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# Screening of Differentially Expressed Genes in Diesel Oil-exposed Marine Fish Using DD-PCR

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## Abstract

**The exploration of genes which expressions are changed by exposure to ecotoxicants or pollutants can provide the important information about the reaction mechanisms in the body as well as adaptation to exterior stimulus or environmental changes. Also they can be developed as biomarkers for the detection of environmental pollution. Differential display polymerase chain reaction (DD-PCR) technique has been usefully used to hunt the clones which expressions are up-regulated or down-regulated by exterior changes and this study aimed to search for those clones in diesel oil-exposed rockfish (*Sebastes schlegeli*) using DD-PCR. The RNA isolated from liver of 20 ppb diesel oil-exposed rockfish was used for screening of the differentially displayed genes and total 44 differentially expressed genes (DEG) are detected then their nucleotide sequences were analyzed. The present data provided the general information about the effect of diesel oil contamination on marine organism and further more the primary step in development of new biomarkers for marine environmental pollution or ecotoxicological stresses.**

**Keywords:** *Sebastes schlegeli*, Diesel, Differential gene expression, Biomarker

The representative environmental stress in marine ecosystem is anthropogenic contamination such as sewage containing persistent organic pollutants and a variety of toxic chemicals from land runoff. In coastal area extrinsic endocrine disrupting materials such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides were widely distributed. According to Koh *et al.* (2001)[1], the concentration of chlorinated compounds such as PCBs and dichlorodiphenyl trichloroethanes (DDTs) among the organic pollutants in Korean coastal environment was relatively lower than those in other developed countries. However, the record on PAHs which are toxic compounds and derived from oil spills by ship accident or oil-contaminated soil showed a higher concentration in Korean coastal regions. DNA damage induced at early life stage of an organism inhibits the development to adult, which might cause disturbance of ecosystem along the food chain as well as serious economic loss in fisheries. In addition, the extinction of a species in marine ecosystem may affect its community. Therefore, it is considerably necessary to detect the biological effects of pollutant on marine organisms under low level of contamination as well as the health status of marine ecosystem.

Korean rockfish, *Sebastes schlegeli*, distribute in shallow coastal area of Korea and Japan. In Korea, this species is one of the typical cultural fishes so it is important both in market and table. In this study we choose the rockfish because of its economic value as food resource and considerable easiness on handling and obtainment as well.

Differential display PCR (DD-PCR) is a technique in which mRNA expressed by a cell population are reverse transcribed and then amplified by many separate polymerase chain reactions[2]. Arbitrary PCR primers and conditions are chosen so that any given reaction yields a limited number of amplified cDNA fragments, permitting their visualization following gel electrophoresis. The relatively simple procedure allows identification of genes that are differentially expressed in different cell populations. In this study, we aimed to explore the ecotoxicant-responsive genes in rockfish, *Sebastes schlegeli*, which is a relatively abundant representative coastal species in the South Sea, using DD-PCR technique and to obtain the molecular-level information investigating the changes of gene expressions in fish affected by exposure to diesel oil.

## Screening of Differentially Expressed Genes

DD-PCR has been developed to identify and isolate differentially expressed genes[2] and extensively applied to various range of differential gene expression analyses because of its effectiveness and convenience. One of the merits of this technique is requirement of

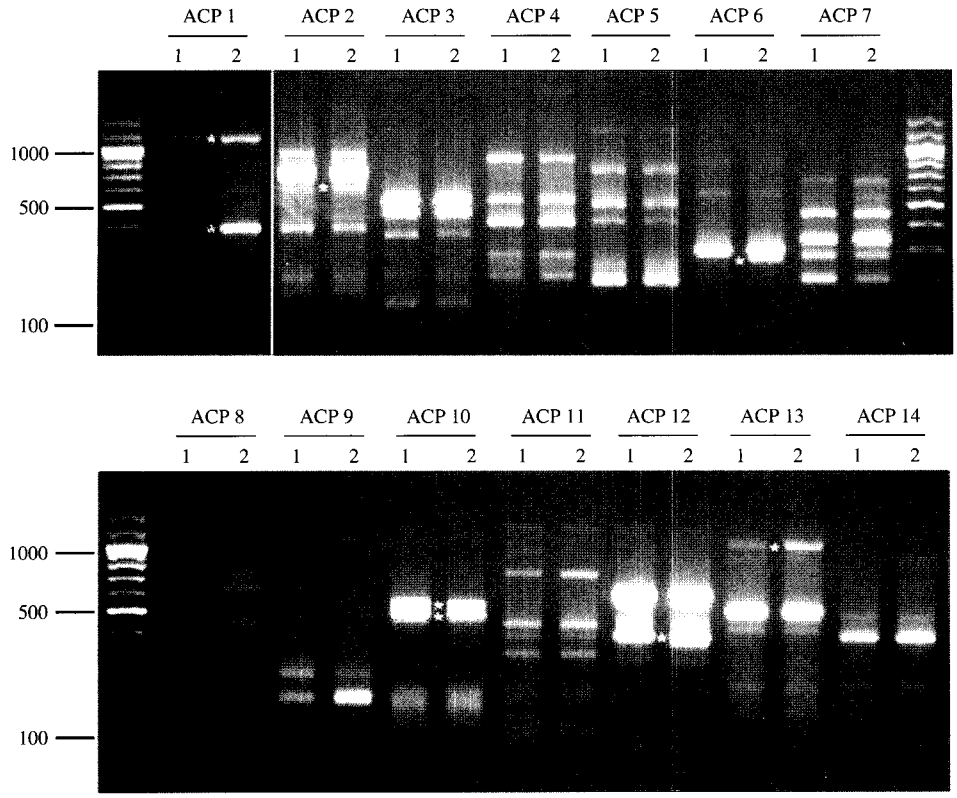


**Fig. 1.** Differential display of mRNA from *Sebastes schlegeli* exposed to diesel oil (primer set 1-14). M, DNA ladder marker; 1, non-exposed control fish; 2, diesel oil-exposed fish. Arrow and number indicate the differentially expressed genes.

only small amount of RNA since this technique is PCR-based. However, relatively high chance to have false-positive acts as a major handicap in this technique. Many efforts have been attempted to improve the specificity of DD-PCR[3,4]. Recently epochal modification to eliminate false-positive has been developed by increasing the annealing specificity with specially designed annealing control primer (ACP) system[5,6]. In this study, we applied the ACP system to identify the differentially expressed genes responding to the chemical contaminant such as diesel oil (Figs. 1, 2). The GeneFishing kit, a commercial brand based on ACP system, has two additional merits, which derived from the high specificity of primers. First is, PCR products could be separated on agarose gel because the improved specificity and sensitivity result only a few amplified product. Second is, no usage of radio isotope. Non-radioactive detection of the products increases convenience and facilities.

This study aimed to discover the genes which transcriptions were changed by the environmental toxicant and to develop a simple and efficient system such as cDNA chip that could be used to assess the contamination at ecotoxicant-impacted sites. As its first step, we obtained 44 fragments whose expressions altered by diesel oil exposure comparing with those of unexposed control using DD-PCR technique (Table 1).

## Differentially Expressed Genes by Exposure to Diesel Oil

Clone SS36-400, which nucleotide sequence showed homology to betaine homocysteine methyltransferase in *Mus musculus* (DDBJ/EMBL/GenBank accession No. BC013515) was repressed at 40 hr post-exposure compared with non-exposed control. According to the recent report, betaine homocysteine methyltransferase (BHMT) was identified as a gene down-regulated in rat liver cirrhosis and most likely involved in dysregulation of homocystein level[7,8]. It has been known that homocysteine levels are elevated in liver cirrhosis and hepatic encephalopathy incorporates neuropsychiatric abnormalities with liver dysfunction. Also it has been focused upon the molecular mechanisms involved in the pathogenesis of hepatic encephalopathy with liver dysfunction incorporating with BHMT and therapy of hepatic encephalopathy to reduce ammonia generation and increase its detoxification[8].

Clone SS16-320 was identified homologous to *Oryzias latipes* warm-temperature-acclimation-related 65 kDa protein-like-protein (AB075199). In similar study using DD-PCR method to identify hepatic

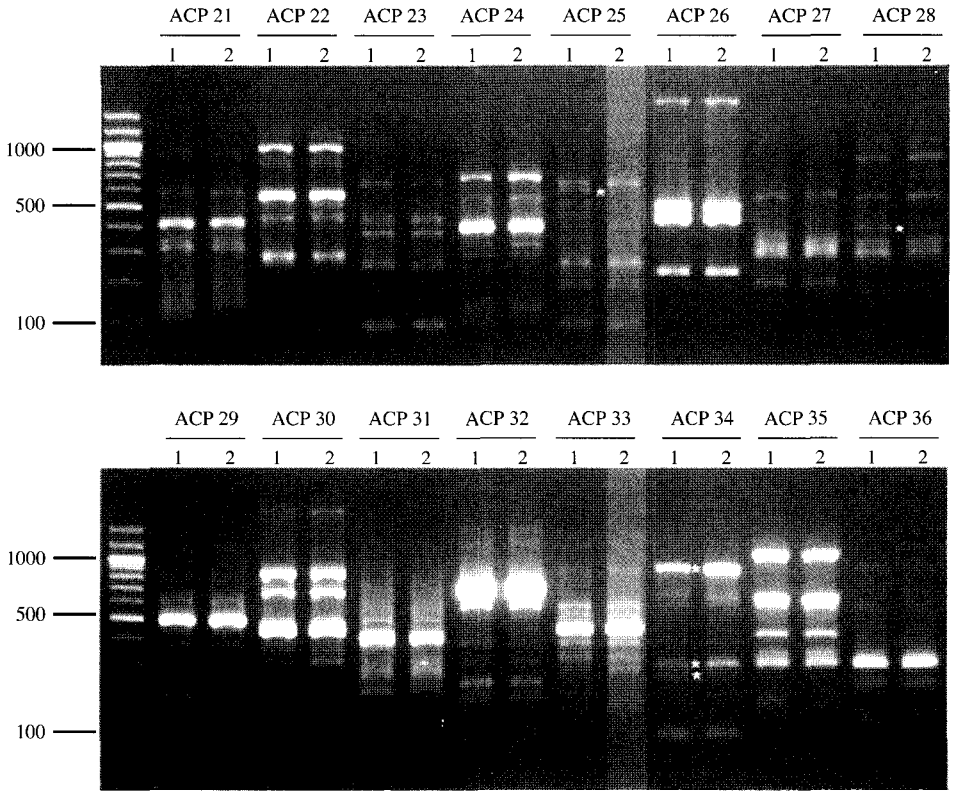


**Fig. 2.** Differential display of mRNA from *Sebastes schlegeli* exposed to diesel oil (primer set 21-36). 1, non-exposed control fish; 2, diesel oil-exposed fish. Arrow and number indicate the differentially expressed genes.

genes responsive to thermal stress in *Fundulus heteroclitus*, genes that differed in expression between fish populations from different thermal environments were discovered and they showed high homology to genes of known function such as glucokinase, serine-threonine kinase 10, glycogen synthase kinase, and warm acclimation-related protein (WAP)[9]. Those genes significantly changed in expression between populations from different thermal environments and especially expression of WAP was eight-fold higher in Southern than in Northern fish, consistent with a previously suggested role for this gene in thermal acclimation or adaptation to extrinsic stresses in fish[9]. In this study, a fragment showing homology to WAP exhibited similar expression pattern by ecotoxicant exposure without thermal change. This result could suggest that exposure to ecotoxicant might cause various damages in the exposed organisms such as incomplete transcription, cellular dysfunction, or metabolic imbalance like as the moment an organism was exposed to chilling or heating stress.

Another clone, SS34-500 showing higher expression in 40 hr diesel exposure, had homology to *Holothuria glaberrima* serum amyloid A protein (AF300706). Serum amyloid A is known as a superfamily of acute-phase proteins. The level of serum amyloid A in blood increases dramatically in response to tissue injury and inflammation, influencing cell adhesion, migration, proliferation and aggregation[10]. In human, serum amyloid A was reported as the precursor to the amyloid A protein found in deposits of reactive amyloid and the amyloid deposition might contribute to the pathogenesis such as Alzheimer's disease[11]. In the independent studies on two heterochonch bivalves (*Mya arenaria, Mactromeris polynyma*) and oyster (*Crassostrea virginica*) exposed to PAHs and high level of organochlorine compound, respectively, phagocytosis by hemocytes in both heterochonch bivalves was significantly suppressed[12]. And the results deduced from *C. virginica* exposed to organochlorine compound suggested that aquatic contaminant could interact rapidly with hemocytes to produce a partially reversible immunotoxicological lesion[13].

The last clone named SS78-300, which level of expression was up-regulated after diesel exposure, showed high similarity to *Dicentrarchus labrax* cytochrome P450 1A (U78316). CYP1 gene family is efficient metabolizers of PAHs and was induced in fish by various PAHs[14]. This enzyme is involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics[15]. CYP1A protein was found to be significantly

**Table 1.** Differentially expressed genes in diesel-exposed *Sebastes schlegeli* identified by DD-PCR.

| Clone | Size (bp) | Putative identity[a] | DD-PCR[b] |
|---|---|---|---|
| SS1-1k | 645 | Gelsolin gene | Up |
| SS1-400 | 393 | Fucolectin-1 | Up |
| SS2-600 | 395 | A stretch regulated skeletal muscle protein | Up |
| SS4-500 | 367 | Piscicida partial coi genes for putative cytochrome C oxidase proteins | Up |
| SS10-500 | 474 | Alpha-crystallin-related protein | Down |
| SS10-400 | 342 | MHC class II antigen gene cluster | Down |
| SS12-800 | 472 | Allatotropin neuropeptide precursor | Up |
| SS13-900 | 749 | Alpha-interferon inducible protein | Up |
| SS14-380 | 309 | Penicillin G acylase precursor | Up |
| SS15-550 | 346 | Ceruloplasmin | Up |
| SS15-380 | 353 | Fructose-1,6-bisphosphate aldolase | Up |
| SS16-320 | 316 | Warm-temperature-acclimation-related-65 kDa-protein-like-protein | Up |
| SS17-800 | 616 | BCL2/adenovirus E1B 19 kDa-interacting protein | Up |
| SS25-800 | 516 | Glycine max beta-carotene hydroxylase | Down |
| SS28-400 | 213 | Translation initiation factor eIF-2B beta subunit gene | Down |
| SS34-500 | 411 | Serum amyloid A protein | Up |
| SS34-800 | 622 | Polyglutamine binding protein variant 4 (PQBP1 gene) | Up |
| SS34-300 | 206 | NAD(P)H dehydrogenase quinone 1 mRNA | Up |
| SS36-400 | 209 | Betaine-homocysteine methyltransferase | Down |
| SS37-300 | 214 | Complement component C3 | Up |
| SS38-600 | 522 | Putative transferrin | Up |
| SS39-500 | 324 | Epidermal lipoxygenase; lipoxygenase-3 | Up |
| SS40-800 | 605 | Arachidonate 5-lipoxygenase | Up |
| SS40-500 | 344 | 15S-lipoxygenase type 2Up | Up |
| SS41-600 | 290 | Complement component C7 | Up |
| SS42-800 | 541 | C1q-like adipose specific protein | Up |
| SS43-300 | 203 | Cytochrome b5 | Up |
| SS44-400 | 342 | Arachidonate 15-lipoxygenase, second type 12-lipoxygenase | Up |
| SS46-400 | 245 | Piscicida trpA gene for putative transposase, clone pRDA33 | Up |
| SS48-500 | 493 | Poly A binding protein | Up |
| SS49-500 | 395 | NADH dehydrogenase | Up |
| SS51-600 | 467 | Alpha-glucosidase | Up |
| SS52-500 | 474 | Proteophosphoglycan | Up |
| SS53-700 | 342 | Antifreeze protein precursor | Up |
| SS54-800 | 472 | Focal adhesion kinase | Up |
| SS60-900 | 749 | BCL2/adenovirus E1B 19 kDa-interacting protei | Up |
| SS69-400 | 309 | Glycine max beta-carotene hydroxylase | Up |
| SS75-500 | 346 | Translation initiation factor eIF-2B beta subunit gene | Up |
| SS77-300 | 353 | Serum amyloid A protein | Up |
| SS78-300 | 261 | Cytochrome P450 1A | Up |
| SS80-400 | 316 | Gnb2l1-prov protein | Up |
| SS89-300 | 354 | Heat shock protein 90 | Up |
| SS91-300 | 342 | G1/S-specific cyclin E1 | Up |
| SS99-300 | 228 | MHC Class I Region | Up |

[a]Basic Local Alignment Search (BlastX) at NCBI
[b]Up- or down-regulated in diesel-exposed rockfish (40 hr)

higher at contaminated sites in response to PAHs and PCBs in the mussel *Mytilus* sp.[16], in the sole *Pleuronectes vetulus* (Miller *et al.*, 2004), and in the salmon *Salmo salar*[17].

## Discussion

In conclusion, we successfully isolate four genes differentially expressed in response to the existence of diesel and the exposure duration. Four genes were presumed to be the *Sebastes* homologues of betaine homocysteine methyltransferase, warm-temperature-acclimation-related protein, serum amyloid A protein, and cytochrome P450 1A, respectively. All those genes are known to be related to pathogenesis, extrinsic stresses, and catalytic metabolites. Considering the expected function of the isolated genes, the ACP-based DD-PCR method might be strong and effective in isolation of the differentially expressed genes and

provided the knowledge regarding gene expressions by toxicant impact. Furthermore, this result might be useful for the development of biomarkers to assess marine environmental stress or contamination.

## Methods

### Animal

Rockfish (500±50 g) were obtained from regional aquaculture farm, away from local pollution sources (Geoje, Korea) and acclimated for 2 days in an aquatic facility. Twenty fish were assigned to filtered seawater containing 20 ppb of diesel oil (commercial oil) and ten control fish were maintained in filtered seawater. Five fish were taken for RNA extraction from the diesel oil-exposed group and control group at 40 hr, respectively. RNAs were extracted from fish livers using Trizol reagent (Sigma) following the manufacturer's instruction. RNAs from the livers of the fish assigned to non-exposed seawater were used as control.

### Differential Display (DD-) PCR and Gene Cloning

The first strand cDNA was synthesized by using moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, WI, USA) following the manufacturer's direction. The total RNA was used as a template and primed with oligo $(dT)_{15}$-ACP (Seegene, Seoul, Korea). Differential display PCR was performed by using GeneFishing DEG kit (Seegene, Seoul, Korea) to identify differentially expressed genes. Manufacturer's direction for ACP-based GeneFishing-PCR was followed. Forty arbitrary ACP primers (A1-20, A41-60) were applied to the PCR. The PCR products were separated in 2% agarose gel stained with EtBr. The differentially expressed PCR products were cut out from the gel and extracted by using QIAquick gel extraction kit (Qiagen). The purified PCR products were cloned into the pGEM-T Easy vector (Promega). Sequencing of positive clones was carried out with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

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