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Aroclor 1254 May Induce Common DNA Effects in Developing *Paralichthys olivaceus* Embryos and Larvae

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Abstract

Polychlorinated biphenyls (PCBs) are persistent pollutants in aquatic environments, often causing the decline or disappearance of wild populations. In this study, we used a random amplified polymorphic DNA (RAPD) assay to evaluate the effects on the genomic DNA of olive flounder embryo and larval stages of exposure to Aroclor 1254 at concentrations of 1, 5, 10, 20, and 40 μg/L. We compared RAPD fingerprints of exposed and non-exposed samples. Polymorphisms were revealed as the presence and/or absence of DNA fragments between the two samples. A dose-dependent increase in the number of polymorphic bands was observed with Aroclor 1254 treatment. Also, RAPD profiles of animals exposed to Aroclor 1254 exhibited an increase in the frequency values (FV) compared to the control. A phenogram constructed using neighbor-joining method indicated that genomic template stability in developing embryo and larval stages was significantly affected at ≥ 5 μg/L. This study suggested that DNA polymorphisms detected by RAPD analysis could be used as an investigative tool for environmental toxicology and as a useful biomarker in early life stages for the detection of potential genotoxicants.

Key words: Aroclor 1254, Genotoxicant, RAPD assay, Embryos, Larva, *Paralichthys olivaceus*

Introduction

Pollutants with genotoxic potential are of great concern to many ecotoxicologists. Once released, these agents have the capability not only to cause morbidity and/or mortality in exposed organisms, but also to affect biological diversity at both intra- and interspecies levels (Atienzar et al., 1999). However, despite growing concerns over the presence of genotoxins in aquatic environments, adequately validated methods that can be used to evaluate genotoxicity and associated toxicity in aquatic organisms under environmentally relevant conditions are lacking. To examine genotoxic impacts, the three classic methods typically used are the micronucleus assay, the comet assay, and 8-oxoguanosine quantification. However, previous studies have demonstrated that in the eel, *Anguilla anguilla*, the micronucleus test was not sufficiently sensitive to detect aquatic pollution, nor was the comet assay sufficiently sensitive in mussel gills (Sanchez-galan et al., 2001; Pruski and Dixon, 2002). Another difficulty linked to the micronucleus test and the comet assay is the necessity to experiment on isolated cells (Cambier et al., 2010). The 8-oxoguanosine method is a sensitive technique for quantifying genotoxic impacts of xenobiotics, but it is a time-consuming and fastidious process (Halliwell, 1999). However, remarkable disagreement remains concerning the values at steady state in liver tissue DNA due to the marked variability of the results obtained by various laboratories (Halliwell, 1999; European Standards Committee on Oxidative DNA Damage (ESCODD), 2002).

Aroclor 1254, a commercial mixture of polychlorinated biphenyl (PCB) congeners, can be carcinogenic, clastogenic,

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and cause birth defects in both animals and humans (Cogliano, 1998; Sargent et al., 1989). While PCBs, such as dioxin, are usually considered to be non-genotoxic carcinogens (Whysner et al., 1998), there remains some uncertainly in this regard. The mechanisms by which PCBs exert their adverse effects are not fully understood and data on their genotoxic properties are controversial (Ross, 2004). Silberhorn et al. (1990) reported that Aroclor 1254 had negative results in the majority of genotoxicity assays. However, Aroclor 1254 produced DNA single-strand breaks in an alkaline elution/rat hepatocyte assay (Sina et al., 1983) and was clastogenic in dove embryos (Peakall et al., 1972). Accordingly, in this study, the genotoxic effect of Aroclor 1254 has been assessed using a random amplified polymorphic DNA (RAPD)-based methodology.

RAPD is currently used for intra-populational polymorphism detection and also enables the detection of genotoxicity after pollutant contamination of animals (Grayson et al., 1999, 2000). Moreover, Atienzar and Jha (2006) suggested that RAPD is a reliable, sensitive, and reproducible assay, and after proper optimization, has the potential to detect a wide range of DNA damage as well as mutations and therefore can be applied to genotoxicity and carcinogenesis studies. In fact, RAPD has been successfully used to detect 'DNA effects' induced by copper, cadmium benzo[*a*]pyrene, and nonylphenol, mainly in aquatic systems (Atienzar et al., 1999, 2001, 2002, 2004; Atienzar and Jha, 2004, 2006; Castano and Becerril, 2004; Cambier et al., 2010). The most significant advantages of the RAPD assay lie in its rapidity, applicability to any organism, and ability to detect a wide range of DNA damage and mutations, including point mutations and large rearrangements (Atienzar et al., 2002). However, in these previous studies, exposure to genotoxins was conducted using concentrations higher than those generally found in environmentally polluted areas.

Some of the most persistent and ubiquitous contaminants are PCBs, although they are found only at low concentrations (Domingo and Bocio, 2007; Brown et al., 2006; Ross, 2004). Recently, the PCB contents of muscle tissues of marine fishes from nine coastal cities of East China were analyzed. The total PCB concentrations in all fish samples varied between 13.3 and 78.3 μg/kg lipid weight (lw), with an average of 35 ± 15 μg/kg lw (Xia et al., 2012). In our previous study on the toxicological effect of Arolcor 1254, the LC_{50} values in olive flounder embryos and larvae were 50.92 μg/L and 3.08 μg/L, respectively, and exposure to concentrations as low as 1 μg/L disrupted the early developmental stages of the fish (Min and Kang, 2013). An expert panel of the European Food Safety Authority (EFSA) concluded that we must improve our understanding of the environmental and human risks associated with PCBs due to their abundance in food and human tissues (Stenberg and Andersson, 2008).

According to Koh et al. (2001), the concentration of chlorinated compounds such as PCBs and dichlorodiphenyl trichloroethane (DDT) among the organic pollutants in Korean coastal environments was lower than in other developed countries. However, polycyclic aromatic hydrocarbons (PAHs), which are toxic compounds derived from oil spills, were present at higher concentrations in Korean coastal regions (Koh et al., 2001). Due to their high persistence and strong lipophilic properties, PCBs have bioaccumulated in aquatic food webs. Although the water solubility of Aroclor 1254 is only 2.7 μg/L, PCBs have been classified as probable human carcinogens by the Environmental Protection Agency (EPA), based on substantial evidence of cancer in animals (Walker, 1994). In fishes, PCBs such as Aroclor 1254 also are transported with lipoproteins into the yolk of developing oocytes (Ungerer and Thomas, 1996). Increased concentrations of PCBs in fish embryos and ovaries have been correlated with decreased viability, embryo toxicity, and embryonic malformations (Black et al., 1988; Weis and Weis, 1989; Min and Kang, 2013).

The development of such *in vivo* test systems is also essential to provide a scientific basis for comparing the relative

Table 1. Test conditions for the acute toxicity tests with olive flounder *Paralichthys olivaceus* embryos and larvae

Test types	Conditions
Toxicity test type	static acute with water renewal
Duration (h)	40 (embryos) / 72 (larvae)
Light quality and photoperiod (L, Light; D, Dark)	ambient laboratory light (12L : 12D)
Water for exposure	filtered and UV-treated
Test chamber size (mL)	500
Test solution volume (mL)	300
Renewal of test solution (h)	every 24
Age of test animals at start of exposure (h)	less than 10 h after fertilized (8~10)
Number of embryos and larvae per chamber	50 (embryos)/ 50 (larvae)
Number of replicates per concentration	3
Feeding regime	not required
Test concentrations	5ea + control
Test acceptability criterion	90% or greater hatching in control

risks of genotoxins (Jha et al., 2000a, 2000b). DNA damage induced in the early life-stage (ELS) of an organism inhibits its development toward adulthood, which might cause disturbances to the ecosystem along the food chain as well as serious economic loss in fisheries. Aquatic toxicologists generally consider ELS toxicity tests to be the most useful for risk assessment because the ELS is a sensitive stage of the fish life cycle during which many critical events take place over a very short span of time (Von Westernhagen, 1988). During this phase, man-made pollutants may exert strong effects on the survival of fish embryos and larvae. Attractive features of this method are that it is a relatively simple bio-indicator, and that it provides good coverage, even for larger marine areas (Cameron and Berg, 1992).

For environmental monitoring purposes, one of the major biological challenges has been to bring potential organisms into continuous routine culture. This is especially important when sound genetic analysis is to be carried out. Olive flounder *Paralichthys olivaceus*, are distributed among coastal areas of Korean and Japan. In Korea, this species is commonly raised in aquaculture, and it is important both in the market and on the table. In this study we chose the olive flounder because of its economic value as a food resource as well as the considerable ease in handling and procurement.

The aims of this study were to detect potential genotoxic effects induced by Aroclor 1254 at environmentally relevant dosages using an RAPD technique, and to confirm the potential for the use of embryos and larvae of the olive flounder *P. olivaceus*, in *in vivo* laboratory studies for biomonitoring of genotoxic risk evaluation.

Materials and Methods

Experimental animals and water conditions

Fertilized eggs of the olive flounder *P. olivaceus* were obtained from hatcheries in Cheju Island, South Korea. The water quality parameters measured for the bioassays conducted were as follows: pH, 8.10 ± 0.2; salinity, 32.70 ± 0.4‰; dissolved oxygen, 6.74 ± 0.84 mg/L; chemical oxygen demand, 1.52 ± 0.008 mg/L. All experiments were conducted at a seawater temperature of 20 ± 0.5°C under a 12-h light/12-h dark cycle, over a 36- or 72-h period. Other pertinent test conditions for the acute toxicity tests are summarized in Table 1.

Chemicals

Aroclor 1254 was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Five solutions (1, 5, 10, 20, and 40 μg/L) were prepared by diluting a stock solution. The stock solution was prepared with seawater that had been filtered through a grade GF/C Whatman filter (Maidstone, UK) and UV-treated.

Embryo and larval toxicity tests

The eggs obtained from the hatchery were approximately at the 8–10-h post-fertilization (blastula) stage. They were transferred directly into clean 500-mL glass beakers containing 0 (control), 1, 5, 10, 20, or 40 μg/L Aroclor 1254 for static exposure. Each Aroclor 1254 concentration (including the control) was carried out in triplicate beakers. Embryonic development was monitored every 3 h, until an endpoint at 40 h, or until hatching was complete. The beaker contents (Aroclor 1254 solutions) were replaced every 24 h throughout the toxicity test (Table 1). Newly hatched larvae were exposed to identical Aroclor 1254 concentrations to maintain the conditions (Table 1). The larvae were monitored every 6 h for up to 72 h.

At each time point during the embryo and larva toxicity tests, from each beaker, 50 organisms were collected, washed with UV-treated seawater, placed in phosphate-buffered saline (PBS), frozen in liquid nitrogen, and then stored at –70°C until subsequent analysis. The entire experiment was repeated in triplicate.

DNA isolation and DNA profiling using RAPD

The genomic DNA (gDNA) of exposed test organisms was extracted using a commercial DNA purification kit (NucloGen Biothechnology, Korea). Briefly, the organisms were lysed using sodium dodecyl sulfate and DNA was extracted by proteinase K digestion, phenol:chloroform extraction, and ethanol precipitation. DNA concentration was measured using a Bio-photometer (Eppendorf AG, Germany). DNA stock solutions were diluted to 10 μg/mL in distilled water and stored at –70°C until analysis.

DNA profiles of *P. olivaceus* were generated with RAPD reactions performed in 20-μL reaction volumes as described previously (Atienzar et al., 2000). The oligonucleotides AP1–AP8 were obtained from an oligo synthesis company (Bioneer Co., Korea; the sequences are provided in Table 2). DNA amplification was performed on a My Cycler™ thermal cycler (Bio-Rad, USA), and the amplification protocol was as follows: 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 2 min at 45°C, and 2 min at 72°C.

Table 2. Sequence of the primers used for RAPD PCR in this study

Primer	Sequence
P1	CAG GCC CTT C
P2	CAA TCG CCG T
P3	AGG GGT CTT G
P4	TCG GCG ATA G
P5	GAA ACG GGT G
P6	AGC CAG CGA A
P7	GTC ACG TAG G
P8	TCC GCT CTG G

Electrophoresis of RAPD reaction products were performed in 2% w/v agarose gels, using a Tris-Borate-EDTA buffer system (1X TBE; 90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA) at 100 volts for 30 min. The gels were stained in a 1X TBE solution containing ethidium bromide (0.015% v/v) for at least 40 min. Gels were visualized under UV light, and photographed using a Gel-doc camera system (Gel-doc XR. Bio-Rad. USA). For comparison, a DNA molecular size marker (100-bp DNA Ladder, Bioneer, Co. Korea) was used.

Data analysis

The RAPD method is relatively new and there is no one standard statistical method used routinely for determining genetic diversity. For comparison, we used neighbor-joining analysis and frequency values (FV). Neighbor-joining analysis (Saitou and Nei, 1987), in which genetic distances are computed for all pairs of individuals, was performed using the NEIGHBOR program in PHYLIP 3.5c (Felenstein, 1993) to generate an unrooted phenogram. A phenogram was constructed based on all individuals examined in this study. In the phenogram, samples from the various concentration groups with similar genotypes can be identified through clustering. Also, in this study, the FVs obtained from calculating band counts of new appearances or disappearances (i.e., mismatched bands compared to the control) in the RAPD profiles using each primer were calculated as follows:

Frequency values of contamination indicative bands (FV) $= (\sum NA + \sum ND)/ NP$

$\sum NA$: the sum of the total number of newly appeared bands in each primer RAPD profile

$\sum ND$: the sum of the total number of disappeared band in each primer RAPD profile

NP: the total number of primers used in the RAPD profile

The frequency values of contamination indicative profiles (FV) were calculated using a modification of a protocol outlined by Theodorakis and Shugart (1997). Treatment groups were compared to the control using a one- or two-way analysis of variance (ANOVA). If the ANOVA was significant ($P < 0.05$), a Duncan's multiple comparison test was used to identify means that were similar and those that were significantly different. For continuously recorded parameters, the median effective concentration (EC_{50}) values of FV (i.e., the statistically derived concentration of a substrate in an environmental medium expected to produce a certain effect in 50% of test organisms in a given population under a defined set of conditions), including upper and lower 50% confidence limits, were determined by Probit analysis using the SPSS/PC+ statistical package.

Results and Discussion

The continuous presence of genotoxic chemicals in aquatic environments is of major concern due to their effects on the health of aquatic biota (Jha, 2004). DNA integrity is one of the biomarkers of pollution, and therefore much effort has been focused on finding rapid, sensitive methods for obtaining information on potential genotoxicity (Sarkar et al., 2006). In fact, several studies have used PCR-based techniques to assess genetic variation and changes in aquatic biota exposed to different contaminants (Theodorakis and Shugart, 1997; Nadig et al., 1998; Ma et al., 2000). In addition, it has been suggested that RAPD assays can detect mutations only if they occur in at least 2% of the DNA (John and Kortenkamp, 2000).

RAPD DNA profiles of embryos and larvae under Aroclor 1254 stress

In this study, RAPD analyses were performed on gDNA pooled from 50 individuals of uncontaminated or Aroclor 1254-contaminated embryos or larvae. gDNA was pooled from 50 individuals to suppress intra-populational genetic polymorphisms that could potentially be revealed by RAPD. Eight different random primers were screened to discriminate controls from contaminated embryos and larvae. Of the eight primers tested, only four gave specific and stable results, and RAPD profiles generated with primers P2 and P6 were especially highly polymorphic. To determine the diversity of organisms exposed to Aroclor 1254, profiles were sorted based on the RAPD fragments at each exposure time (Fig. 1). The RAPD profiles showed substantial differences between unexposed and exposed animals, with apparent changes in the number, size, and intensity of amplified DNA fragments. Asterisks and the letter "d" on the RAPD profile gels of exposed groups indicate some of the obvious differences from the control (Fig. 1).

Analysis of data generated by RAPD profiling

Neighbor-joining analysis assigns a genotype to each examined fish based on RAPD profiles generated by all analyzed primers and further reveals genetic relationships among all examined fish (Nadig et al., 1998). Because there was genetic differentiation among the treatment groups, a phenogram of genetic distance among the groups may be useful in delineating water quality (Fore et al., 1995). In this study, Fig. 2 is a phenogram generated using all primers and shows that the embryos were divides into two groups, designated here as the affected cluster (A), containing embryos affected by Aroclor 1254, and the non-affected cluster (NA). Interestingly, the number of the exposed group belonging to cluster (A) increased in an exposure-time-dependent manner in the embryo stage, increasing from two groups (20 and 40 μg/L) at 12 h, to four groups (5, 10, 20 and 40 μg/L) at 36 h (Fig.

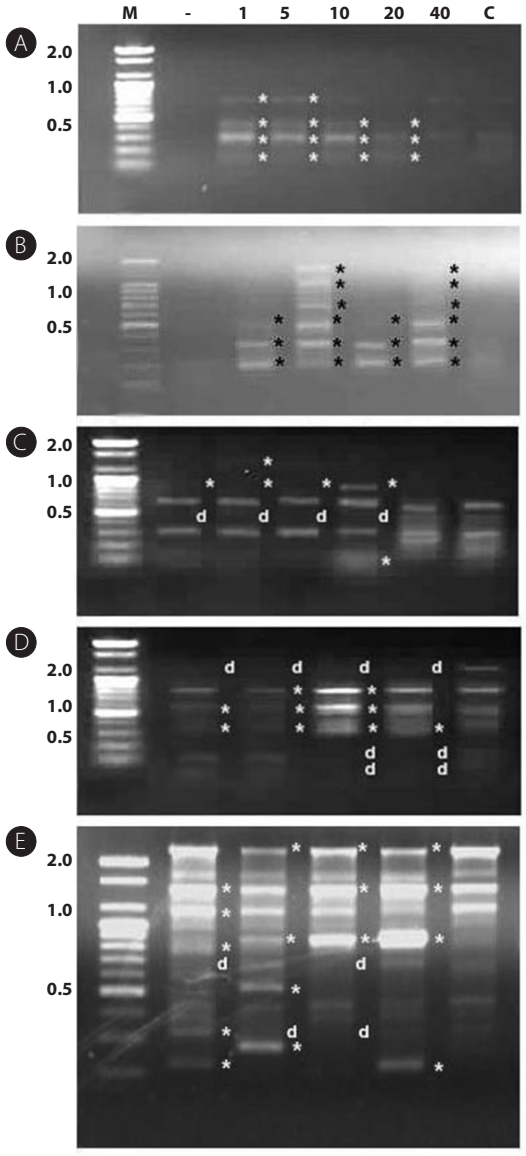

Fig. 1. RAPD profiles obtained with the primers 2 (C), 6 (A, B and E) and 7 (D). DNA extracts form *Paralichthys olivaceus* embryos (A, B, and C) and larvae (D and E) exposed to various concentrations of Aroclor 1254; Control (C), 1, 5, 10, 20 and 40 μg/L for 12 (A), 24 (B), 36 (C), 60 (D) and 72 (E) hours. Selected changes are indicated by a symbol (*, variation in band intensities and appearance of a new band; d, disappearance of a band) in comparison to control patterns in profiles. The 100 bp DNA ladder (M) was used to as DNA molecular weight marker.

2). In the larval stages, the phenogram also indicates two genetic clusters: cluster (NA) (1 and 5 μg/L), was more similar to the control than to cluster (A) (10 and 20 μg/L). The results demonstrate that the genotypes of embryos and larvae were highly variable, indicating that the genetic diversity of Aroclor 1254-contaminated groups can be readily analyzed by RAPD.

The phenetic numerical analysis of RAPD profiles is the most popular way to analyze RAPD in ecotoxicology studies and has been used to determine genetic diversity in nat-

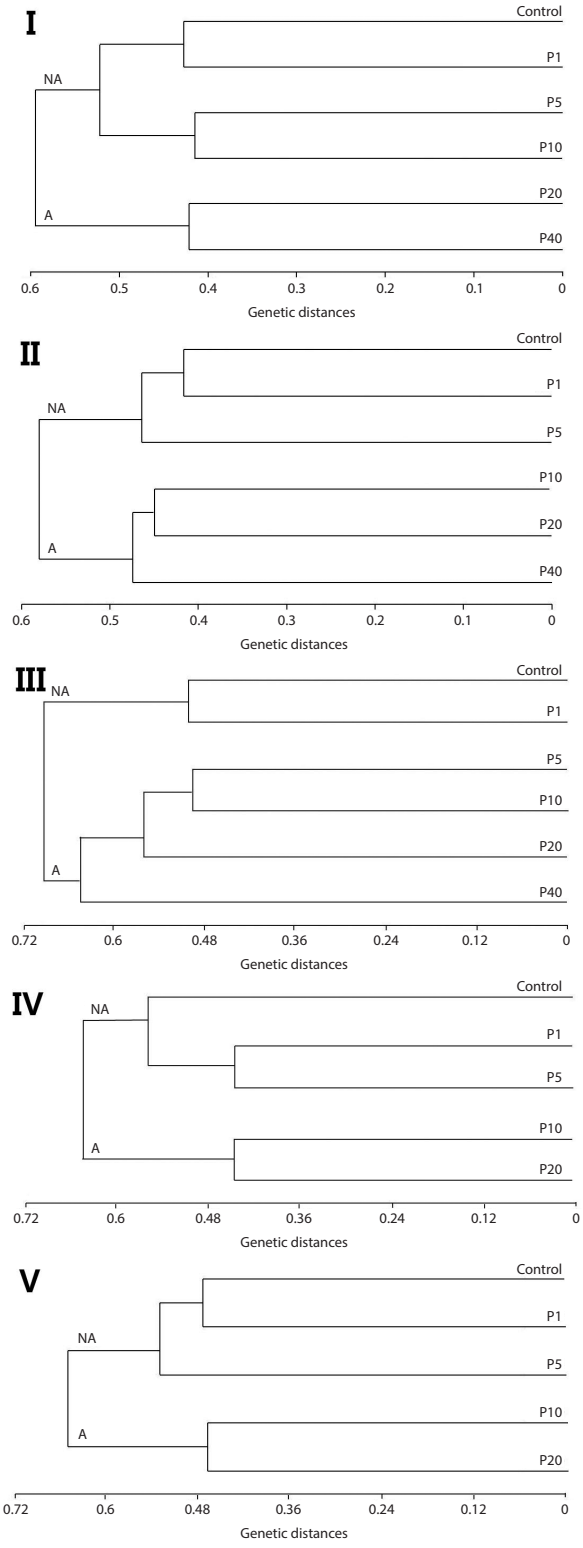

Fig. 2. Phenogram constructed using RAPDPLOT Neighbor joining method incorporating all primers in embryos (I, II, III) and larvae (IV, V) from control and different Arclor 1254 concentrations; 1, 5, 10, 20 and 40 μg/L. Two major clusters of genotype (NA and A) indicates smaller clusters within each of these major groups.

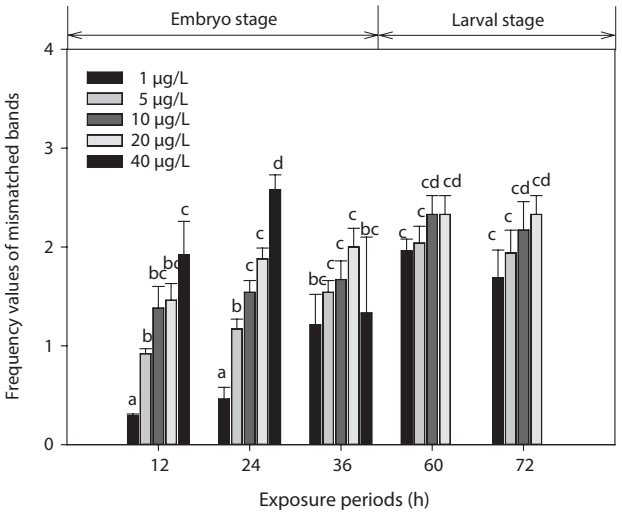

Fig. 3. Frequency values of contamination indicative bands compared to control in the RAPD profiles from embryos and larvae exposed to different Aroclor 1254 concentration for different exposure periods. Values (mean ± standard error) with the same superscript letter are not significantly different (P>0.05).

ural populations exposed to a variety of pollutants (Ross et al., 2002; Turuspekov et al., 2002). On a phenetic basis, the diversity is estimated by comparing the absence/presence of RAPD bands in exposed and non-exposed individuals (i.e., similarity analysis), or by counting their RAPD band numbers and considering band abundances (Maldonado et al., 2003). Modifications in band intensity and lost bands are likely to be due to one or a combination of the following events: (1) changes in oligonucleotide priming sites due to genomic rearrangements (more likely) and/or point mutations and DNA damage in the primer binding sites (less likely), and (2) interactions of DNA polymerase in organisms with damaged DNA (Liu et al., 2005). Thus, the FVs of these DNA polymorphisms could be used for the detection of genotoxic effects. In this study, changes in RAPD profiles were expressed as increases in FV (a measure reflecting the obvious changes in DNA patterns generated from toxicant-exposed organisms) in relation to profiles obtained from control organisms (Fig. 3).

The EC_{50} values of FVs determined for embryos and larvae exposed to different concentrations of Aroclor 1254 are shown in Table 3. FVs significantly increased in a dose-dependent manner in developing embryos exposed to Aroclor 1254, but in the larval stage, there was no significant difference among the concentration groups (Fig. 3). Although FVs in larvae were not significantly different between the groups, a high FV was sustained until 72 h. This result suggests that during ELS, changes to gDNA stability may be an effect of the genotoxic potential of Aroclor 1254. This result also suggests that gDNA stability in the larval stage may be more sensitive than in the embryonic stage. As mentioned in our previous study (Min and Kang, 2013), our results demonstrate that Aroclor 1254 disrupted the early development stages of olive flounder, with higher toxicity in the larvae than in the developing, unhatched embryos.

Several studies have used ELS marine organisms to detect genomic instability caused by pollutants (Jha et al., 2000a, 2000b). Atienzar et al. (2002) suggested that 4-n-Nonylphenol and 17β-estradiol induced DNA effects in larvae of the barnacle *Elminius modestus*, and these specific modifications in RAPD patterns may arise as a consequence of hot spot DNA damage, DNA adduct formation, and point mutations or genomic rearrangements. Jha et al. (2000a) demonstrated that tributyltin oxide induced potential genotoxic effects in the ELS of the marine mussel *Mytilus edulis*. These previous results are in agreement with our study. These data indicate that it may be possible to relate DNA damage from the pooled embryos and larvae to similar damage in DNA isolated from the organs, that is, the liver, gills, and blood cells, of individual fish. In this study, using a genotoxicity assay *in vivo* based on the larval stage of olive flounder, we have evaluated the potential genotoxic hazard of Aroclor 1254. The results obtained from this study provide circumstantial evidence of the mechanisms mediating some of the DNA changes. The developmental (ELS) toxicity results in our previous study confirmed that Aroclor 1254 caused toxic responses in the larvae of olive flounder (Min and Kang, 2013). The developmental toxicity results complemented the genotoxic response at lower levels of biological organization, as more sensitive and robust end points. The cytotoxic effects can be detected alterations on a specific mechanism at DNA level using the cytogenetic assays, which would have direct implications for the developmental, and subsequently reproductive, success of the exposed organism (Jha et al., 2000b).

Table 3. The value of EC_{50} of the frequency values (FV) determined by Probit analysis in olive flounder *Paralichthys olivaceus* embryos and larvae exposed to Aroclor 1254

	Embryo stage			Larval stage	
Exposure period (hours)	12 h	24 h	36 h	60 h	72 h
EC_{50} (μg/L)	28.67	21.52	21.46	8.88	10.81
95% confidence upper limit (μg/L)	40.28	29.91	46.64	16.58	18.94
95% confidence lower limit (μg/L)	20.79	14.96	7.73	0.45	0.08

PCBs were not genotoxic in the bacterium *Salmonella typhimurium* or V79 Chinese hamster cells *in vitro*; only in rat liver did lower chlorinated congeners lead to covalently modified macromolecules including proteins and DNA (Knerr and Schrenk, 2006). However, in *in vitro* tests using the fish cell line RTG-2 from the rainbow trout, the comet assay and the micronucleus test showed clear genotoxic damage after PCB exposure (Marabini et al., 2011). Several mechanisms could account for the genotoxic potential of PCBs. The cause of the genotoxic damage from PCBs may be oxidative stress, which could give rise to reactive oxygen species (ROS) production, lipid peroxidation, loss of glutathione (GSH) content and reduced superoxide dismutase (SOD) levels, with significant amounts of DNA oxidized bases (Marabini et al., 2011). A previous study has linked PCBs such as Aroclor 1254 and their hydroxylated metabolites (OH-PCBs) with ROS production (Strathmann et al., 2006). The PCBs, metabolized by the CYP450 system, reportedly formed dihydroxylated metabolites such as catechols and hydroquinones. These were oxidized to semiquinones, which can damage DNA directly (Schlezinger et al., 2006). Previous studies have also demonstrated that PCBs cause double-strand DNA breaks in yeast and bluegill sunfish, and have suggested that this is secondary to oxidative stress (Theodorakis et al., 1992; Appelgren et al., 1999; Tharappel et al., 2002).

In the field of "genetic-ecotoxicology" or "eco-genotoxicology", there are few well-validated tools for linking genetic damage to end points of direct importance to the ecological viability of marine populations (Anderson et al., 1994; Jha et al., 2000a). This study will provide a useful strategy in which first, RAPD analysis can be used as a screening method for potential genotoxic effects and, second, phenetic numerical analysis can be applied to measure DNA instability resulting from pollutants. These techniques are therefore being adopted as sensitive methods for the detection of induced genetic damage at the molecular level in both embryos and larvae of aquatic organisms.

In conclusion, our results indicate that Aroclor 1254 increased DNA damage in the embryo and larval stages (ELS) of olive flounder, as assessed by RAPD analysis. We suggest that DNA polymorphisms detected using RAPD analysis could be used for investigation of environmental toxicology and as a useful biomarker in an early warning system. In addition, we established this method under laboratory conditions using ELS and provided evidence of a reproducible and sensitive model for the detection of genotoxicants.

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