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Amplification and cloning of cDNAS of cytochrome P4501A1 and metallothionein genes from *Sparus aurata* Linnaeus, 1758 and *Liza aurata* (Risso, 1810) by Race-PCR

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ABSTRACT

The biotransformation of xenobiotics found in marine ecosystems is catalysed by inducible systems, a property that makes them useful as early-warning biomarkers of environmental pollution. We have focused our study on cytochrome P4501A1 (*CYP* 1A1) as a phase I response against organic aromatic xenobiotics, and metallothionein (*MT*), which reflects pollution by transition metals. The high homology existing between the sequences of both genes, already cloned in different fish species, enabled us to design degenerate oligos to amplify by RT-PCR specific sequences of *CYP* 1A1 and *MT* genes in two teleost fish species of the Spanish South Atlantic littoral, the gilthead seabream, *Sparus aurata* Linnaeus, 1758, and the grey mullet, *Liza aurata* (Risso 1810). To this end, the expression of both genes was previously induced by intraperitoneal injection with Aroclor 1254 (*CYP* 1A1) and CdCl2 (*MT*). The specific sequences for both genes amplified by RT-PCR with degenerate oligos were subsequently used to design new, specific oligos to obtain by Race-PCR (Rapid Amplification of cDNA Ends) the complete cDNAs coding for both genes in both fish species, which were subsequently cloned and sequenced. The coding sequences and the corresponding proteins were compared with those already obtained in other fish species. We are currently developing homologous probes for mRNA quantification using molecular biology techniques in both fish species, to be employed as molecular biomarkers of pollution in the Spanish South Atlantic littoral.

Key words: Biomonitoring, biomarkers, pollution, mRNA, cDNA, polymerase chain reaction, sequencing, Spanish littoral.

RESUMEN

Amplificación y clonación de cDNAs de los genes del citochromo P4501A1 y la metalotioneína de* Sparus aurata *Linnaeus, 1758 y* Liza aurata *(Risso, 1810) por Race-PCR

*La biotransformación de xenobióticos presentes en ecosistemas marinos está catalizada por varios sistemas inducibles, lo que permite su uso como biomarcadores de alerta temprana de contaminación ambiental. Este estudio se ha centrado en el citocromo P4501A1 (*CYP *1A1), como respuesta de fase I contra xenobióticos orgánicos aromáticos, y la metalotioneína (*MT*), que refleja la contaminación por metales de transición. La alta homología existente entre las secuencias de ambos genes previamente clonados en diferentes especies de peces permitió diseñar oligos degenerados para amplificar por RT-PCR secuencias específicas de los genes* CYP *1A1 y* MT *en dos especies de peces teleósteos del litoral suratlántico español, la dorada* Sparus aurata *Linnaeus, 1758 y la lisa* Liza aurata *(Risso, 1810). Para ello, la expresión de ambos genes se indujo por inyección intraperitoneal con Aroclor 1254 (*CYP *1A1) y CdCl2 (*MT*). Las secuencias específicas de ambos*

*genes, amplificadas por RT-PCR con tales oligos, se usaron posteriormente para diseñar nuevos oligos específicos; éstos se usaron para amplificar por Race-PCR (*Rapid Amplification of cDNA Ends*) los cDNAs completos que codifican tales genes en ambas especies de peces, que fueron posteriormente clonados y secuenciados. Las secuencias codificantes y las proteínas correspondientes se han comparado con las obtenidas en otras especies de peces. Actualmente se desarrollan sondas homólogas para cuantificar por métodos de biología molecular los mRNAs específicos en ambos peces, para su uso como biomarcadores moleculares de contaminación en el litoral suratlántico español.*

Palabras clave: *Biomonitorización, biomarcadores, contaminación, mRNA, cDNA, reacción en cadena de polímero, secuencias, litoral español.*

INTRODUCTION

Aquatic toxicology is an essential part of environmental toxicology, since water covers 70 % of the Earth (Timbrell, 1991; Sheehan, 1994). Some 11 000 chemicals –used in industry, mining or agriculture– are produced in sufficient amounts to pose environmental threats (Sheehan, 1994). Many are toxic, due to their interference with various vital processes (Timbrell, 1991). These exogenous compounds, or xenobiotics, finally reach aquatic ecosystems by natural processes or accidental or intentional release. Ecotoxicology uses the responses of sentinel organisms, called bioindicators, to assess the presence of pollutants (Livingstone, 1993; Timbrell, Draper and Waterfield, 1994; López-Barea, 1995). Organisms and populations alike respond to stressors by changing several parameters, known as biomarkers, at different organisational levels (Livingstone, 1993; López-Barea, 1995). Molecular biomarkers indicate the presence of xenobiotics and their biological effects. Unlike biomarkers at higher organisational levels, these respond very early, while the damages are still reversible. Thus, they are highly useful as early warnings, which make it possible to adopt corrective measures before organisms, communities or ecosystems suffer irreversible damages (López-Barea, 1995). Most xenobiotics are lipophilic, facilitating membrane passage, distribution and storage, although hindering elimination. Before excretion, xenobiotics should be biotransformed into water-soluble derivatives through biochemical reactions (Buhler and Williams, 1988; Timbrell, 1991). The first step in conversion, or phase I, is usually catalysed by mono-oxygenases, most of which catalyse oxidations. In phase II, their products are subsequently coupled to endogenous metabolites (Buhler and Williams, 1988; Timbrell, 1991).

To develop new molecular biomarkers of pollution, we focused on two biotransforming systems found in most organisms. Cytochrome P4501A1 oxidises organic pollutants (e.g. PAHs, PCBs, pesticides) and endogenous compounds (e.g. steroids and fatty acids). It is a mixed-function oxidase, using one O atom to oxygenate the xenobiotic, while another O atom is reduced to H_2O by NADPH. It is a huge microsomal protein, with 50 KDa and a home-group per subunit (Payne *et al.,* 1987; Nebert, Nelson and Feyereisen, 1989; Nebert, Petersen and Fornace, 1990; Goksøyr and Förlin, 1992). Metallothioneins are low molecular-weight proteins (6 KDa) which trap transition metals with their abundant Cys residues (Kagi and Kogima, 1987; Kagi and Schaffer, 1988; Kille *et al.,* 1992). These two systems are induced by specific pollutants: P4501A1 by aromatic compounds (Goksøyr and Förlin, 1992), and *MT* by transition metals (Kille *et al.*, 1992). Thus, they are useful as pollution biomarkers (Livingstone, 1993; Timbrell, Draper and Waterfield, 1994; López-Barea, 1995).

In induction, a chemical enhances expression of one or several genes, increasing mRNA levels, which once translated represent new protein(s), with the subsequent increase of enzymatic activity. Such a process makes it possible for several levels to quantify induction: i) measuring mRNA, ii) immunologically assessing protein, and iii) enzymatic activity assays. Both P450 1A1 and metallothioneins are well established as biomarkers at enzyme or protein levels (Goksøyr and Förlin, 1992; Kille *et al.,* 1992; Livingstone, 1993; Timbrell, Draper and Waterfield, 1994; López-Barea, 1995); nevertheless, denaturation or proteolisis complicates this quantification. Enzyme immunoassays provide an excellent alternative approach, since denatured proteins usually maintain their epitopes intact (Goksøyr and Förlin, 1992; Kille *et al.,* 1992); however, not every laboratory can obtain the anti-

bodies needed for ELISA. Quantification of specific mRNAs of these two genes could be a better alternative, probably more simple to perform than enzymatic assays or ELISA. Such a technique could also allow the analysis of induction at shorter times, thus being more useful as early warnings of pollution.

The present paper intends to specifically quantify, at mRNA level, the induction of gene coding for cytochrome P4501A1 and metallothionein, to compare the sensitivity of this method with ELISA quantification and catalytical assays. We cloned the cDNAs of *CYP* 1A1 and *MT* genes in two autochthonous fish from the South Atlantic Spanish littoral, the gilthead seabream, *Sparus aurata,* Linnaeus, 1758, and the grey mullet, *Liza aurata* (Risso, 1810). After cloning, they were sequenced to develop homologous probes for specific quantification of induction at mRNA level.

MATERIALS AND METHODS

Fish species and conditions for maintenance and exposure

As bioindicators of pollution, we selected two species of teleost fish typical of the Spanish South Atlantic littoral, *S. aurata* and *L. aurata,* using 200-g and 150-g specimens, respectively. Fish were maintained at 21 °C in tanks with 4 000 l of seawater and constant aeration at 300 % daily water renewal. Fish were fed twice daily (Dibaq-Diproteg, Madrid, Spain) at 0.5 % of the total fish weight per tank. For induction, fish were anaesthetised with quinaldine sulfate (10 mg/l in a 20-l tank) and intraperitoneally injected with Aroclor 1254 (1 g/kg) or $CdCl_2$ (0.5 mg/kg) for specific induction of P4501A1 or *MT*, respectively (Heilman *et al.,* 1988; Kille, Stephens and Kay, 1991). After 48 h, three fish from each species and treatment group were netted, and their spinal cords were sectioned; then their livers were removed, washed in 0.9 % saline solution, frozen into liquid N_2 ground in a cold mortar, and frozen at –80 °C until they were used for the corresponding experiments.

As a positive control group in the initial experiments, the rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), was also used, since the gene coding for its P4501A1 and *MT* had already been cloned and sequenced (Heilman *et al.,* 1988; Kille, Stephens and Kay, 1991). *O. mykiss* weight was also 200 g, and the fish were maintained at 15 °C as described above, in fresh water. The *O. mykiss* group was fed once a day with the same feed at the same portions used for the other groups, and induced with Aroclor 1254 (0.5 g/kg) or $CdCl_2$(0.5 mg/kg).

Total RNA isolation

RNA was isolated from liver samples using the Tri Reagent-RNA, DNA and Protein Isolation Kit (Molecular Research Center, Cincinnati, OH, USA), starting from 100 mg of frozen liver samples and following the steps described in the manufacturer's protocol. Final DNA samples were resuspended in DEPC-treated water at 2-5 mg/ml final concentrations; its purity was spectrophotometrically determined at 260 and 280 nm (Sambrook, Fritsch and Maniatis, 1989).

Oligonucleotide design

Oligonucleotides for the different types of PCR were designed with the programs Oligo 4.05 (National Biosciences, Plymouth, Minnesota, USA) and PrimerSelect 1.0 (DNAStar, Madison, Wisconsin, USA). Oligos were synthesised by Operon (Alameda, California, USA) or Biosource International (Camarillo, California, USA). Figure 1 shows the degenerate oligos (MetX1-MetX2 and MetX3-MetX4) designed for initial amplification of portions of cDNA specific for *MT* in *S. aurata* and *L. aurata*, the degeneration code being shown in the lower part of the figure. Figure 2 shows the degenerate oligos designed for the initial amplification of sections of cDNA specific for P4501A1 in both fish species (P450x1-P450x2 and P450x3-P450x4). Figures 3 and 4 show the specific oligos designed for amplification by Race-PCR of the complete cDNAs coding for *MT* and P4501A1, respectively, in *S. aurata* and *L. aurata.*

cDNA synthesis, RT-PCR, and partial DNA sequencing

The cDNA synthesis was carried out with the GeneAmp RNA PCR Kit (Perkin Elmer, Norwalk,

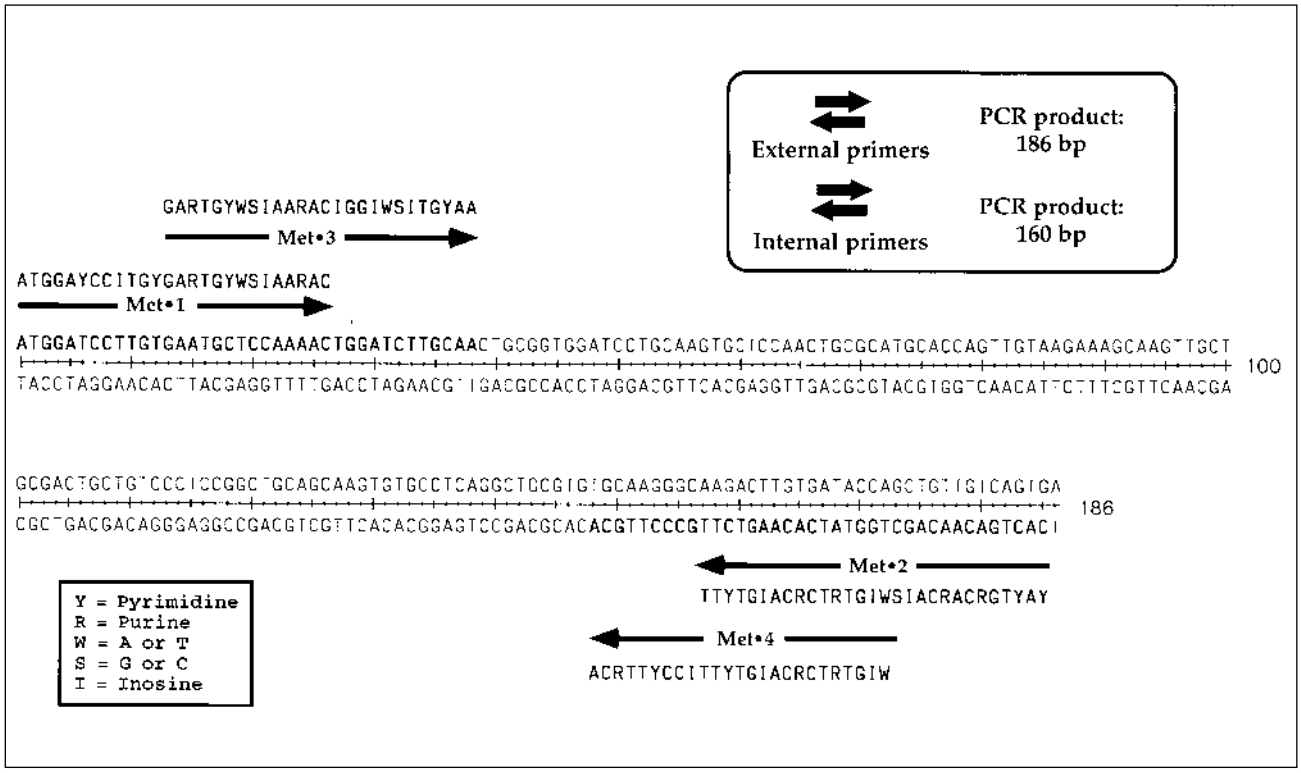


Figure 1. Degenerate primers for initial amplification of specific metallothionein cDNAs in *S. aurata* and *L. aurata*

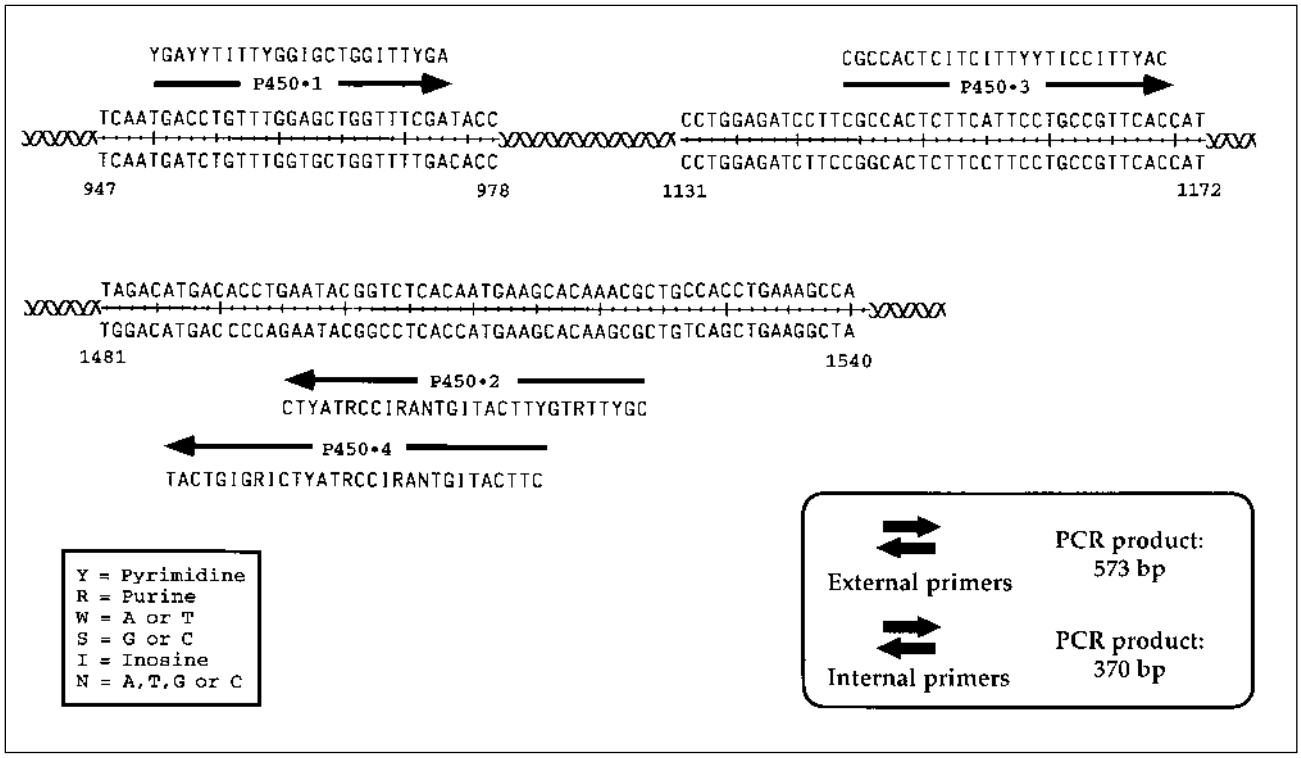


Figure 2. Degenerate primers for initial amplification of specific *CYP* 1A1 cDNAs in *S. aurata* and *L. aurata*

Connecticut, USA) using random hexanucleotides as primers for the retrotranscriptase reaction. Figure 5 summarises this process (see Results and Discussion for further details). The PCR was car-

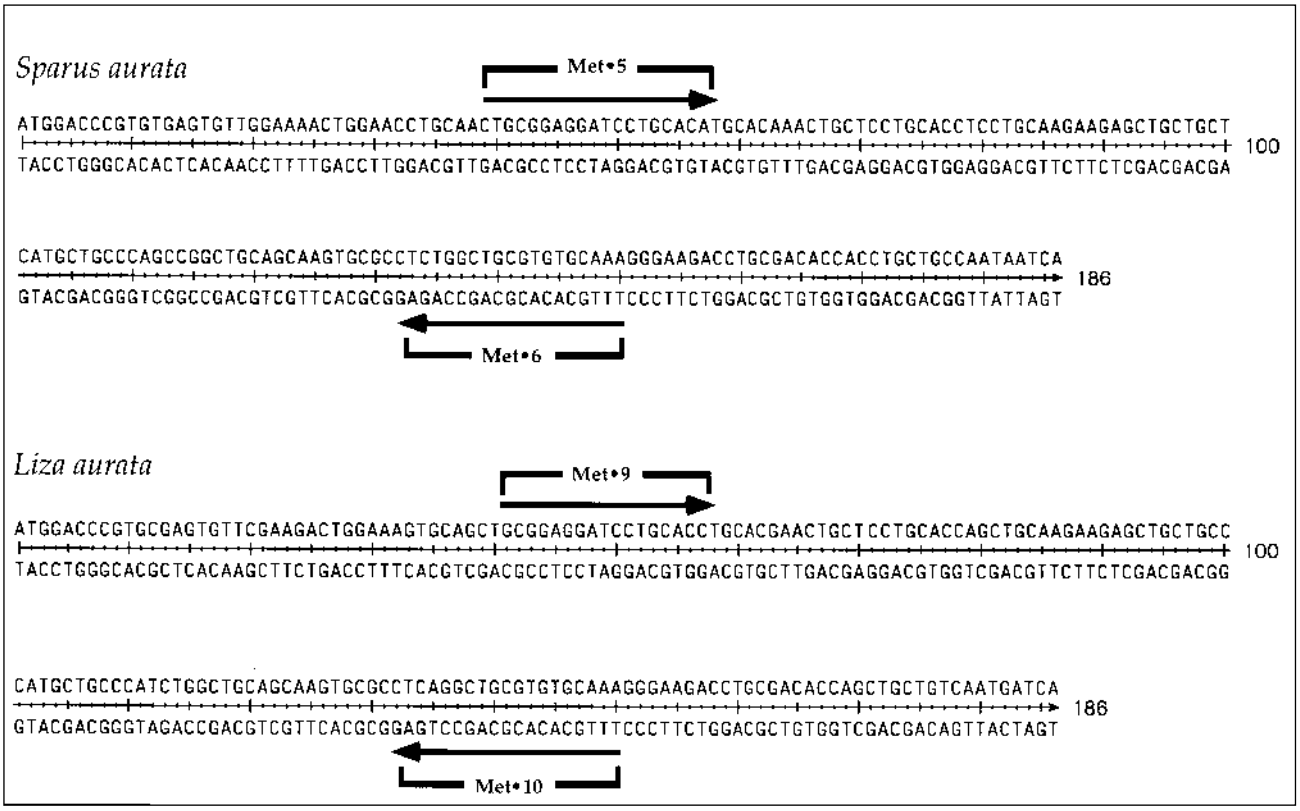


Figure 3. Specific primers for amplification by Race-PCR of the complete cDNAs coding for metallothionein in *S. aurata* and *L. aurata*

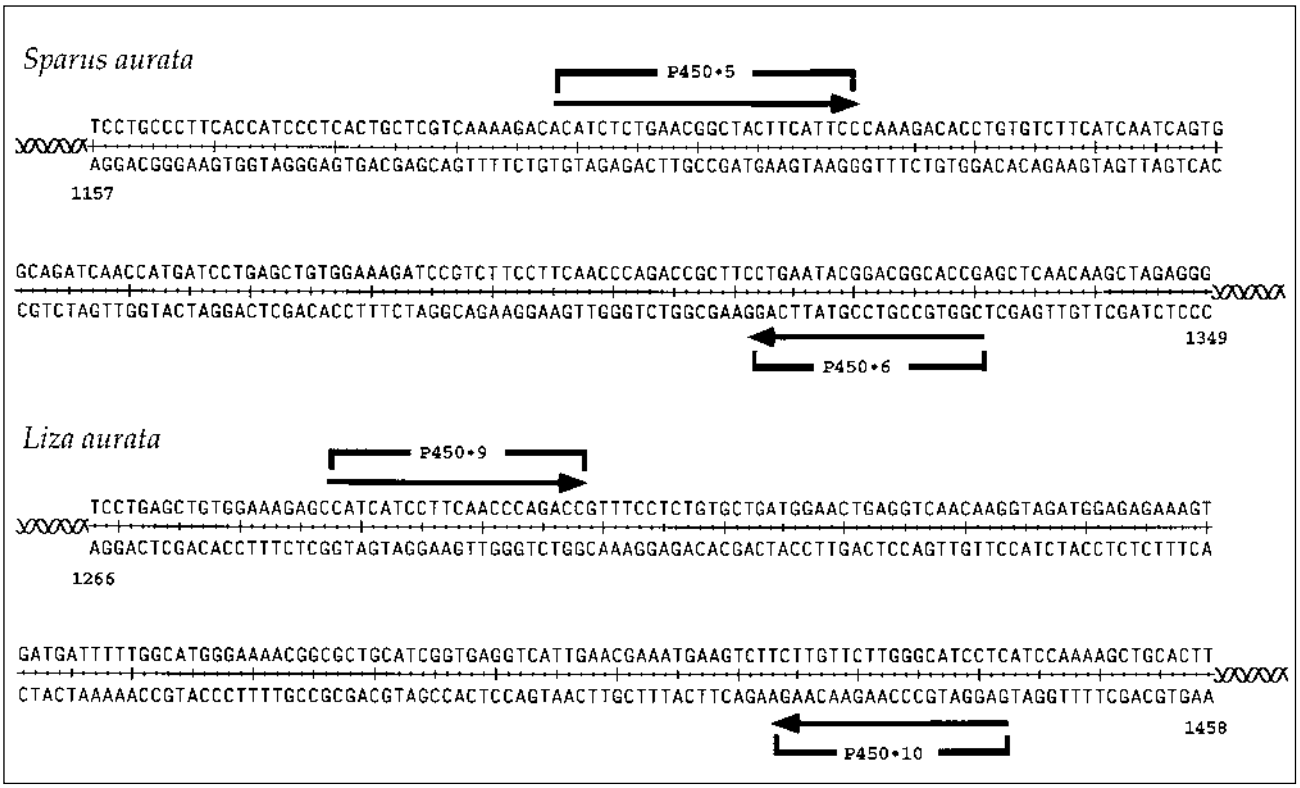


Figure 4. Specific primers for amplification by Race-PCR of the complete cDNAs coding for P4501A1 in *S. aurata* and *L. aurata*

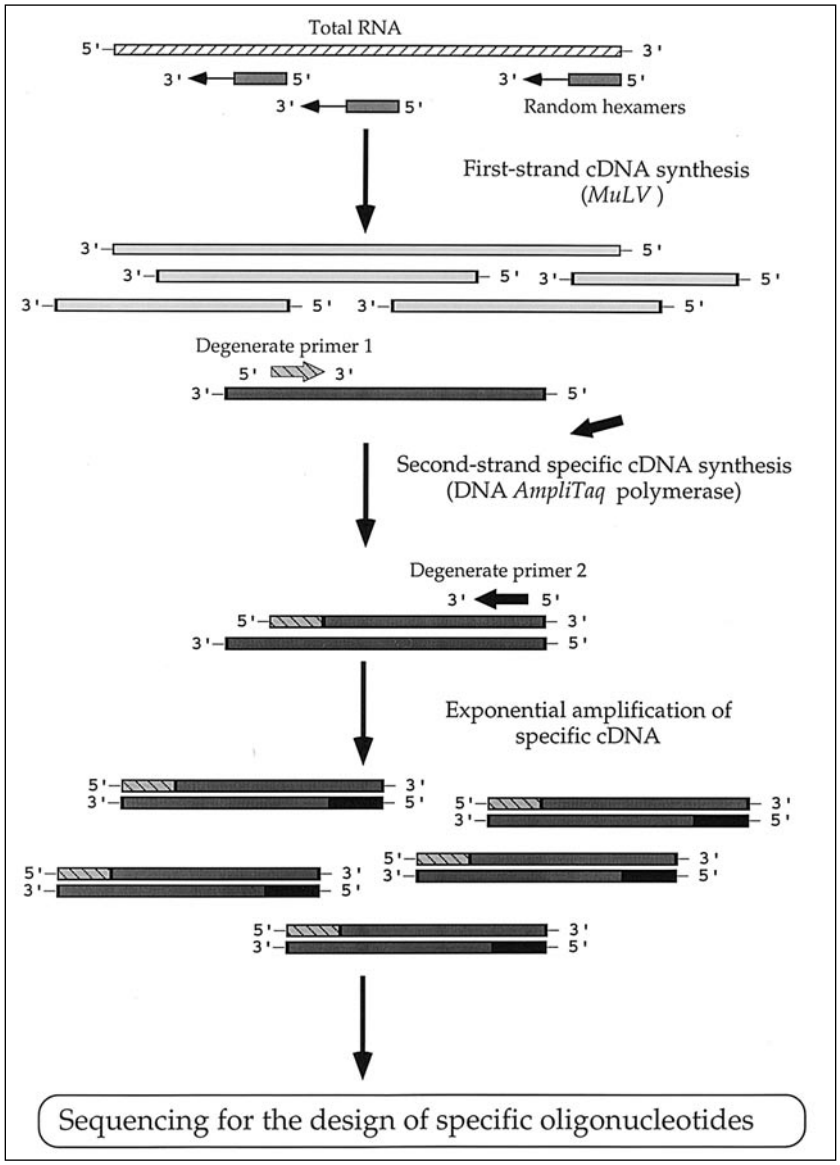


Figure 5. RT-PCR technique

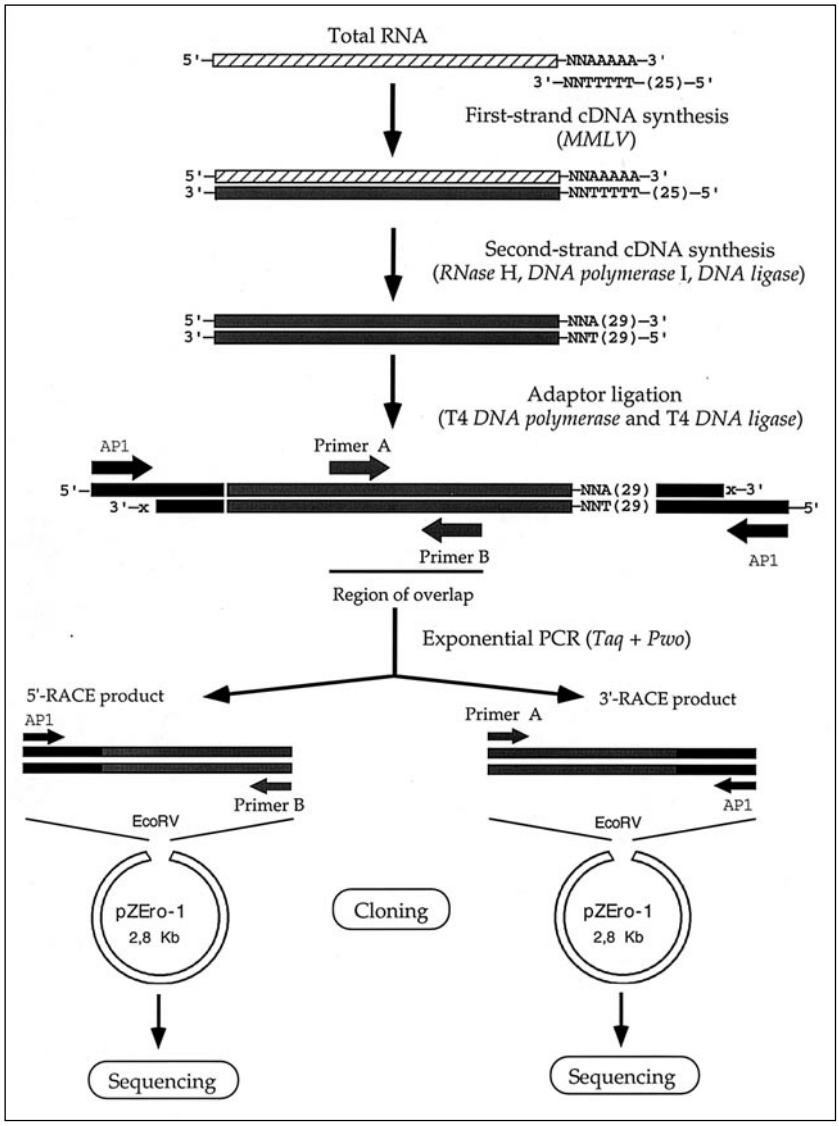
ried out in a Perkin Elmer Thermal Cycler (mod. 480). First, 30 asymmetric PCR cycles were carried out using the 5'-primer. Once the target sequence had thus been asymmetrically amplified, 35 additional cycles were performed after adding the 3'-primer. This second symmetrical PCR exponentially amplified the target sequence generated previously. These amplifications were carried out using degenerate oligos designed from previously published and highly conserved fish sequences for *CYP* 1A1 and *MT* cDNAs.

The amplicons obtained in this initial PCR were directly sequenced with the dideoxy procedure of Sanger, Nicklen and Coulson (1977), using the ABIPrism Dye Terminator Cycle Sequencing Ready Reaction Kit in the ABIPrism 373 Stretch Laser-induced Fluorescence automatic sequencer, from Perkin Elmer/Applied Biosystems (Foster City, California, USA). Both the direct and the reverse strands of cDNA were sequenced.

Synthesis of the complete cDNA (Race-PCR) and cloning

For complete cDNA isolation, we followed the protocol of the Marathon cDNA Amplification Kit (Clontech, Palo Alto, California, USA) as described by Chenchick, Moqadam and Siebert (1995), using specific internal oligos. Figure 6 summarises this process (see Results and Discussion for further details).

Figure 6. Race-PCR strategy



The 5' and 3' ends of cDNA form both *CYP 1A1* and *MT* genes were cloned following the protocol of the Zero Background Cloning Kit (Invitrogen, Carlsbad, California, USA) as described by Bernard *et al.* (1994). The cloning vector pZEro-1 contains the *ccdB* gene fused at the carboxyl end of the protein *lacZa*, whose expression is lethal for *E. coli*. The insertion within the vector polylinker of a DNA fragment alters the correct expression of the *lacZa–ccdB* fusion protein, thus allowing only the growth of the positive recombinants.

The direct and reverse sequencing of these clones enabled us to obtain complete sequences of both *CYP* 1A1 and *MT* cDNAs from two teleost fish species characteristic of the Spanish South Atlantic littoral, *S. aurata* and *L. aurata*, initially selected as bioindicators of environmental pollution.

RESULTS AND DISCUSSION

Oligonucleotide design and amplification of the complete cDNAs coding for cytochrome P4501A1 and metallothioneins

The high homology found in the sequences of *CYP* 1A1 and *MT* genes cloned in the fish species already studied was used to design degenerated oligos in order to amplify (by RT-PCR) cDNA sequences specific for such genes in the two species studied. They were subsequently used to design fully homologous oligos for amplifying the complete cDNAs. The degenerate metallothionein oligos were designed starting from the sequence described for the *O. mykiss MT-A* cDNA (Kille *et al.*, 1992), focusing on those fragments near its 5' and

3' ends having high homology with other teleost fish in which it had been previously cloned, e.g. *Noemacheilus barbatulus*, *Esox lucius* and *Platichthys flesus* (Kille, Stephens and Kay, 1991). As shown in figure 1, two external degenerate oligos (Met1-Met2) were designed to amplify a 186-bp PCR product, including most of the *MT*-coding region. Two nested oligos (Met×3-Met×4) were also prepared for a second PCR of the initial products. Their main goal was to increase the probability of successful amplification. In other words, the first PCR sometimes generates no specific product, which is revealed only after the second nested PCR. Additionally, the generation of such a 160 bp product would further confirm that the amplicon included sequences specific for the *MT* gene. Cytochrome P4501A1 oligos were prepared based on the sequence already published of the *CYP* 1A1 cDNAs from *O. mykiss* (Heilman *et al.,* 1988) and plaice (Leaver, Pirrit and George, 1993). As shown in figure 2, the oligos were designed at the 3' portion of the coding region, highly conserved in the organisms where the *CYP* 1A1 cDNA had been sequenced, since this region codes for the heme-binding region of the apoprotein. The two external degenerate oligos (P450×1-P450×2) would amplify a 573 bp product, and a 370 bp product the secondary PCR using the nested degenerate oligos (P450×3-P450×4).

Figure 5 summarises the strategy used for the initial amplification by RT-PCR of some specific sequences for *MT* and *CYP* 1A1 cDNAs. Starting from total RNA, the first cDNA chain was generated by using murine leukemia virus retrotranscriptase and random hexanucleotides as primers. Several copies of the second cDNA chain were generated subsequently in a first asymmetric (arithmetic) PCR, using the degenerate 5' external oligo complementary of the 3' –end of the desired sequence in the presence of AmpliTaq DNA polymerase. Exponential cDNA amplification was finally obtained upon addition of the other 3' degenerate oligo (i.e. symmetric PCR). The products of these initial PCRs were subsequently reamplified with the corresponding nested primer couples, to increase specificity and additionally confirm the identity of the amplified sequences. After agarose gel electrophoresis, DNA bands of the expected sizes were always observed. These products were subsequently sequenced and, as shown in figures 3 and 4, they contained sequences clearly homologous to the *O. mykiss CYP* 1A1 and *MT* cDNAs, yet different between them and with respect to those of *O. mykiss*, used as the positive control (Heilman *et al.*, 1988; Kille, Stephens and Kay, 1991).

Starting from those specific sequences, new specific primers were designed to amplify, with Race-PCR (Rapid Amplification of cDNA Ends), the complete cDNAs from both *S. aurata* and *L. aurata*. Figure 3 shows that the internal sequences of the *MT* cDNAs made it possible to design two homologous primer couples to obtain the complete cDNA of *MT* gene in *S. aurata* (MetX5-MetX6) and *L. aurata* (MetX9-MetX10). In close parallelism, figure 4 shows the design of two additional homologous primer couples to amplify the complete *CYP* 1A1 cDNA of *S. aurata* (P450×5-P450×6) and *L. aurata* (P450×9-P450×10).

The complete cDNA of any gene can be obtained by Race-PCR (Chenchick, Maqadam and Siebert, 1995), provided that a short internal sequence is known; figure 6 summarises the strategy used. Starting from total RNA, a complete cDNA library was prepared with Molowney virus retrotranscriptase, using as first DNA chain primer a poly-T oligo complementary of the poly-A tail present in most eukaryotic mRNAs. The second DNA chain was obtained upon addition of RNAse H, which nicked the RNA chain in the RNA-DNA hybrid yielding small fragments with 3'-OH ends ready to be used as primers for cDNA synthesis by the DNA polymerase I, which may degrade the fragments found ahead with its 5'm3' exonuclease activity. Finally, DNA ligase sealed any remaining nicks in the new cDNA chain. Once obtained the double stranded (ds) cDNAs, T4 DNA polymerase generated blunt ends. T4 DNA ligase was then used to attach special Marathon cDNA adaptors on both cDNA ends. This effectively generated an adaptor-linked cDNA population. Such a population was essentially a library of uncloned ds cDNAs. Different cDNAs could be amplified from such a library, using the appropriate homologous internal primers. The specificity of the Marathon Race reaction is greatly enhanced by the lack of an AP1 on the adaptor-ligated cDNA library. This site is created only on the cDNA of interest after the extension from the internal gene-specific primer during the first Race cycle. Additionally, the amino group on the Marathon cDNA adaptor efficiently blocks any extension of the 3' end of the adaptor-ligated ds cDNA library. The result is a more efficient and

highly specific Race amplification of the desired product instead of the full cDNA population (PCR (Chenchick, Maqadam and Siebert, 1995). Therefore, this strategy generates less background amplification than the standard Race approach.

As shown in figure 6, PCR amplification was carried out with a mixture of DNA polymerase (Taq and Pwo) that generates long PCR products with high fidelity. Using one internal homologous oligo and AP1, one of the cDNA region (e.g. 5') was amplified. Using the other internal homologous oligo and AP1, the other region (e.g. 3') of cDNA was obtained. Two such products may overlap and could be fused together, generating the complete cDNA, or else being independently cloned and/or sequenced. In our case, the 5' and 3' products of each cDNA were amplified with *Ultma* DNA polymerase and cloned in the strain Top10F' of *E. coli* using the vector pZEro-1 digested with *Eco*R V. Five clones, carrying an insert of appropriate size, were sequenced independently to obtain the full length of both *MT* and *CYP* 1A1 cDNAs, both in *S. aurata* and *L. aurata*.

Full-length sequences of *MT* and *CYP* 1A1 cDNAs in *S. aurata* and *L. aurata*

Figure 7 shows the complete cDNA sequence of metallothionein gene in both teleost fish studied, *S. aurata* and *L. aurata*, as well as their deduced amino acid sequences. In *S. aurata*, metallothionein cDNA contains 416 nucleotide pairs with an untranslated 3' end and a poly-A tail. An open-reading frame extends from position 58 to nucleotide 240 (183 bp), which encodes a protein with 60 amino acids and 20 cysteine residues, thus corresponding to 33 % of the total amino acid content. In *L. aurata*, *MT* cDNA contains 412 bp, with an open-reading frame extending from nucleotide 50 to 233, encoding another 60 amino acids *MT*. Figure 8 shows the alignment of cDNA and protein sequences in *S. aurata* and *L. aurata*, compared with those of *O. mykiss* (Kille, Stephens and Kay, 1991). On the nucleotide sequence, the newly isolated cDNAs show 91 % homology, compared to a 77 % homology with *O. mykiss MT* cDNA. The proteins encoded by both *S. aurata* and *L. aurata MT*

Gilthead seabream (*Sparus aurata*)

```
ctgagactgaagacgcaatcacatctcacgaagagaaacctcgacatcacctgaaaaATGGACCCTTGCGAGTGCTCTAAGACTGGAACC   90
                                                          Met Asp Pro Cys Glu Cys Ser Lys Thr Gly Thr

TGCAACTGCGGAGGATCCTGCACATGCACAAACTGCTCCTGCACCTCCTGCAAGAAGAGCTGCTGCTCATGCTGCCCAGCCGGCTGCAGC  180
Cys Asn Cys Gly Gly Ser Cys Thr Cys Thr Asn Cys Ser Cys Thr Ser Cys Lys Lys Ser Cys Cys Ser Cys Cys Pro Ala Gly Cys Ser

AAGTGCGCCTCTGGCTGCGTGTGCAAAGGGAAGACGTGCGACACTAGCTGCTGCCAGTGAggagtctgcaacatcagctgcttgctgccc  270
Lys Cys Ala Ser Gly Cys Val Cys Lys Gly Lys Thr Cys Asp Thr Ser Cys Cys Gln  •

ttgtgatggcgcctgtgtgtgacccatttaacatctattgcagtagcaaatgtccagagaagtctattttgtacctgtcttcagtgttga  360

aataaacatgtttccttaaagcagaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  416
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Grey mullet (*Liza aurata*)

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cggacgaatcagcttctaactcaacgaacgaacaagccatcatctgaaaaATGGACCCTTGCGAGTGCTCCAAGACTGGAAAGTGCAGCT   90
                                                          Met Asp Pro Cys Glu Cys Ser Lys Thr Gly Lys Cys Ser

GCGGAGGATCCTGCACCTGCACGAACTGCTCCTGCACCAGCTGCAAGAAGAGCTGCTGCCCATGCTGCCCATCTGGCTGCAGCAAGTGCG  180
Cys Gly Gly Ser Cys Thr Cys Thr Asn Cys Ser Cys Thr Ser Cys Lys Lys Ser Cys Cys Pro Cys Cys Pro Ser Gly Cys Ser Lys Cys

CCTCAGGCTGCGTGTGCAAAGGGAAGACATGCGACACCAGCTGCTGTCAGTGAggatccgaggatccagtagcaccggcgcccttctgac  270
Ala Ser Gly Cys Val Cys Lys Gly Lys Thr Cys Asp Thr Ser Cys Cys Gln  •

cttgtgatggagcccgtgcgctacgctgaataaaaattgcagatgttagagtagtccattttctacattgcttttgtgttgaaataaaaa  360

tgtgttggcatgggaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  412
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Figure 7. Sequence of complete cDNA and translated peptide of metallothionein in *S. aurata* and *L. aurata*

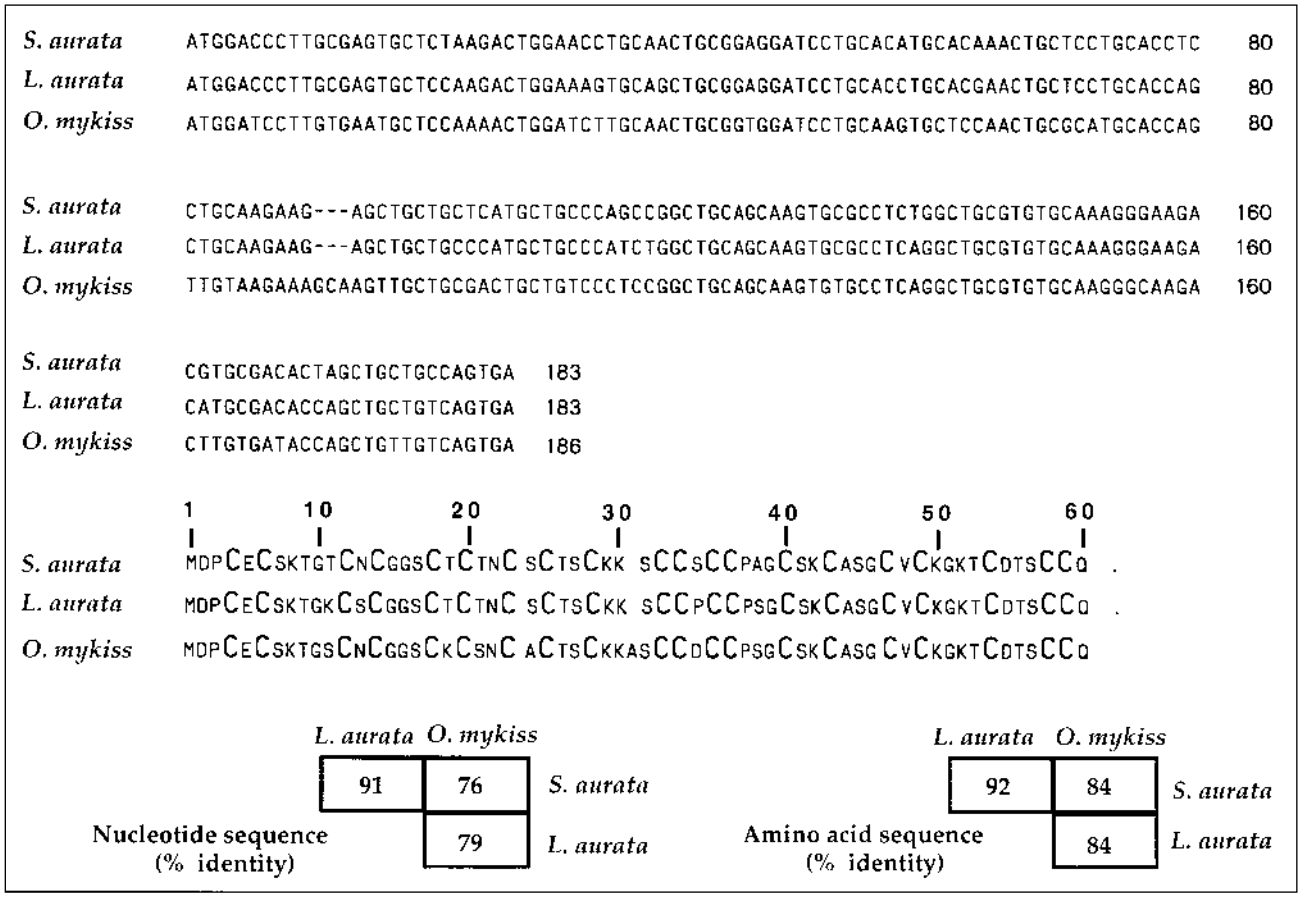

Figure 8. Alignment of *S. aurata*, *L. aurata* and *O. mykiss* metallothionein nucleotide and amino acid sequences. The percentages of nucleotide and amino acid sequence identities are also shown

cDNAs are 60 amino acids long with the same 20 cysteine residues fully conserved, as shown in the lower part of figure 8. Protein *MT* sequences from both fish show 92 % homology, compared with 84 % homology with their *O. mykiss* counterpart. *S. aurata* and *L. aurata MT*s both lack the Ala-31 residue, characteristic of *O. mykiss metA* gene product, but absent in all other fish metallothionein cDNAs sequenced to date (Kille, Stephens and Kay, 1991; Kille *et al.,* 1992).

Figure 9 shows the protein sequence of both cytochromes P4501A1 from *S. aurata* and *L. aurata*, superimposed on that of the *O. mykiss* counterpart (Heilman *et al.,* 1988); the nucleotide sequences are not shown due to their extreme length. Nevertheless, the *S. aurata CYP* 1A1 cDNA is 2 095 nucleotides long and displays an open-reading frame extending from nucleotide 131 to position 1 696 (1 566 bp). The *CYP* 1A1 cDNA from *L. aurata* is significantly longer, with 2 519 base pairs and an open-reading frame extending from position 114 to 1 679 (again 1 566 bp). The higher length of *L. aurata CYP* 1A1 cDNA is due to the much longer sequence existing between the termination codon and the poly A tail. figure 9 also summarises the homologies, both for nucleotides and the protein sequences, between the two teleost fish species studied and *O. mykiss.* At the nucleotide level, the *L. aurata CYP* 1A1 cDNA shows 83 % homology with that of *S. aurata*, but only 75 % homology when compared with *O. mykiss* cDNA. Cytochromes P4501A1 from both *S. aurata* and *L. aurata* are 522 amino acids long and show 85 % homology between them, compared with a homology of only 81 % with their *O. mykiss* protein counterpart. Figure 9 indicates, with different grey intensities, the degree of homology in different regions of the P4501A1 protein. Maximum homology is observed between amino acids 380 and 438, which correspond to the highly conserved region that was used initially for designing the degenerate primers for the initial amplification of specific *CYP* 1A1 sequences.

In conclusion, by using known sequences of gene encoding for metallothionein and cytochro-

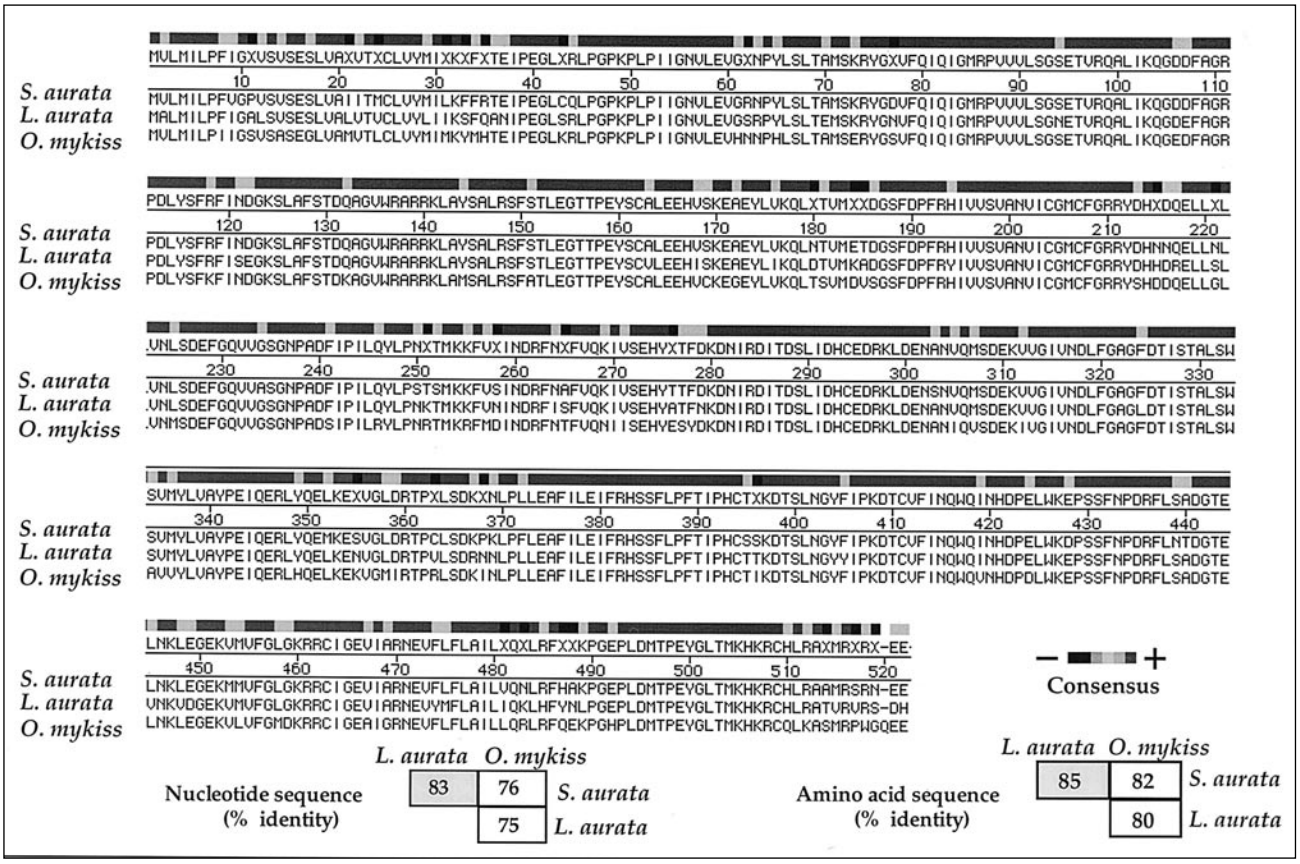

Figure 9. Alignment of *S. aurata*, *L. aurata* and *O. mykiss* P4501A1 amino acid sequences. The percentages of nucleotide and amino acid sequence identities are also shown

me P4501A1 in different fish species (Kille, Stephens and Kay, 1991; Kille *et al.,* 1992; Heilman *et al.,* 1988; Leaver, Pirrit and George, 1993), we have succeeded, with complex PCR strategies, in obtaining full-length cDNAs for both the *MT* and the *CYP* 1A1 genes in both *S. aurata* and *L. aurata*. Such sequences will be subsequently used to validate the quantification of the specific mRNAs as early-warning biomarkers for environmental pollution.

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