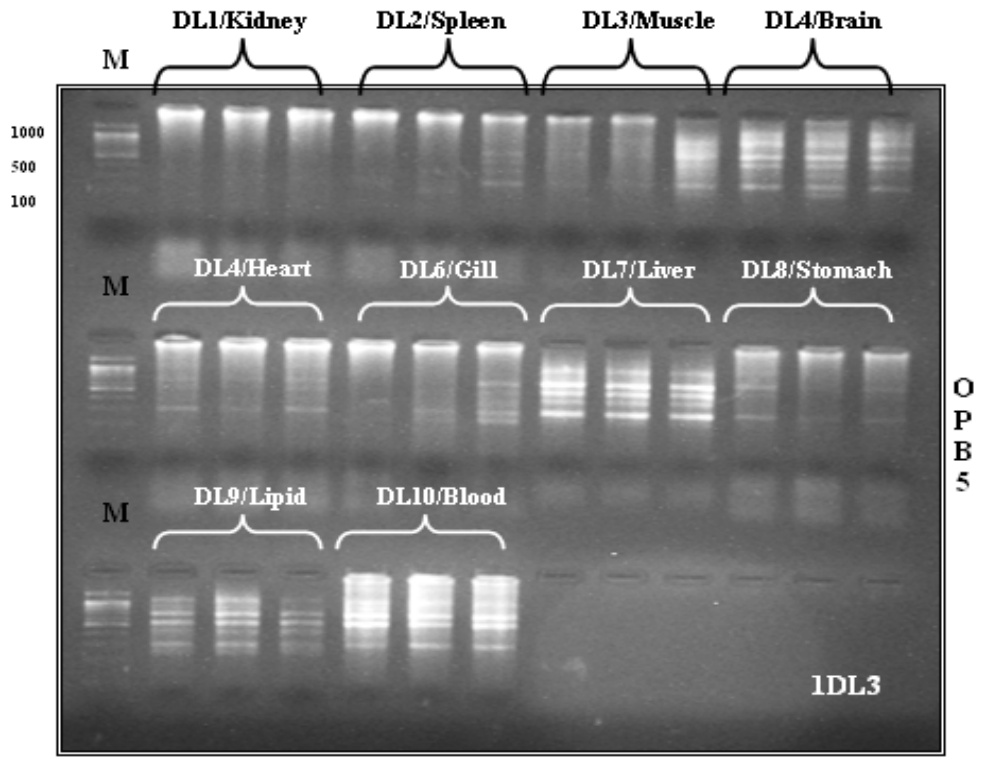

Figure 3.9 Triplicate RAMD Profiling of dissected tissues of 1.*Dicentrarchus labrax* using OPB-5 primer. **M:** DNA size marker.

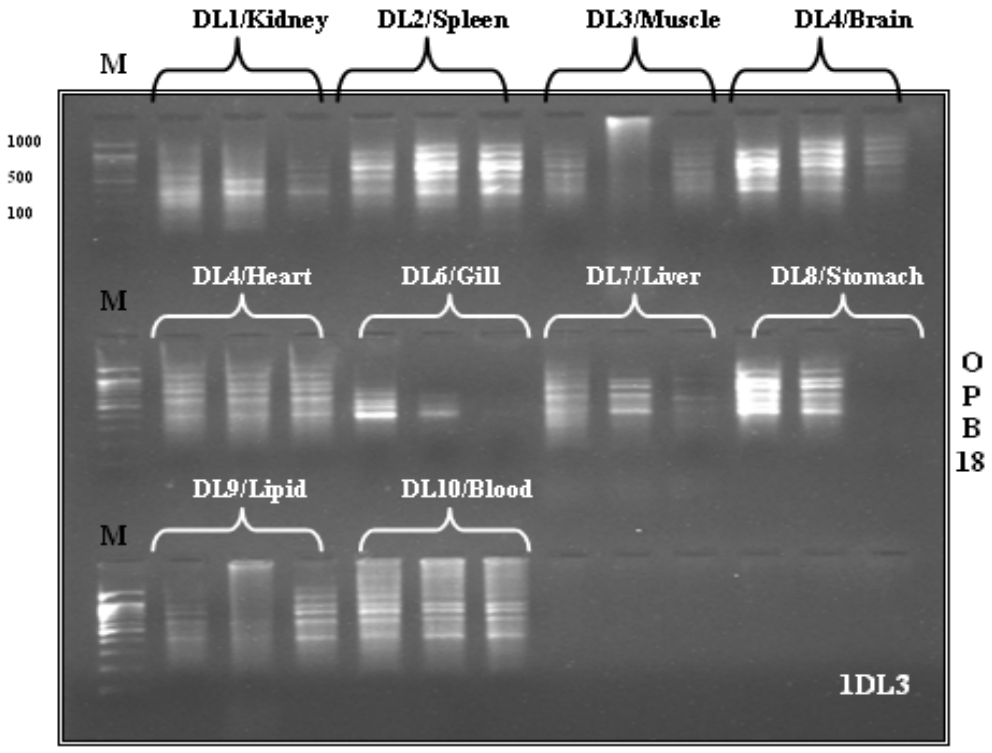

Figure 3.10 Triplicate RAMD Profiling of dissected tissues of 1.*Dicentrarchus labrax* using OPB-18 primer. **M:** DNA size marker.

Most tissues of the two cultured fish seem to be highly heterogeneous, what about the free-living marine fish. We have done some experiments with two *Pomatomus saltatrix* (Bluefish) fish were sampled from Marmara Sea in winter 2008 and their various organs' DNA. Again high heterogeneity in RAPD profiles can be seen.

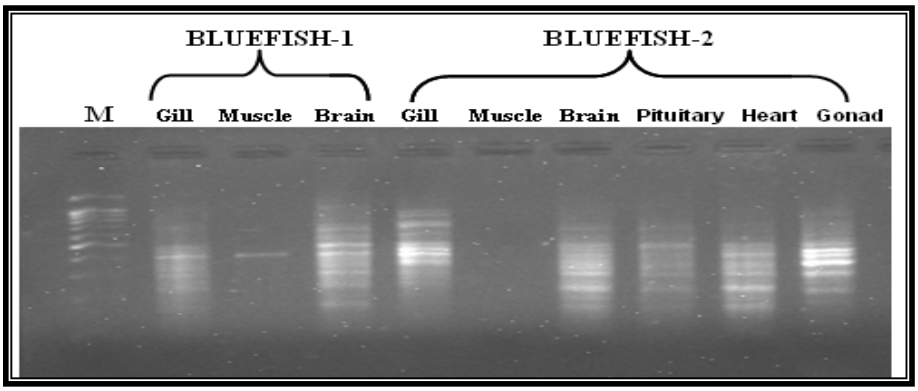

Figure 3.11 Comparative tissue-specific RAPD profiles of *Pomatomus saltatrix* various organs and tissues with OPB-7 primer. **M:** DNA size marker.

When we further do triplicate RAMD-PCR with two primers OPB-6 and OPB-18, we obtained more homogeneity in tissue level for *Pomatomus saltatrix* (Figure 3.12-3.13).

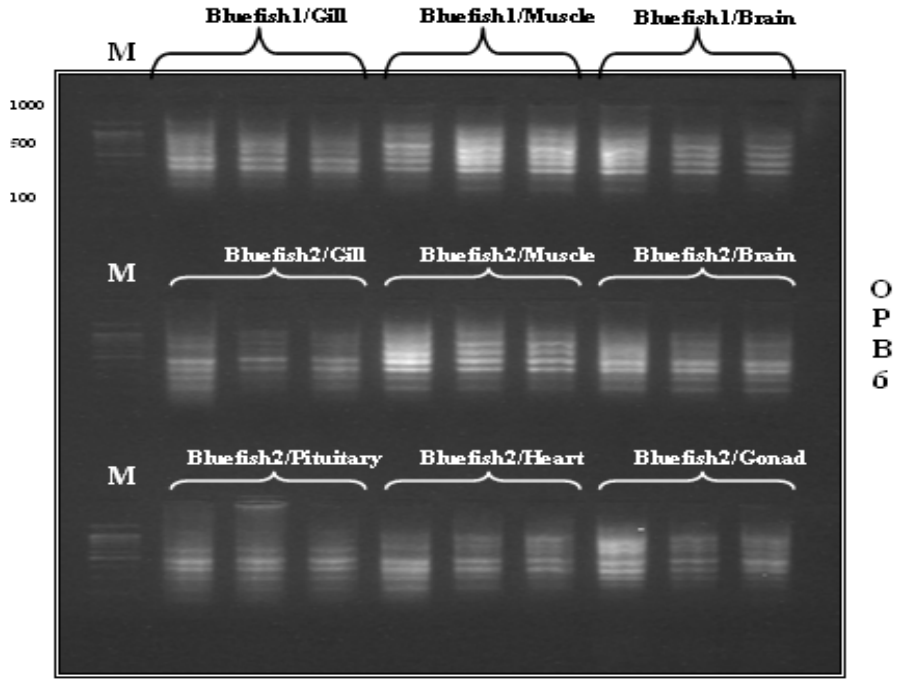

Figure 3.12 Triplicate RAMD Profiling of dissected tissues of 1. and 2. samples of *Pomatomus saltatrix* using OPB-6 primer. **M:** DNA size marker.

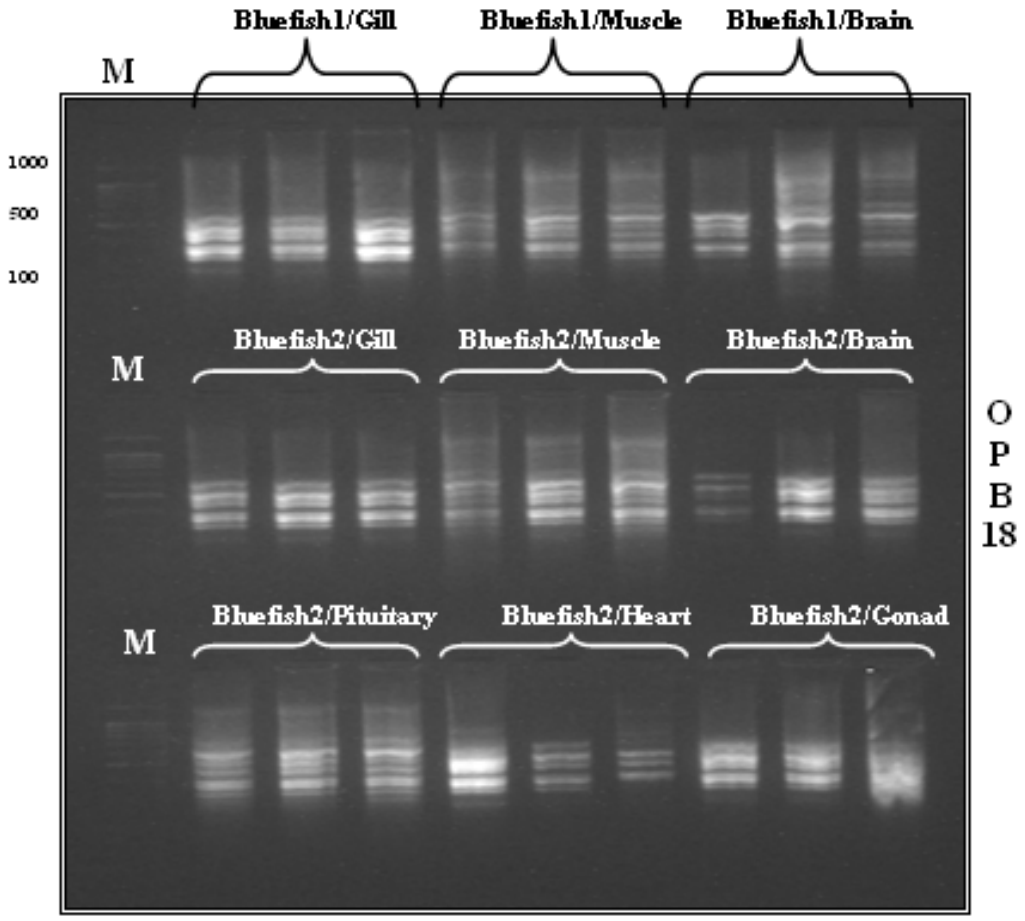

Figure 3.13 Triplicate RAMD Profiling of dissected tissues of 1. and 2. samples of *Pomatomus saltatrix* using OPB-18 primer. **M:** DNA size marker.

In the next part of the work the organs that show the most prominent and the highest number of differences in RAMD profiles were chosen. These are spleen, muscle, gill and brain tissues. The mosaicism levels have been shown with OPB-5 as triplicates for the same conditions for every component within the triplicates.

Triplicate RAMD-PCR profiles of various tissues are given in the Figure 3.14. The arrows are indicating the altered profiles that can be seen as gain / loss of the bands or increase and decrease in the band intensities.

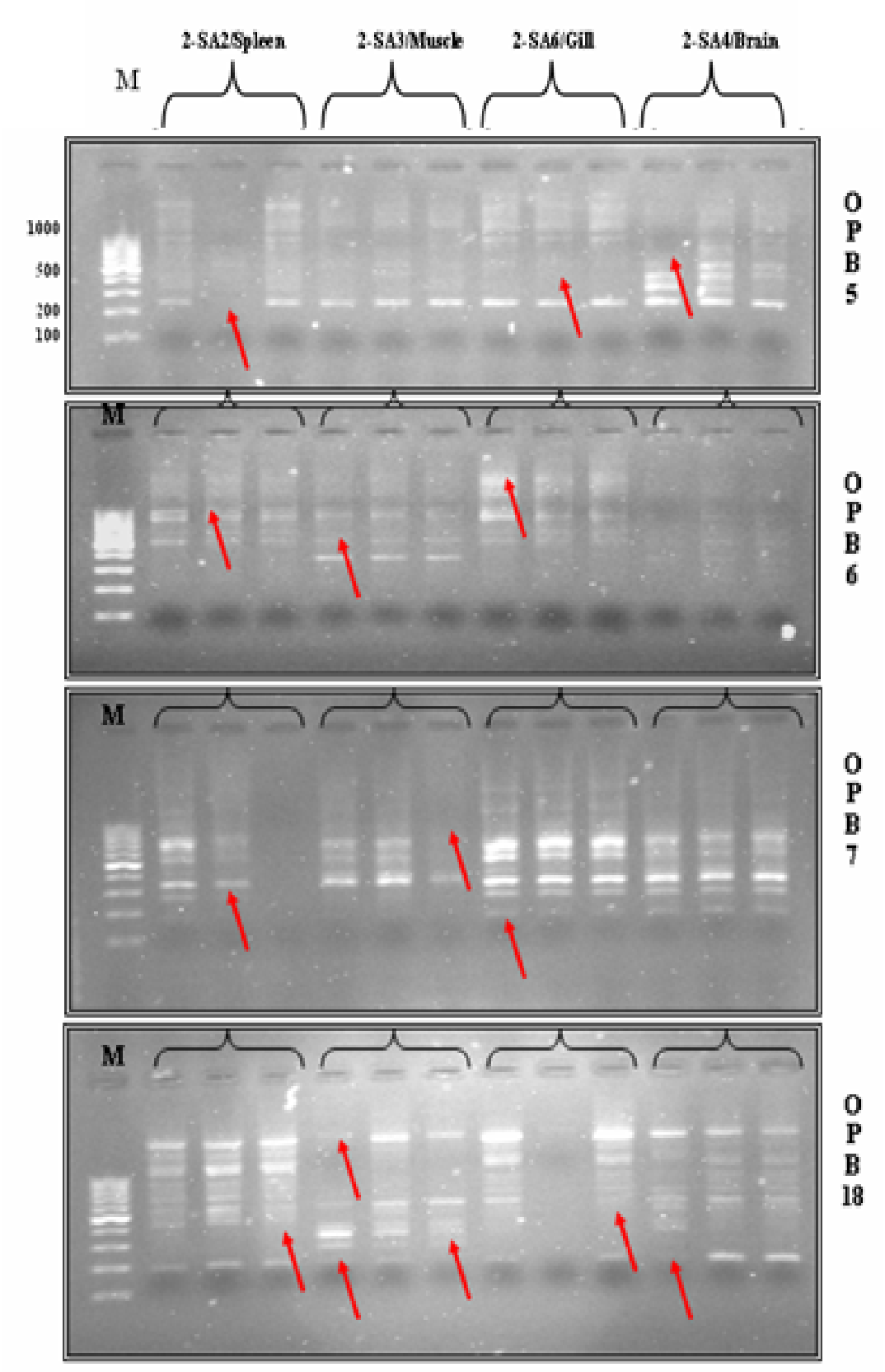

Figure 3.14 Triplicate RAMD Profiling of spleen, muscle, gill and brain tissues of 2.*Sparus auratus* using OPB-5, OPB-6, OPB-7 and OPB-18 primers. **M**: DNA size marker.

3.4.3 Somatic Mosaicism Detection

In this part of the work brain, liver and muscle tissues will be focused because these tissue and organs have an importance in two aspects. Firstly important for toxicological evaluations and secondly the muscle tissue is important as it is the edible portion of the fish.

To make the previous level of variation more clearly seen, we have done a 10 replicate mix for RAMD of each tissue and organ sample. Figures (3.15- 3.19) with indicated primers show the level of somatic mosaicism in a more distinguishing way.

In this part, figures are organized in the same tissue level for different samples using different primers. The differences band profiles can be seen clearly and these differences may be caused by mutations, chromosomal rearrangements, modifications and different epigenetic factors.

In Figure 3-15 and Figure 3.16, many different windows (OPB-5, OPB-7 and OPB-18) have been used to show the genetic variation in brain tissue. It was not that possible to show changes in brain tissue for different members of *Sparus auratus* and *Dicentrarchus labrax*.

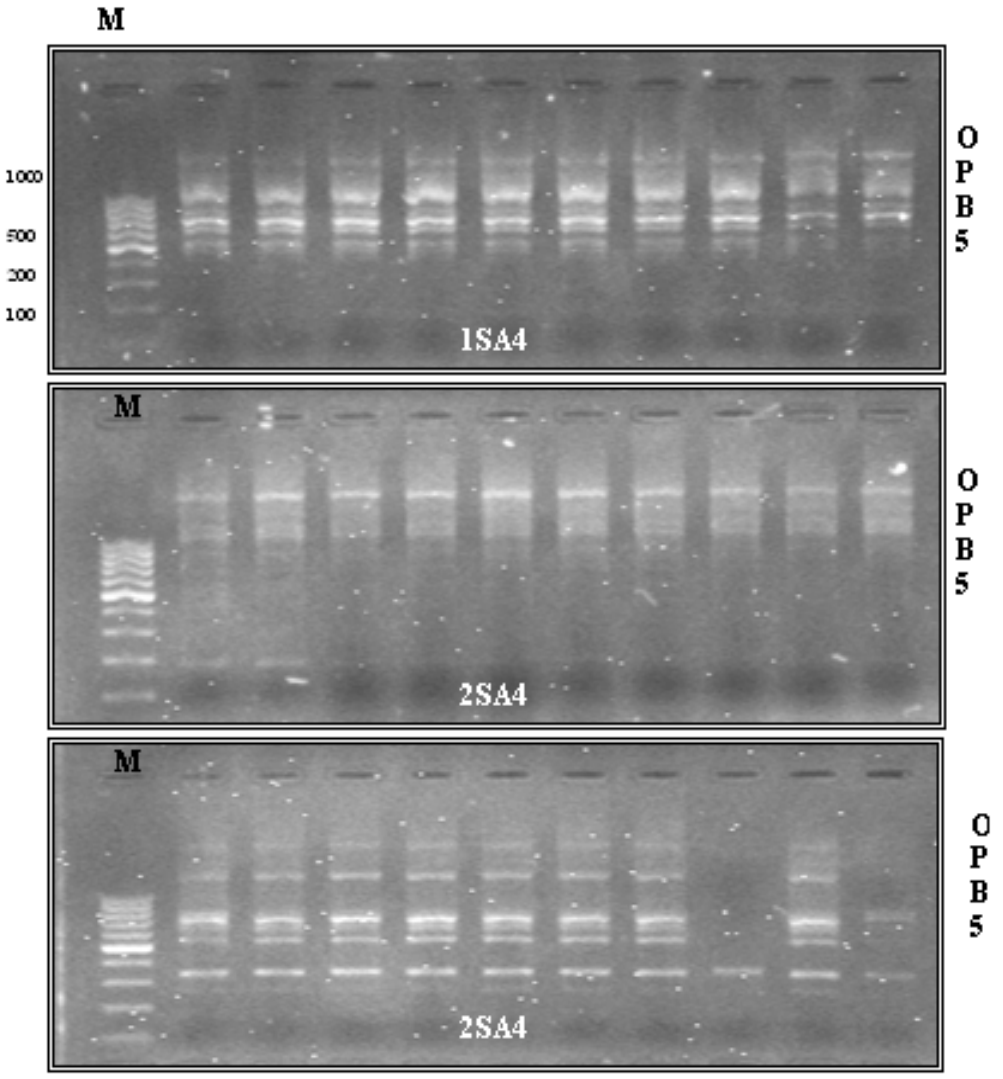

Figure 3.15 Decaplet RAMD Profiles of brain tissue samples of *Sparus auratus* with OPB-5 and OPB-7 primer. **M**: DNA size marker.

Nearly no alterations are seen on the Figure 3.15 and 3.16. This can mean *Dicentrarchus labrax* may have protection mechanism about brain tissue. This mechanism can be blood-brain barrier mechanism. The blood-brain barrier is impervious to many toxicants and can protect the brain from accumulation of substances present in blood. In fish, toxicants can enter the blood stream from the diet and across the gills from the external environment. But circumvention of the blood-brain barrier can occur if toxins are taken up via nerves that innervate water-exposed sensory organs. Nerve terminals in lateral line, olfactory and gustatory systems, have all been highlighted as routes of uptake for waterborne metals (68-74).

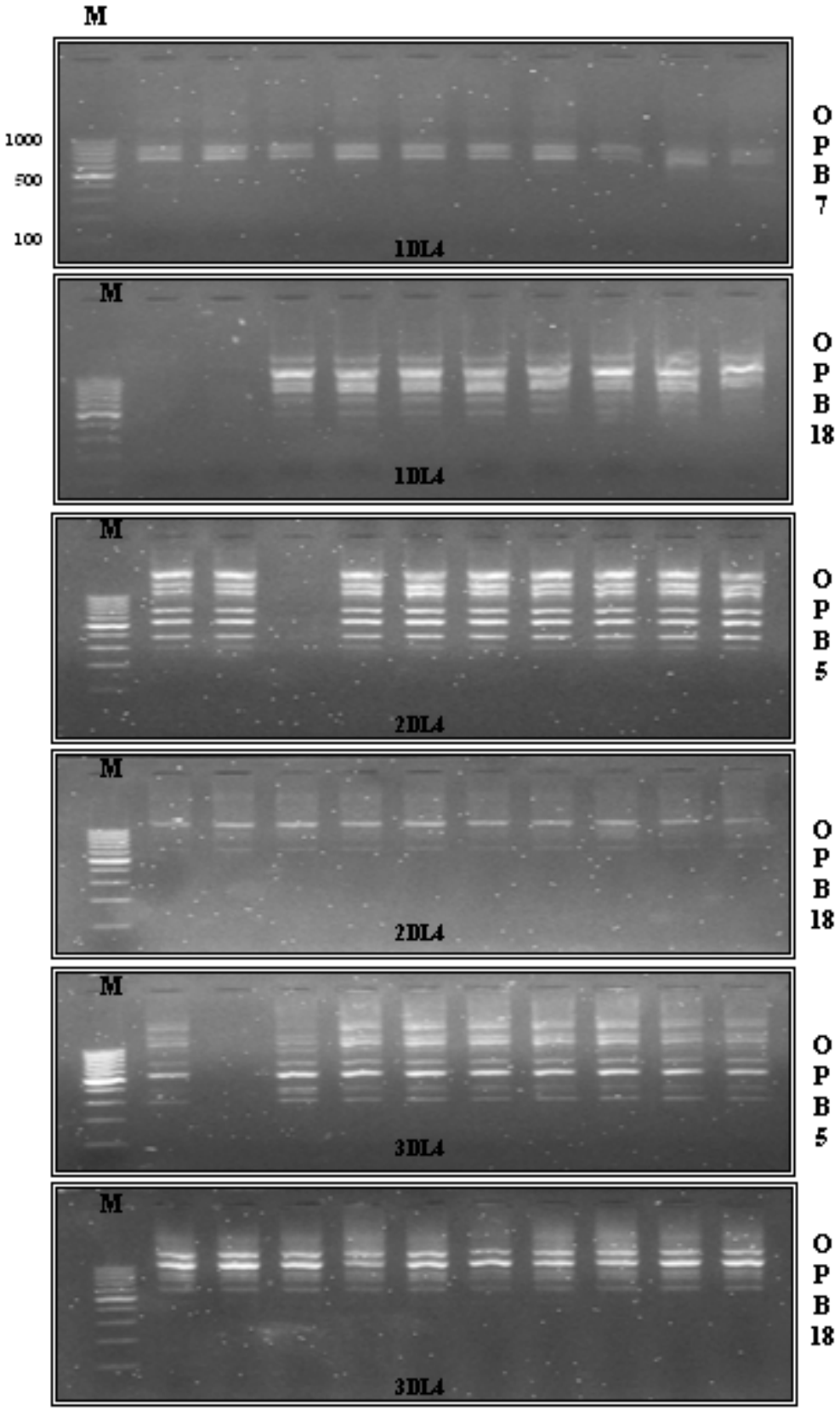

Figure 3.16 Decaplet RAMD Profiles of brain tissue samples of *Dicentrarchus labrax* with OPB-5, OPB-7 and OPB-18 primers. **M**: DNA size marker.

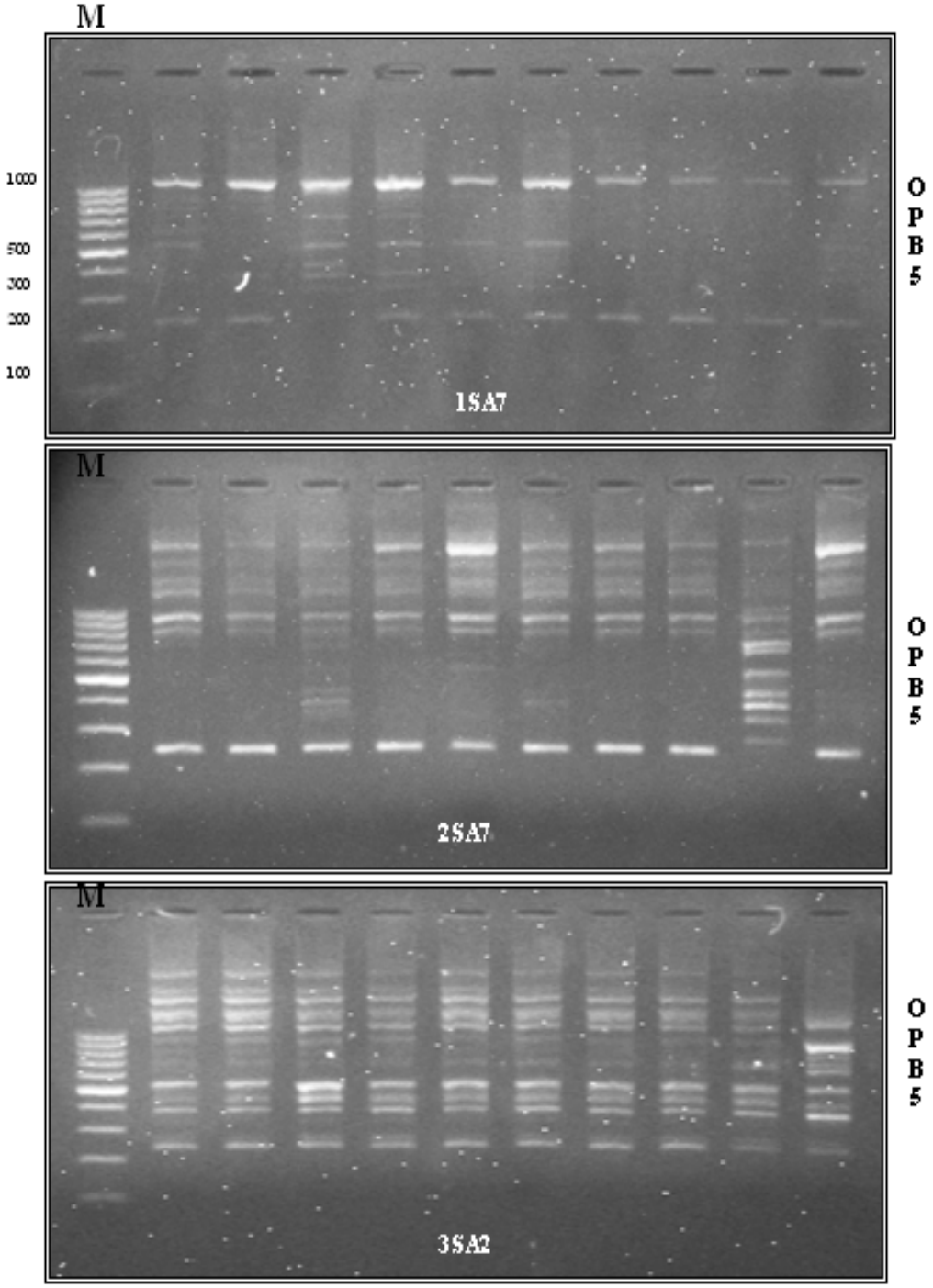

Figure 3.17 Decaplet RAMD Profiles of liver tissue samples of *Sparus auratus* with OPB-5 primer. **M**: DNA size marker.

More changes have seen in different members of *Sparus auratus* liver tissue when compared with brain tissue but still not that mosaic when we remember it is most important detoxifying organ prone to every toxic attack.

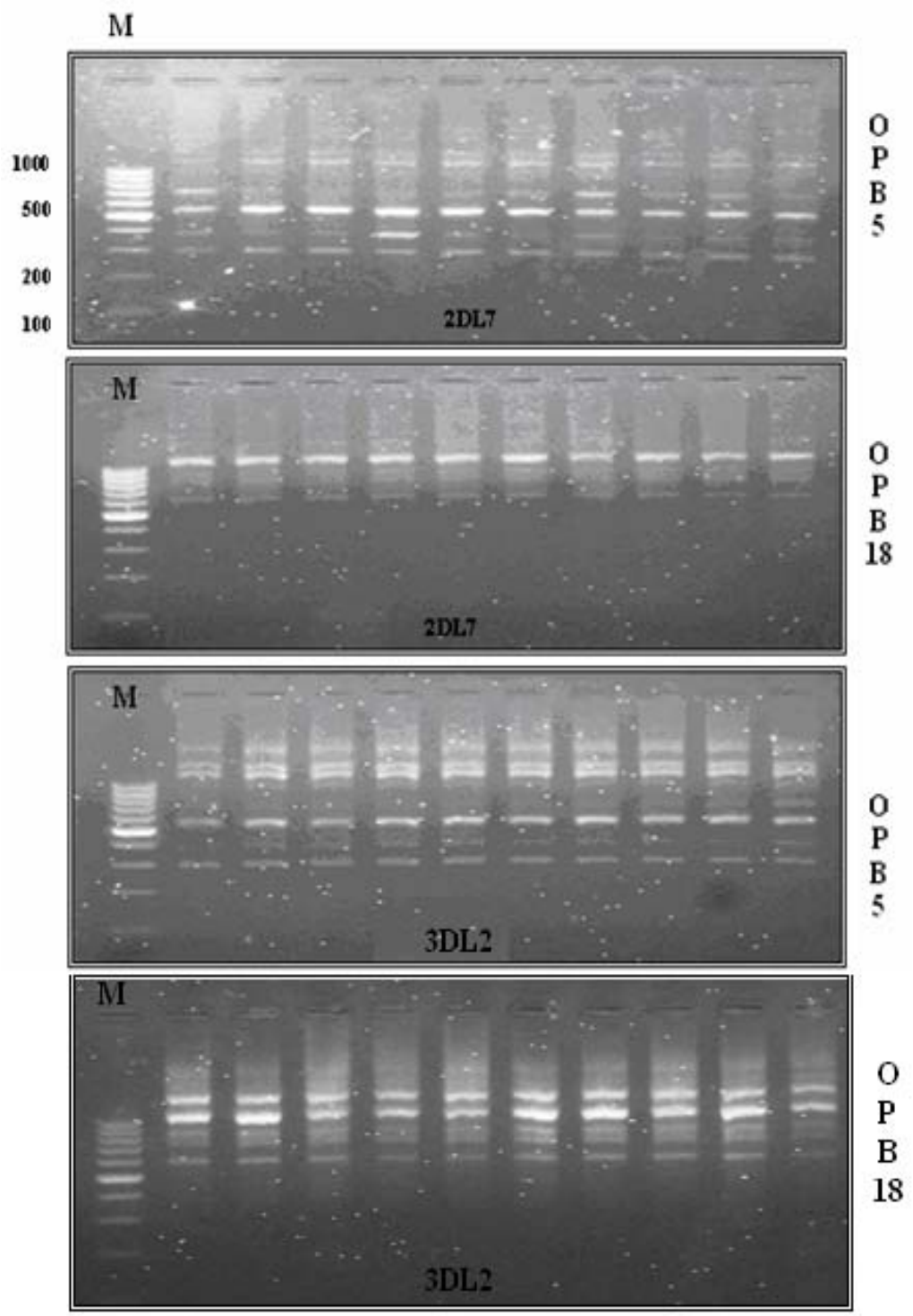

Figure 3.18 Decaplet RAMD Profiles of liver tissue samples of *Dicentrarchus labrax* with OPB-5 and OPB-18 primers. **M**: DNA size marker.

There is little change in *Dicentrarchus labrax* liver tissue samples. Variations are indicated with the arrows in Figure 3.18, but there is no significant difference as in the liver tissue of *Sparus aurarus* (Figure 3.17).

There are significant differences in 3.*Sparus auratus* and 2.*Dicentrarchus labrax* muscle RAMD profiles (Figure 3.19). These two samples have been collected from the same fish farm at the same time. There can be an undetermined chemical exposure that can affect the fish samples at this season. Besides, no alterations have been detected on the 3.*Dicentrarchus labrax* RAMD profile. This fish sample's RAMD profiles were produced with two different primers, OPB-5 and OPB-18.

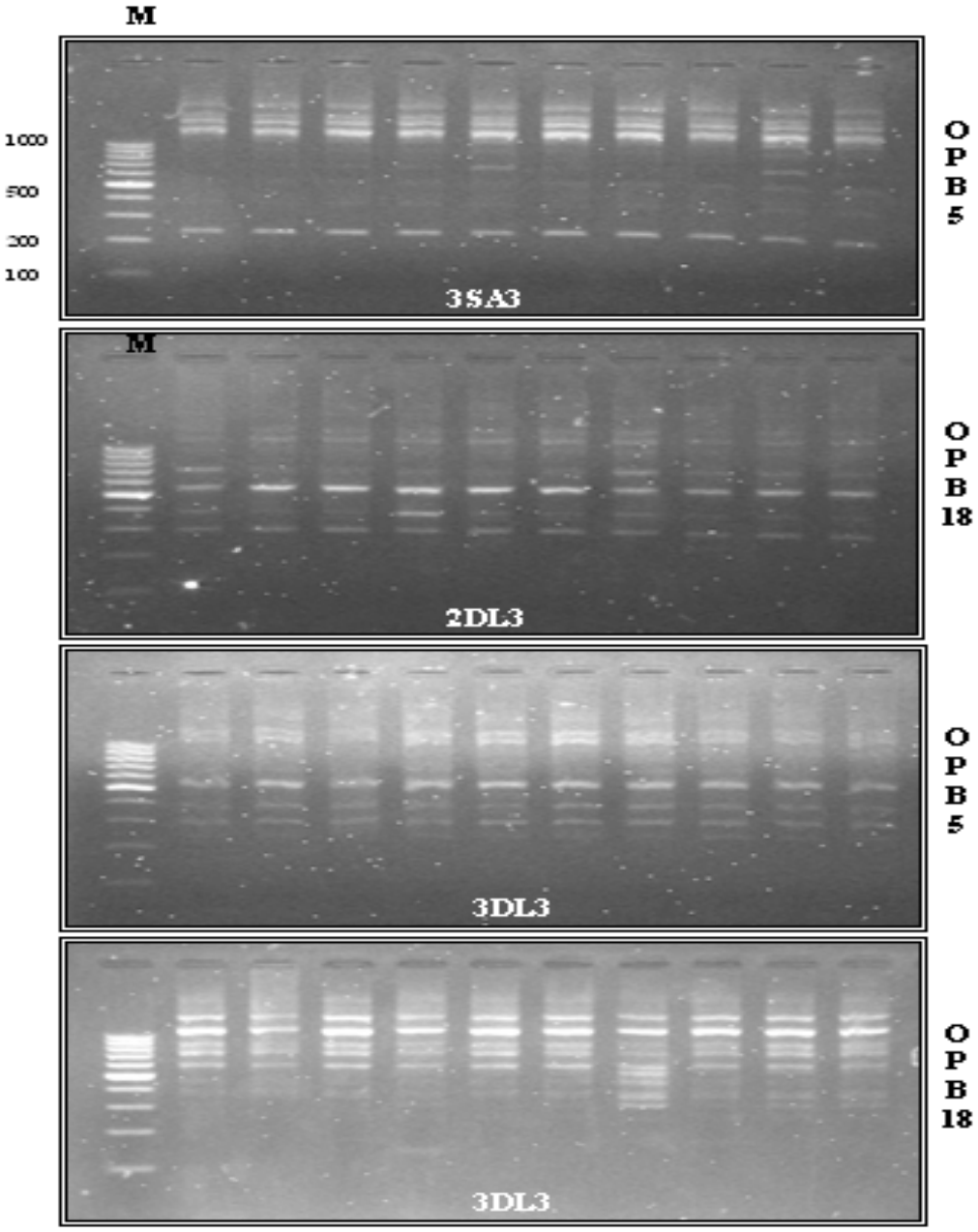

Figure 3.19 Decaplet RAMD Profiles of muscle tissue samples for *Sparus auratus* and *Dicentrarchus labrax* with OPB-5 and OPB-18 primers. **M**: DNA size marker.

Muscle tissue have conserved parts detected and less conserved when compared with the other two organs looking at different patterns detected from the windows of OPB 5 and OPB 18.

According to the RAMD profiles of above mentioned tissues, the results of our work have revealed certain peculiarities. As a whole, the somatic modifications of DNA can be caught randomly. Most often, the modifications were observed in the DNA isolated from the *Sparus auratus* brain, liver and muscle tissues. It could be noted that the majority of additional or shifted bands in the amplification spectra are major; they have been well reproduced in the same DNA samples in repeated experiments, which is evidence of the reliability of results.

Somatic mosaicism may result from mutations of the nuclear genome, modified number of chromosomes, various epigenetic modifications like methylation and the modification of extranuclear genetic material. If mutations appear in the primer annealing sites, there can be a gain or loss of amplification fragments; if mutations occured between the sites of primer annealing, like deletions and insertions, the mobility of amplification fragments are affected.

The modification of mobility or gain/loss of DNA fragments in the amplification spectra of tested samples may also be associated with replication errors or mutations affecting the regions of complementary DNA binding with the oligonucleotide primers

The present study focused on the identification of somatic mosaicism cases in humans with the help of polymerase chain reaction using random primers (RAPD PCR). It was demonstrated that this method was effective for detection of somatic mutations (74).

The nature of the somatic variation in the RAPD profiles for the tissues examined remained unclear. The change of the DNA fragments mobility, or the fragments gain/loss in the RAPD profile could be caused by genomic rearrangements, or the mutations involving the sites of the complementary binding between the DNA and oligonucleotide primers.

Based on the data obtained the following specific features can marked: (1) even in case of the analysis of a small number of DNA samples it is possible to find the primers detecting somatic differences; (2) these differences are distinguishes only in some individuals; (3) no preferred variation of the RAPD profiles for any tissue (organ) is observed. It is evident that the features listed require confirmation using larger sample of DNA specimens. New information on the nature of somatic variation can be yielded by means of cloning and sequencing of amplification products (75).

3.4.4 Gonadal Mosaicism Detection

Gonadal mosaicism or germline mosaicism is a special form of mosaicism, where some gametes, i.e. either sperm or oocytes, carry a mutation, but the rest are normal. The cause is usually a mutation that occurred in an early cell that gave rise to all or part of the gonadal tissue.

Gonadal mosaicism detection has a different importance as the mutations in the gonadal tissue are inherited to the next generations causing a deterioration of the coming generations. The same systematic for gonadal mosaicism detection has been applied with 10 replicates of RAMD-PCR. Figure 3.14 and 3.15 shows the RAMD profile of gonadal tissue samples.

OPB-5 is a good candidate for detecting the different band profiles for *Sparus auratus* samples. OPB-7 primer did not catch up differences, as no alterations seem on the RAMD profile (Figure 3.20).

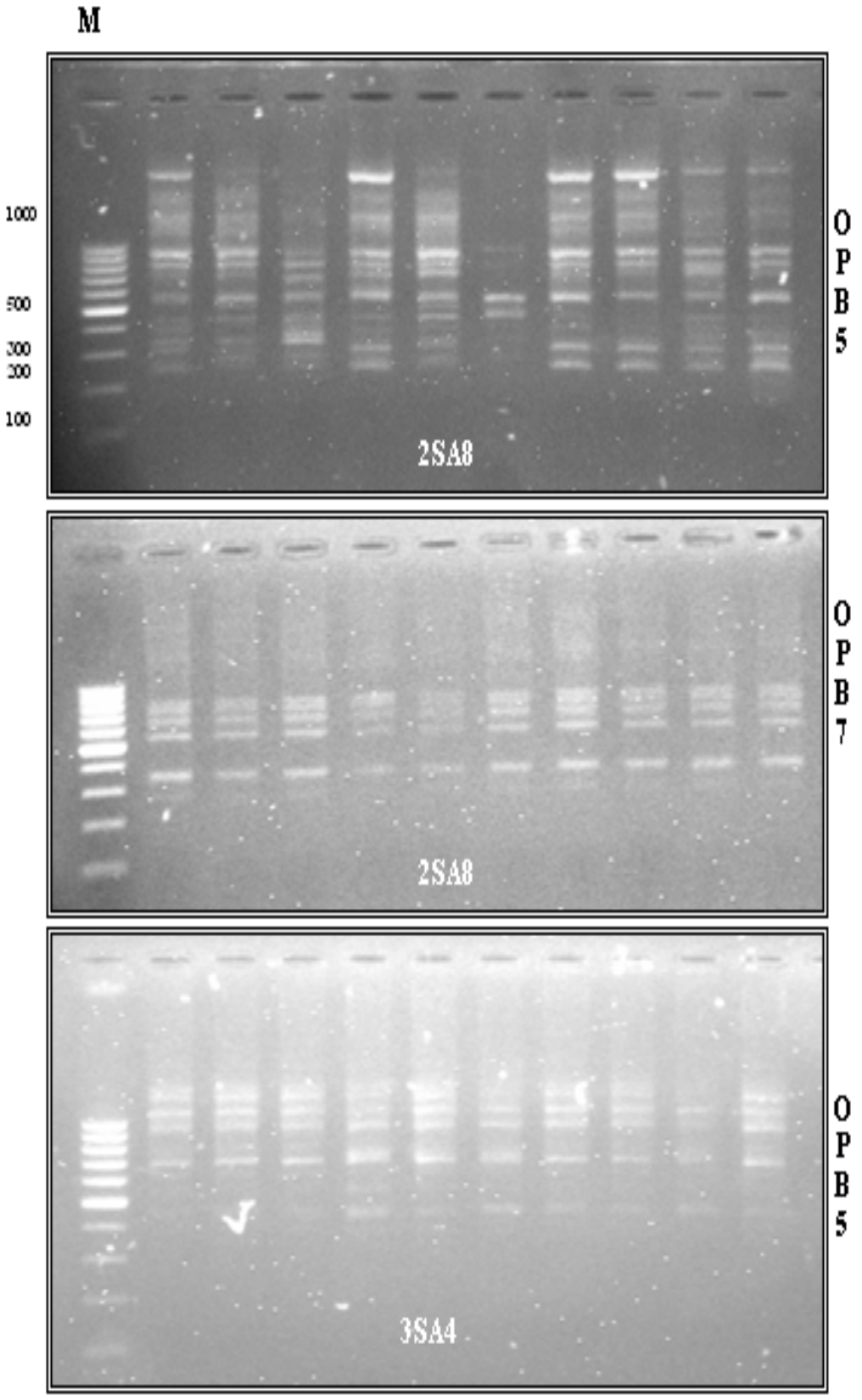

Figure 3.20 Decaplet RAMD Profiles of gonad tissue samples of *Sparus auratus* with OPB-5 and OPB-7 primer. **M:** DNA size marker.

For also *Dicentrarchus labrax* gonad tissue samples, OPB-5 primer is a good candidate for indicating the different band profiles. OPB-18 primer did not show significant differences (Figure 3.21).

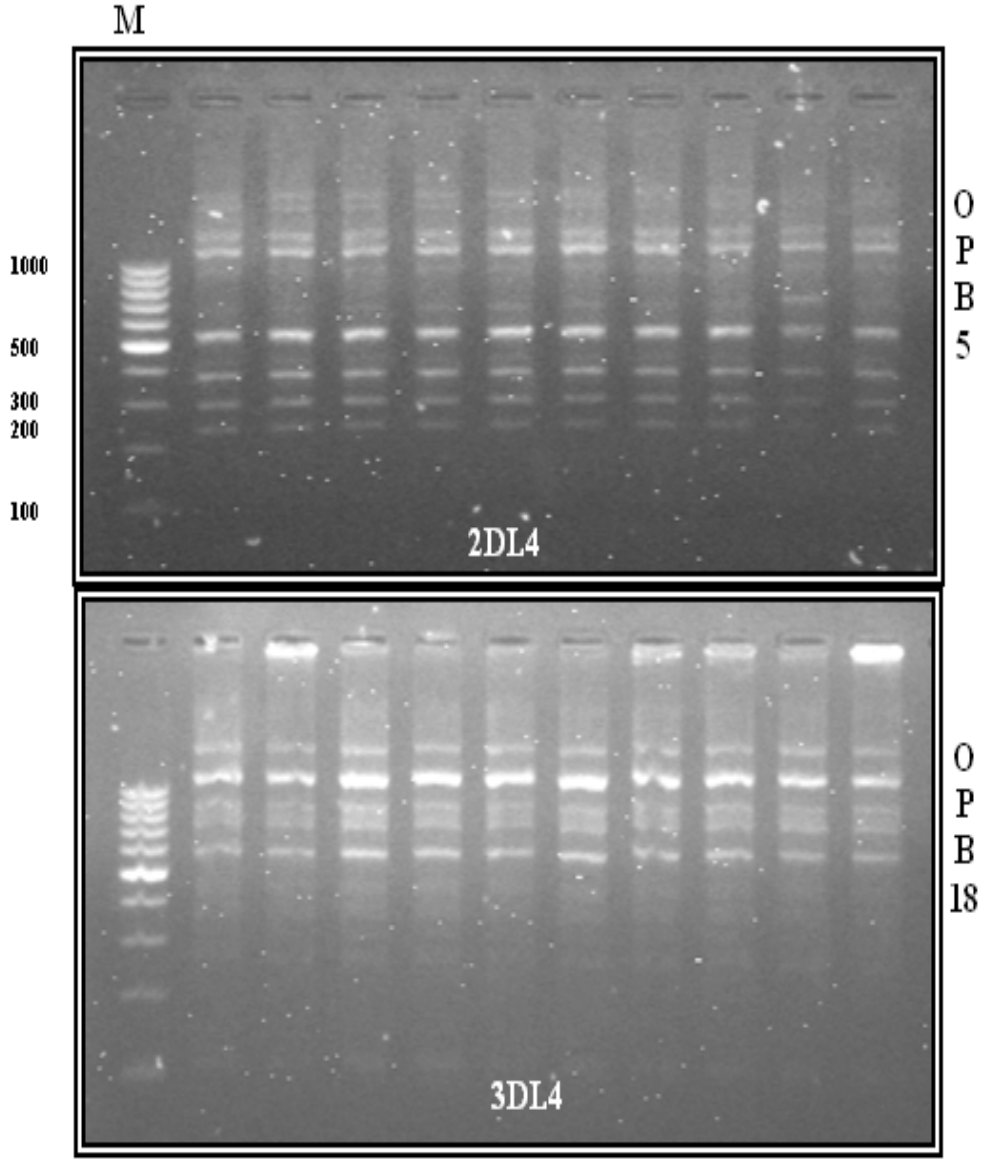

Figure 3.21 Decaplet RAMD Profiles of gonad tissue samples of *Dicentrarchus labrax* with OPB-5 and OPB-18 primer. **M**: DNA size marker.

Sample number is too small to discuss the results. Variations caught by the same primer in both fish species is the most important achievement. The most prominent changes are observed in *Sparus auratus* gonad samples, but still it is early to conclude, unless the sample number is increased.

3.5 RAPD and RT-RAPD Comparative Results

In eukaryotic species gene expression can also be regulated at the RNA level. These gene expression mechanisms are; alternative splicing, RNA editing, RNA stability, RNA interference, general regulation of translation and translational regulation of specific mRNAs.

Analysis of eukaryotic structural genes in the late 1970s revealed that they are not always colinear with their functional mRNAs. Instead, coding sequences, called exons, are interrupted by intervening sequences or introns. Transcription produces the entire gene product; introns are later removed or excised and exons are connected together or spliced. This phenomenon is termed RNA splicing. It is a common genetic phenomenon in eukaryotes.

One very important biological advantage of introns in eukaryotes is the phenomenon of alternative splicing. Alternative splicing refers to the phenomenon that pre-mRNA can be spliced in more than one way. In most cases, large sections of the coding regions are the same resulting in two alternative versions of a protein that have similar functions. Nevertheless, there will be enough differences in amino acid sequences to provide each protein with its own unique characteristics.

The term RNA editing refers to a change in the nucleotide sequence of an RNA molecule. It involves additions or deletion of particular bases or a conversion of one type of base to another. RNA editing can have various effects on mRNAs like generating start or stop codons and changing the coding sequence of a polypeptide.

Stability of RNAs varies considerably. The stability of mRNA can be regulated so that its half-life is shortened or lengthened. This will greatly influence the mRNA concentration and consequently gene expression. Factors that can affect mRNA stability include length of the polyA tail and destabilizing elements.

RNA transcripts have different functions. Once they are made, RNA transcripts play different functional roles. Well over 90% of all genes are structural genes

producing mRNA and translated into a protein product. The RNA transcripts from nonstructural genes are not translated. They do have various important cellular functions. They can still confer traits. In some cases, the RNA transcript becomes part of a complex that contains protein subunits, for example ribosomes, spliceosomes and signal recognition particles (28).

Genome-wide screening showed various levels of changes in the whole genome seen from different windows, but we are not sure these changes are in the genes that encode proteins or not. Proteins can be structural and functional and any change in their 3-D structure due to the encoding genetic defect can have varying degrees of effects on the organism. RAPD with RT-PCR extension will show us the cDNA changes detectable by the same RAPD primers used.

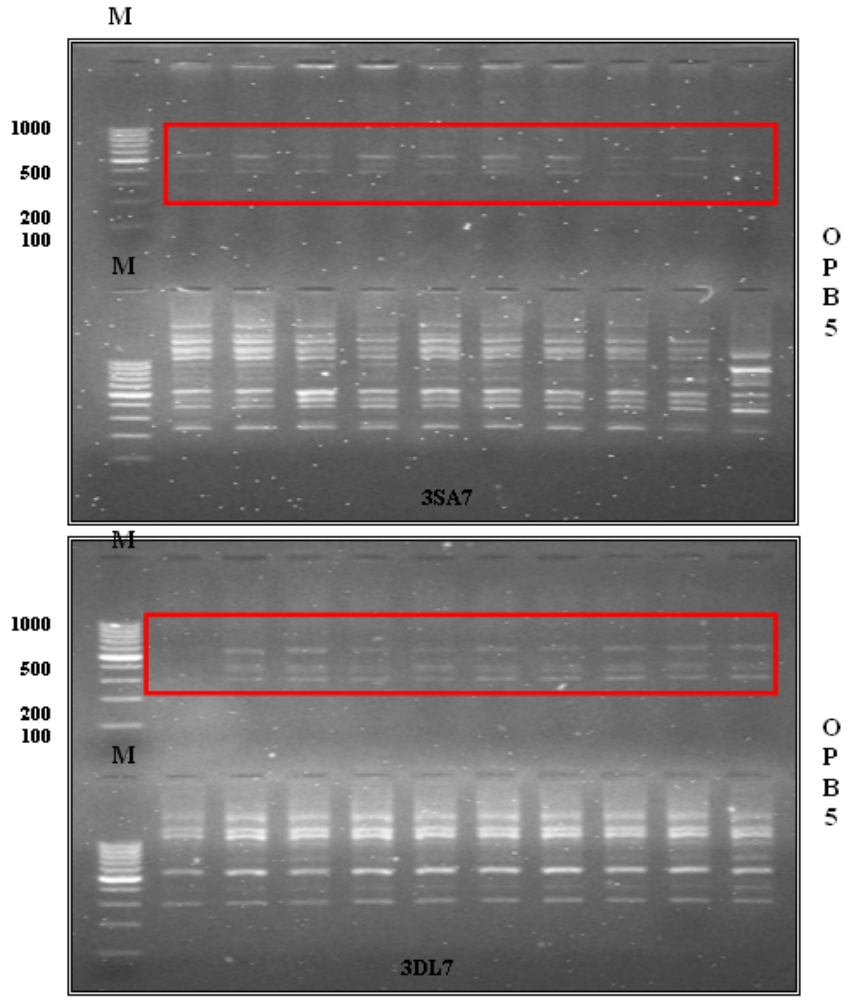

Figure 3.22 Decaplet RAMD and RT-RAPD profiles of liver tissues of 3.*Sparus auratus* and 3.*Dicentrarchus labrax* using OPB-5 primer. **M:** DNA Size Marker.

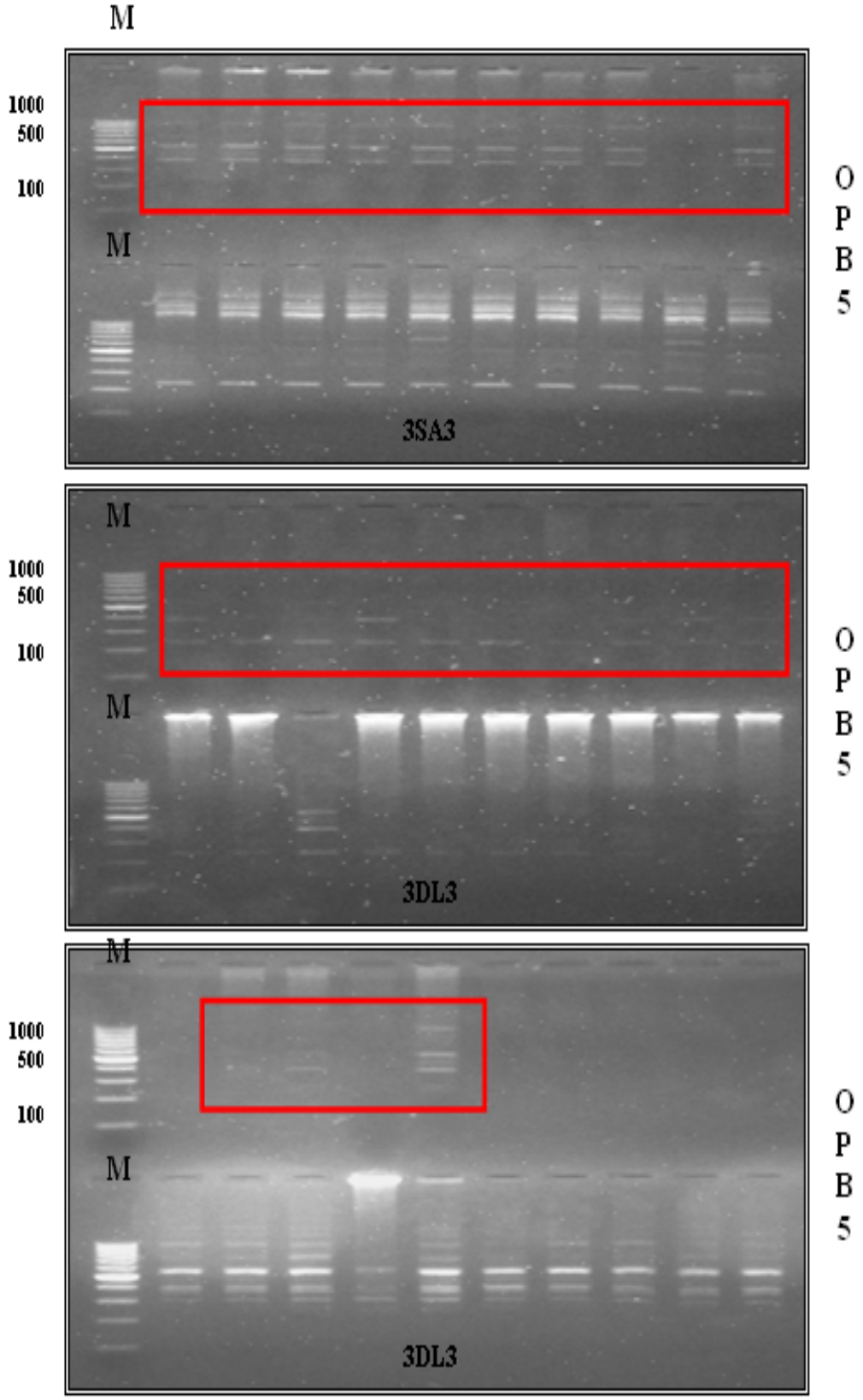

Figure 3.23 Decaplet RAMD and RT-RAPD profiles of brain tissues of 3.*Sparus auratus* and 3.*Dicentrarchus labrax* using OPB-5 primer. **M:** DNA Size Marker**.**

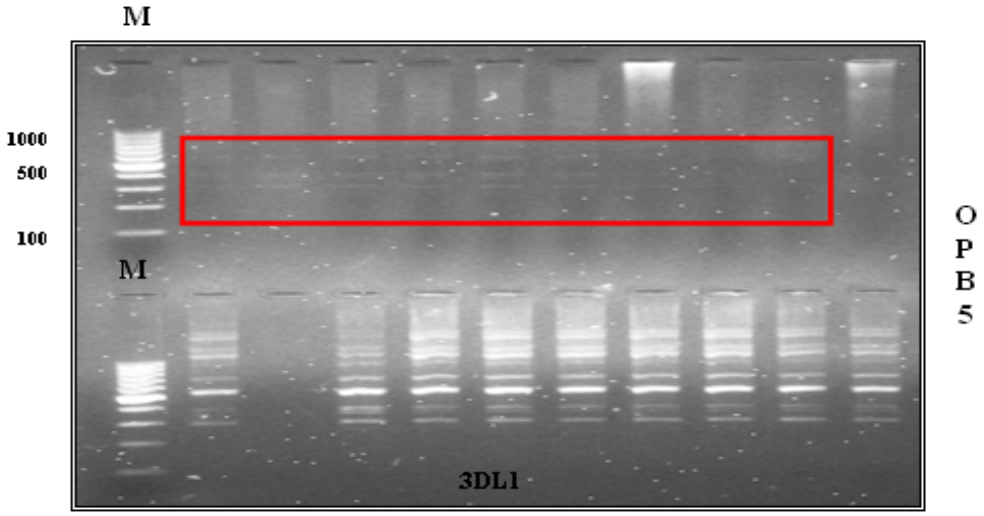

Figure 3.24 Decaplet RAMD and RT-RAPD profiles of brain tissues of 3.*Dicentrarchus labrax* using OPB-5 primer. **M:** DNA Size Marker.

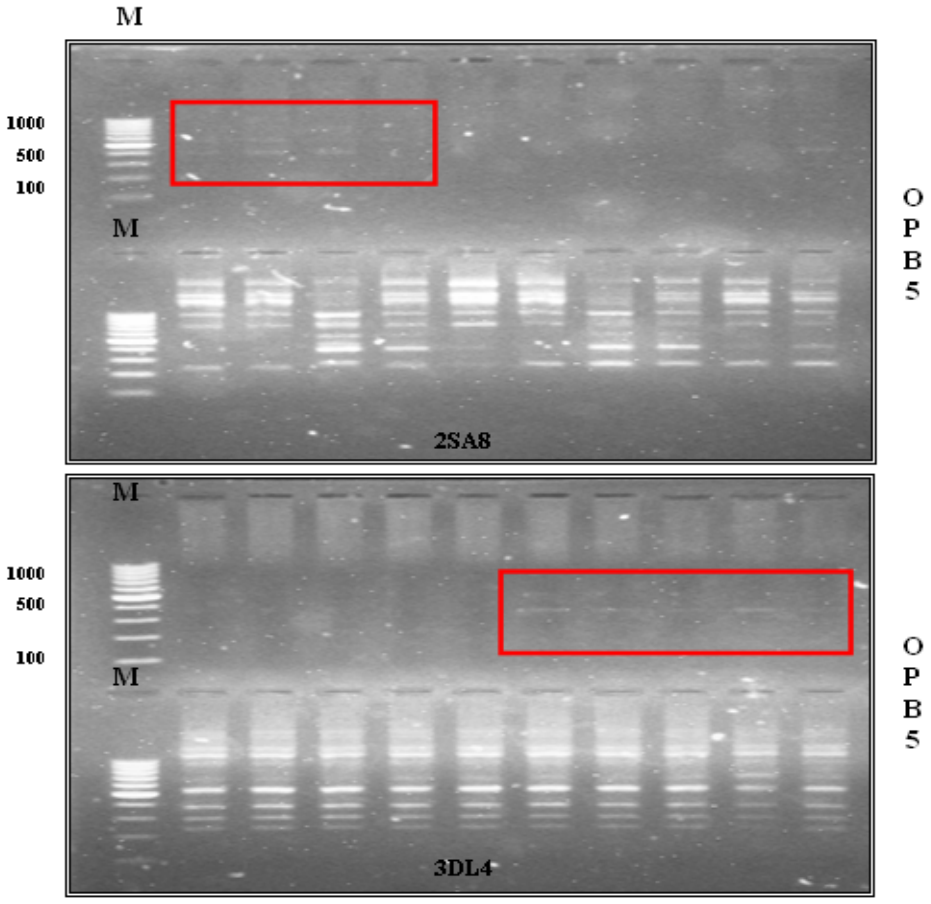

Figure 3.25 Decaplet RAMD and RT-RAPD profiles of gonad tissues of 2.*Sparus auratus* and 3.*Dicentrarchus labrax* using OPB-5 primer. **M:** DNA Size Marker.

We are giving our RT-RAPD results in this context. We have tried two primers, OPB5 and OPB18 that gave the best results for DNA profiles for both fish, but only OPB5 made amplification, this has an important but unraveled discussion about the gene sequences' common properties. We have worked on four tissues: brain, liver, muscle and gonad that gave RT-RAPD results with both different and similar band profiles that might be an indication for tissue-specific and/or house-keeping gene expressions. And also 10 replicate profile changes were detected in a few cases which can be detection of mosaicism in the gene level or in RNA level at either regulation of RNA processing or RNA mutation.

CHAPTER 4

CONCLUSION

Fish aquaculture is very important for food industry and economy, and should be enhanced for this sake with different scientific innovations. Genotyping is an important tool in selection and maintenance of domesticated fish strains. Molecular techniques based on the polymerase chain reaction are widely used for assessment of genetic variations between and within strains, species, populations, etc. In our thesis we applied DNA and RNA-based molecular biology and genetics tools to unravel various DNA and RNA-related or facts about fish-aquaculture in Turkey.

Despite of the developments of sensitive and specific biomarkers, the environmental surveillance and monitoring of fish populations is still quite expensive and routine applications especially at DNA level are not widespread. Because of the high number of individuals needed and the complexity of variables analysed, therefore evaluation of results is very difficult. For this reason studies mostly used confined populations, or been based on laboratory studies where reliable controls are available and the exposure situation can be defined. It is not exactly possible to find a reliably controlled system to assess the changes, variation at the DNA level, especially if you are trying to monitor the adverse effects of pollution on DNA of fish and fish populations. Observable genetic variations at the DNA level may depend on many parameters that can be hardly controllable. Innate genetic variation should be differentiated from the acquired variation. Different organs and tissues of fish should be evaluated for their genetic variation to be used for target organ biomonitoring by RAPD-PCR based genotoxicity assays.

In this study, brain, muscle, liver and gonad tissues of three different fish samples for both *Sparus auratus* and *Dicentrarchus labrax* have been evaluated for genetic variation at the organ levels to make background knowledge for further genotoxicity, target organ toxicity and ecotoxicological monitorings. Brain tissue DNA for both fish samples found to be the most conserved and stable for variation assessed by RAPD-PCR done for 10 replicates of the same tissue DNA used in the same mastermix to be a control for the expected mosaic pattern of DNA. Each 10 replicate for liver and especially muscle and gonad tissue showed significant variation though theoretically with the same DNA no change was expected, though for brain variation was comparably lower than the other tissues most probably blood brain barrier's protective role for various DNA damaging exposures.

Different types of DNA mutations and lesions can induce the same type of alterations in RAPD profiles (e.g. variation in band intensity, band appearance and disappearance). Thus, other methods measuring genotoxic endpoints, such as detection of DNA adducts, gene mutations or cytogenetic effects, are required for the identification of changes at DNA level. In the field of eco-genotoxicology, we propose using RAPD in conjunction with parameters because there should be higher levels of biological organization such as growth, fitness and reproduction, as our studies have demonstrated a link between these parameters and changes in RAPD profiles in given aquatic organisms.

The effectiveness of RAPD in detecting polymorphism between and among different fish populations, their applicability in population studies, and the establishment of genetic relationships among fish populations has been demonstrated.

The RAPD assay is very reliable. The assay presents a number of advantages for the detection of genotoxic effects. And last but not the least, the RAPD method has the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA breakage) as well as mutations (point mutations, large rearrangements). In the field of cancer research, the RAPD assay and related techniques allow the cloning of genomic alterations and simultaneous detection, without previous knowledge of the altered region.

A large number of new technologies and assays are developing to profile the gene expression pattern either following exposure to environmental contaminants or during the process of malignant development. The RAPD based techniques encourage a great hope for future and would continue to complement other new and well-established techniques in genotoxicity, population genetics and carcinogenesis studies.

It is important to mention the fact that data results from RAPD assays can be extended to further dissect traits in a more refined way to exactly knowledge on specific genes and genetic pathways using other molecular methodologies. There is also the opportunity and need to study sequences of specific polymorphic bands, to determine the genes detected by RAPD experiments. Further studies with other molecular methodologies are essential to clarify and confirm genetic relationships among fish species depicted using RAPDs (76-79).

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