RAPD-PCR analysis for molecular characterization and genotoxic studies of a new marine fish cell line derived from *Dicentrarchus labrax*

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Abstract Continuous cell lines could provide an important tool for studying epidemiology, toxicology, cellular physiology and the host–pathogen interactions. Random amplified polymorphic deoxyribonucleic acid analysis by PCR (RAPD-PCR) was used for the molecular characterization of *Dicentrarchus labrax* embryonic cells (DLEC) as a possible tool to detect DNA alterations in environmental genotoxic studies. We studied the DNA pattern of the DLEC fish cell line, a fibroblast-like cell line derived from European sea bass. From a total of 15 primers only six showed good discriminatory power for the amplification process on DNA samples collected from cells by three different methods (organic extraction, salting-out method and chelating agent extraction). The results obtained show that the cell line chosen for this study could be used as a possible tool for the detection of potential genotoxicity of numerous chemical compounds.

Keywords DLEC cell line · RAPD-PCR technique · GTS % · Genotoxicity

Introduction

Environmental pollutants such as nanoparticles, Pharmaceuticals and Personal Care Products (PPCPs), metals, pesticides, pharmacological agents and other molecules pose serious risks to many aquatic organisms and cause genotoxic damage as well as the possible development of cancer both in man and other organisms (Stahl 1991; De Flora et al. 1993). The increasing presence of genotoxic chemicals in the aquatic environment has led to the development of both in vivo and in vitro assays for target species. The fish population represents an important level of aquatic ecosystems that can be threatened by increased environmental pollution. Practicality and cost are also important factors that have to be taken into consideration in developing laboratory tests (Girling et al. 2000).

Fish cells have many functions that are similar to those of mammalian cells, but they also have many advantages over mammalian cells (Bols and Lee 1991). For example, they can be cultured at room temperature (20–28 °C) and can be directly exposed to environmental samples of different osmolarity. In addition they greatly facilitate the collection of more

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useful in vitro data for toxicity tests. In fact, over 150 cell lines have been established from fish (Fryer and Lannan 1994; Sobhana et al. 2009). However, most of these cell lines are derived from freshwater fish species. The study on marine fish cell lines has developed rapidly in recent years and numerous cell lines from tissues of commercially important marine fish have been described (Buonocore et al. 2005; Parameswaran et al. 2007; Zhou et al. 2007; Huang et al. 2009; Fan et al. 2010). The advantages of these cell lines with respect to mammalian cells are that they are standardizable, easy to handle with relatively low variability, more convenient, and less laborious to use.

Several bioassays have been recommended for assessing the toxicological effects on cellular growth rate and viability (Sauvant et al. 1997). Therefore, the detection of genotoxic effects using in vitro cell systems can be extremely useful in risk assessment procedures. In vitro culture of fish cells provides an important tool for studying cytotoxicity, genotoxicity, gene regulation, virology and tumorigenesis. The first fish cell line, RTG-2, was established from rainbow trout gonads (Wolf and Quimby 1962). In addition, to the avoidance of using animals, the advantages of using in vitro assays are related mainly to cost, versatility, volume of waste, and laboratory facilities required. There are some widely used methods for the characterization of cell lines. The random amplified polymorphic deoxyribonucleic acid analysis by PCR (RAPD-PCR) appears to be a reliable method. This technique involves the amplification of random segments of genomic DNA, using short arbitrary primers without the requirement of previous knowledge of genomic deoxyribonucleic acid (DNA) (Welsh and McClelland 1990; Williams et al. 1990), and has been used in species and strain identification (Bardakci and Skibinski 1994; Cocconcelli et al. 1995), genetic diversity analysis (Koh et al. 1999), genetic marker-assisted breeding (Liu et al. 1999), detection of genetic variation (Keshava et al. 1999) and genotoxicity evaluation of environmental pollutants (Rocco et al. 2010, 2011, 2012). It has also been used in the characterization of fish, including RTG-2, mammalian and insect cells lines (Ferrero et al. 1998; Stacey et al. 1992; Kawai and Mitsuhashi 1997; Perry et al. 2001).

Genotoxic agents not only disrupt the integrity of the genome but also directly or indirectly affect the expression of DNA (Shugart and Theodorakis 1994). These effects lead to an increase in the incidence of

different types of gene mutations and, in the long-term, result in genetic variability of the exposed populations. In this context, random amplified polymorphism DNA (RAPD) analysis, developed by Williams et al. (1990) and Welsh and McClelland (1990), is a powerful technique that involves the amplification of random segments of genomic DNA using PCR. Changes in the DNA fingerprint (i.e. band patterns) reflect DNA alterations in the genome ranging from single base changes (point mutations) to complex chromosomal rearrangements (Atienzar et al. 1999, 2002). Thus, DNA fingerprinting offers a useful biomarker assay in ecotoxicology (Savva 1998; Rocco et al. 2010, 2012).

In the present study we tested the applicability of RAPD-PCR analysis for the molecular characterization of the marine fish cell line DLEC and we present the final protocol used to obtain a stable and specific fingerprint for this cell line. We also report on the application of PCR based DNA fingerprinting procedures in mutation detection and discuss their application to ecotoxicological studies. The findings of this study demonstrate that the use of RAPD-PCR technique with DLEC fish cell line is suitable as an in vitro screening assay in environmental genotoxicity testing.

Materials and methods

Cell lines used

We first used DLEC, a continuous adherent cell line, derived from early embryos of the European sea bass *Dicentrarchus labrax* L. obtained by Buonocore et al. (2005). Embryo cells (2- to 12-h old) were obtained by passing approximately 1,000 embryos through a 100-mesh sieve with a pestle in Leibovitz's L15 medium (GIBCO, Grand Island, NY, USA), and the cellular suspension was then washed twice by centrifuging at 380g in RPMI (LONZA, Visp, Switzerland). Resulting cells were cultured at 18 °C in Leibovitz's L15 medium containing 5 % fetal calf serum (FCS) (GIBCO) and 10 % supernatant fraction of the embryo homogenate. After 8 weeks the culture medium was replaced with Leibovitz's L15 medium containing 10 % FCS and the DLEC cells started to proliferate. Subsequently, they were continuously cultured until the 50th passage without evident changes in their morphology.

We also used RTG-2 cells (LGC Standards, Sesto San Giovanni, Italy), an established fibroblastic cell

line already in use in our laboratories, derived from rainbow trout (*O. mykiss*). They were grown in Leibovitz's L15 medium, supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1.25 µg/ml fungizone, and 2 mM L-glutamine (GIBCO), in tissue culture flasks (Cellstar) and incubated at 20 ± 1 °C.

Genomic DNA extraction

Cells were grown under controlled conditions and were then dissociated with trypsin–EDTA 200 mg/l (BioWhittaker, Lonza), collected in phosphate-buffered saline solution (PBS 1X, Lonza), divided into aliquots, and centrifuged at 1,000 rpm for 15 min.

Three different approaches were used to isolate genomic DNA from the cell pellet: organic extraction, salting-out method and chelating agent extraction. The genomic DNA extraction was performed on about 2×10^6 cells for the three different methods.

Approach I: Phenol extraction

Cells were pelleted and then diluted in 600 µl of extraction buffer: 100 mM Tris–HCl (Sigma-Aldrich), pH 8.0, 50 mM EDTA (Carlo Erba, Rome, Italy) pH 8.0, 500 mM NaCl (Carlo Erba) with 0.5 % SDS (Sigma-Aldrich, St. Louis, MO, USA) (Dellaporta et al. 1983), and incubated at 65 °C in a water bath for 20 min; 180 µl 5 M potassium acetate (Sigma-Aldrich) was added and the mixture centrifuged at 13,000 rpm for 15 min to purify DNA; the supernatant was transferred to a 1.5 ml graduated microcentrifuge tube and incubated with proteinase K and RNase (ROCHE, Milan, Italy) at 37 °C for 2 h. Digested proteins were extracted with Tris-buffered phenol (Invitrogen, Carlsbad, CA, USA), and the DNA (aqueous phase) was precipitated with isopropanol (Carlo Erba) and then with 70 % ethanol (a modification of the procedure of Sambrook et al. 1989) (Carlo Erba).

Approach II: Salting-out

Cells were pelleted and then diluted in 600 µl of extraction buffer: 10 mM Tris–HCl pH 8.0, 2 mM Na₂EDTA (Sigma-Aldrich), pH 8.2 (Miller et al. 1988), 500 mM NaCl with 0.5 % SDS (Dellaporta et al. 1983), and incubated at 65 °C in a water bath for

20 min. The procedure of DNA extraction and purification is the same as described for method I, but phenol:chloroform extraction was replaced by high-salt precipitation of proteins with saturated NaCl according to Cheng et al. (1995).

Approach III: Chelating agent extraction

DNA was extracted using Chelex chelating resin (Chelex 100 Resin, Biorad, Hercules, CA, USA) as described by Walsh et al. (1991), under the conditions suggested by the manufacturer. Cells were pelleted at 2,800 rpm for 5 min. The cell clamp was diluted with 500 µl of sterile distilled water, and 200 µl of 20 % Chelex solution was added. After incubating at 55 °C for 10–15 min, the suspension was vortexed and boiled for 1 min in a water bath. After a 10 min centrifugation at 13,000 rpm, the supernatant was transferred to a 1.5 ml microcentrifuge tube, ready to be used for PCR amplification.

The sizes and integrity of the DNA fragments obtained in each extraction procedure were evaluated in 2 % agarose (LONZA) by gel electrophoresis and finally DNA was diluted with sterile distilled water and its final concentration and purity were measured at 260 nm and by the ratio of OD₂₆₀/OD₂₈₀.

DNA amplification

Fifteen commercial primers with sizes from 10 to 12 bases, with variable nucleotide proportion (G–C content above 60–70 %), were used for the amplification process (Table 1). The purified oligonucleotides were supplied by PRIMM (Milan, Italy). Amplification was performed in 25 µl reaction volumes containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 200 µM of each dNTP and 12.5 µl of Taq DNA Polymerase (Roche). The RAPD-PCR protocol consisted of an initial denaturing step of 2 min at 95 °C, followed by 45 cycles at 95 °C for 1 min (denaturation), 36 °C for 1 min (annealing of primers), and 72 °C for 2 min (extension). Cycling was concluded with a final extension at 72 °C for 4 min, and then held indefinitely at 4 °C. Optimization of amplification conditions was carried out by ranging the template DNA from 10 to 100 ng, the primers from 5 to 10 pmol/µl, MgCl₂ from 0.5 to 5.0 mM, and temperature of annealing primers from 32 to 42 °C. A negative control, containing all reaction ingredients

except for template DNA, was included for each amplification. The thermal cycler used was a Mastercycler personal (Eppendorf, Hamburg, Germany). All amplifications were done in triplicate and on different days. Ten microliters of amplification products were separated electrophoretically in a 2 % agarose gel using Tris–borate–EDTA (TBE 1X), Sigma-Aldrich buffer system (Lonza) for about 1 h at 130 V. Fractionated bands were detected by ethidium bromide fluorescence under UV light and photographed with a Digital camera (Coolpix 950, Nikon, Tokyo, Japan).

Chemical reagents and exposure of cells

The reference genotoxic compounds, benzene (99 % purity) and hydrogen peroxide (99 % purity) were purchased from Sigma-Aldrich. Stock solutions and dilutions of the test substances were prepared in dimethylsulfoxide (Carlo Erba) (DMSO 1 %) as vehicle solvent and were added to the culture medium to give a final DMSO concentration (v/v) of 0.01 %.

Confluent cultures were trypsinized, centrifuged and cells were counted in a hemocytometer to give a cell suspension of $0.05/0.06 \times 10^6$ cells ml^{-1} . From this suspension, a volume of 3 ml was transferred into flasks (Cellstar, VWR International pbi SpA, Milan, Italy). After 24 h, the culture medium was removed from the flasks and the attached cells were washed twice with

5 ml of phosphate-buffered saline (PBS 1X) containing Ca^{2+} and Mg^{2+} . Then, 5 ml of L-15 culture medium containing the various dilutions of the test compounds (10 $\mu\text{l}/\text{ml}$ of benzene and 25 μM of hydrogen peroxide) in constant volumes of vehicle solvent were added and duplicate cultures were incubated for 2, 24 and 48 h. Negative controls contained no toxicant, but only DMSO at a final concentration of 0.01 %. At the end of incubation the cells were digested with trypsin and washed with PBS 1X. They were then fixed with ice-cold 75 % ethanol at 4 °C overnight and used for RAPD-PCR analysis of DNA damage.

Estimate of genomic template stability

The polymorphic pattern generated by RAPD-PCR profiles by using the selected primers allowed the calculation of Genomic Template Stability (GTS, %) as follows:

$$\text{GTS} = (1 - a/n) \times 100$$

where a is the average number of polymorphic bands detected in benzene and H_2O_2 cells and n the number of total bands in the non-treated cells. Polymorphisms in RAPD profiles included disappearance of a band and appearance of a new band with respect to the control profile. To compare the sensitivity of genomic template stability, changes in these values were calculated as a percentage of their control. The

Table 1 Primers with relative sequences and length used for the amplification reactions

	Primer identification	Primer sequences (3' → 5')	Length (bp)	Number of polymorphic bands
	D-4	5'-CTGTAGCATC-3'	10	5
	D-8	5'-CCAAGTCGACA-3'	11	3
	C-95	5'-CGGCCACTGT-3'	10	3
	C-96	5'-AGCACTGTCA-3'	10	6
	TRNA-1	5'-AGTCCGGTGCTCTA-3'	14	5
	ALU-2	5'-GACCCGCACC-3'	10	4
	S-91	5'-TGCCCCGTCGT-3'	10	4
	S-228	5'-GGACGCGGTT-3'	10	4
	S-237	5'-ACCGGCTTGT-3'	10	5
The first nine primers gave a small number of polymorphic bands (data not shown). The last six primers were chosen for the high number of polymorphic bands (above seven)	RAPD analysis primer 1	5'-GGTGCGGGAA-3'	10	11
	RAPD analysis primer 2	5'-GTTTCGCTCC-3'	10	10
	RAPD analysis primer 3	5'-GTAGACCCGT-3'	10	7
	RAPD analysis primer 4	5'-AAGAGCCCGT-3'	10	8
	RAPD analysis primer 5	5'-AACGCCGAAC-3'	10	7
	RAPD analysis primer 6	5'-CCCCTCAGCA-3'	10	10

Fig. 1 Comparison of genomic DNA extracted by three different methods, phenol:chloroform (*P*), salting out (*S*) and Chelex resin (*C*). Genomic DNA was made from the same culture preparations. In all the three cases, genomic bands showed similar molecular weights (above 3,000 bp), although different fluorescence intensities were obtained. *M* = 100 bp molecular weight marker

statistical analyses were carried out using the package software SPSS 9.05 for Windows (Liu et al. 2007; Rocco et al. 2011).

Results

Genomic DNA extraction methods

The suitability of the extraction methods was evaluated on the basis of the extract purity, the integrity and efficiency of the genome amplification, and the yield obtained with each one. Concentration and purity of DNA extracts are usually measured at OD₂₆₀ and by the 260 nm/280 nm absorbance ratio. Chelex extractions gave OD₂₆₀/OD₂₈₀ Index Values above 2, whereas the values obtained with salting-out and phenol–chloroform were above 1.50–1.70; these values indicate the high and acceptable DNA purity grade of the extraction. The yield obtained was different for each method: about 0.00292 ng of DNA per cell by salting-out, about 0.00145 ng of DNA per cell by

phenol–chloroform and about 0.1954 ng of DNA per cell by Chelex extraction. The integrity of the genomic DNA extracted by each procedure from the same charged amount of DNA is illustrated in Fig. 1.

DNA amplification by RAPD-PCR

The purity and integrity of the DNA template are crucial for good RAPD analysis (Zhou et al. 1997; Zhiyi and Haowen 2004; Sharma et al. 2010). Electrophoresis indicated that the size of DNA obtained from DLEC by the three different methods was the same. Even if the use of Chelex resin gave good results in terms of concentration of DNA, in the scanning of a complete genomic DNA pool (RAPD-PCR technique), this last extraction method is not recommended for the high number of undistinguishable bands (Fig. 2).

For the RAPD-PCR analysis, the random primer set that gave reproducible results, was RAPD Ready To Go, obtained from GE Healthcare Bio-Sciences Corp. (USA) (Table 1). The selection of the primers to obtain a specific band pattern depends on the number of bands obtained with each primer and their intra- and inter-genomic repeatability. It was found that conditions of 40 ng of DNA template, 5 pmol/μl for primers, 2 mM of magnesium ion, and 36 °C of annealing temperature were optimal, and were thus adopted in all the amplifications. An annealing temperature of 36 °C gave better results than the others tested. The primers selected for the band pattern were oligonucleotides from 10 bases (Table 1), which generated a number of bands above 10, from 100 to 1,500 bp in size. The result that all the primers can generate some distinct bands indicates that this set of primers has a high efficiency to amplify DLEC genomic DNA under this PCR condition, and they are available as genetic markers.

Genotoxicity potential of chemical reagents used

Eighteen amplification patterns were identified in our experimental settings. In the amplifications carried out with genomic extracts derived from different cell passages (from 50 to 100), no differences were observed in the band patterns. The RAPD-PCR pattern obtained was specific and stable (Fig. 3). The specificity of the pattern was tested by amplifying genomes extracted from the RTG-2 cell line with all six selected primers (RAPD analysis primers: 1–6)

Fig. 2 DNA efficiency in RAPD-PCR amplification coming from the three different extraction procedures applied to the same culture, by using the six selected primers (1 = RAPD analysis primer 1; 2 = RAPD analysis primer 2; 3 = RAPD

analysis primer 3; 4 = RAPD analysis primer 4; 5 = RAPD analysis primer 5; 6 = RAPD analysis primer 6). *P* = phenol:chloroform, *S* = Salting out and *C* = Chelex extraction. *M* = 100 bp molecular weight marker

(Fig. 4b). Obviously the results obtained reflect differences between DLEC and RTG-2 fingerprints. The RAPD patterns generated by the benzene and H_2O_2 -exposed cells were different with respect to the control groups. The differences in RAPD patterns refer to band intensity, loss of normal bands and appearance of new bands as compared with the control. These effects manifested quite rapidly and at low genotoxic agent concentrations. Besides the number of appearing/disappearing RAPD bands correlated positively with increasing exposure time. The RAPD pattern was performed twice with each chosen primer, confirming the variation of bands. The results of this experiment clearly demonstrate the potential of the DLEC cell line to be used for predicting the toxicity induced by environmental pollutants.

Estimate of genomic template stability

Changes in the RAPD patterns are expressed as decreases in GTS, a measure reflecting the change in the number of RAPD profiles generated by benzene and H_2O_2 , in relation to profiles obtained from the non-treated cells. As an example GTS values referred to primer 1 are illustrated in Fig. 5.

Discussion

In recent years DNA profiling through the RAPD-PCR technique has been used for the analysis of molecular characterization of several model organisms. A constant pattern in the DNA banding is essential when an organism or cell line is used to detect DNA alterations produced by environmental genotoxic chemicals. Evidently, RAPD-PCR technology is reproducible, rapid and sensitive and it can be used for examination and estimation of genomic variation in genotoxic studies.

Some extraction methods, such as phenol–chloroform and salting-out, are more suitable than others, such as Chelex extraction, in the RAPD-PCR technique because the grade of purity, integrity, and effectiveness is very high. Although the yield obtained with Chelex extraction is higher, our results do not indicate its use.

To demonstrate the actual efficiency of the RAPD-PCR technique in the molecular characterization of the marine DLEC cell line we have highlighted the reproducibility of the electrophoretic profile generated by the amplification of DNA with the primers selected. The band pattern established using RAPD analysis primers: 1–6 was specific and stable in the DLEC cell

Fig. 3 RAPD-PCR fingerprints obtained from DLEC cell line generated by three selected primers, RAPD analysis primer 1 (lanes 2 and 3), RAPD analysis primer 3 (lanes 4 and 5) and RAPD analysis primer 6 (lanes 6 and 7), proved to be stable for two different genomic extracts, named A and B. In all cases, the electrophoretic patterns are identical for the same primer. *M* = 100 bp molecular weight marker

line. Under the selected conditions, the fingerprinting obtained was clear. It was formed by a sufficient number of bands (between 250 and 1,500 bp) in the product amplified using this primer set. The observation that we obtained similar fingerprinting profiles in all the genomic DNA extracts indicates the degree of

their homology and provides referable data for the RAPD analysis of other cell lines. These primers can be useful for the identification of other cell lines, for the detection of cross-contamination among them, and for the characterization of the molecular variations after treatment with environmental contaminants.

The traditional method of identifying DNA damage is gradually being replaced by molecular studies that are more reliable (Sharma et al. 2010). Molecular genetics have provided a good number of innovative techniques to measure genotoxicity.

The second aim of our research was to standardize the use of RAPD-PCR technique to investigate the eventual genotoxicity of pollutants utilizing the DLEC cells as in vitro experimental model. The difference in the electrophoretic pattern was also analyzed from a quantitative point of view, considering the reduction of the percentage of GTS in relation to untreated cells. To reach this goal it was essential to perform the technique on cells exposed to substances already considered to be genotoxic.

Benzene is a chemical intermediate used for the synthesis of pesticides, dyes, and plastic resins, a component of commercial gasoline, and is present in the air, food, feed stuffs, tobacco, and pyrolysis products. According to several studies, benzene produces a variety of tumors, gland carcinomas, oral cavity carcinomas, hepatocarcinomas, and possibly, mammary carcinomas. Studies on the mechanisms of the carcinogenic action of benzene have focused on its conversion

Fig. 4 Electrophoretic pattern of amplification of DNA isolated from DLEC (a) and RTG-2 (b) cell lines. RAPD-PCR analysis was performed using six selected primers (1 = RAPD analysis primer 1; 2 = RAPD analysis primer 2; 3 = RAPD analysis primer 3; 4 = RAPD analysis primer 4; 5 = RAPD analysis primer 5; 6 = RAPD analysis primer 6). Different random products were obtained for each cell line. *M* = 100 bp molecular weight marker

Fig. 5 Changes of percentage of GTS (genome template stability) in DLEC cells after exposition to benzene and H_2O_2 . Black = non-treated cells, White = benzene treated cells, Gray = H_2O_2 treated cells

to toxic metabolites and its ability to damage DNA (Arfellini et al. 1985; Reddy et al. 1994). Several environmental contaminants or their metabolites may exert toxic effects through the mechanism of oxidative stress (Winston and Di Giulio 1991). Hydrogen peroxide (H_2O_2) is a common reactive oxygen intermediate generated by various forms of oxidative stress. ROS induce both cytotoxic and mutagenic damage (Cadet et al. 1999). Hydrogen peroxide (H_2O_2), one of the main ROS, is known to cause DNA damage in various cell types (Sun-Yee et al. 2000). It is a natural source of oxidative damage in cells, causing a spectrum of DNA lesions, including single and double strand breaks. DNA damage due to H_2O_2 results from the production of the hydroxyl radical in the presence of transition metal ions such as iron via the Fenton reaction, in which H_2O_2 is

reduced in the presence of ferrous ions: $H_2O_2 + Fe^{2+} \rightarrow OH^\cdot + OH^- + Fe^{3+}$ (Collins 1999).

The results of our study support the use of RAPD-PCR analysis as an effective tool in species identification and in cross-contamination tests among different cell lines. Moreover, the results of our studies carried out in the laboratory using mutagenic products (benzene and H_2O_2) support the use of the DLEC cell line for the detection of mutations induced by the presence of chemical mutagens or pollutants in the aquatic environment by RAPD-PCR.

As suggested by Liu et al. (2005), modifications of 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 mainly to genomic rearrangements and less likely to point mutations; (2) DNA damage in the primer binding sites; and (3) interactions of DNA polymerase in the test organism with damaged DNA (Table 2). On the other hand, the appearance of new DNA bands occurs because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural changes or because some changes in the DNA sequence have occurred due to mutations, large deletions, and/or homologous recombination (Atienzar et al. 1999). Appearance of new bands may also be the result of genomic template instability related to the level of DNA damage, the efficiency of DNA repair and replication (Atienzar et al. 1999).

Moreover, other studies have reported that RAPD analysis or amplified fragment length polymorphism was more sensitive than classic tests such as the comet and micronucleus assay, since RAPD analysis is capable of detecting temporary DNA changes at lower concentrations of pollutants that may not finally manifest themselves as mutations (Liu et al. 2005; Atienzar and Jha 2006). As suggested by Liu et al.

Table 2 Changes in number and intensity of the bands in the RAPD-PCR profile (from Liu et al. 2005)

Bands variation	DNA damage	Genomic events
Appearance of bands	Point mutation Rearrangements	New site of primer annealing
Disappearance of bands	DNA adducts Point mutations Rearrangements Breaks in the double helix	Lack association between DNA and primers Loss of the primer binding sites Dissociation of enzyme/DNA
Variation of band intensity	DNA adducts Point mutations	Reduced polymerization of DNA New site of primer annealing

(2005), RAPD analysis in conjunction with other biomarkers such as growth parameters, etc., could prove to be a powerful ecotoxicological tool. The method is rapid, non-radioactive and applicable to any organism with the potential of detecting a wide range of DNA damage and mutations including point mutations and large rearrangements (Unyayar et al. 2006; Hagger et al. 2005). This assay has been successfully applied to study the effect of contaminants on population genetics and can be adapted to different stresses (Liu et al. 2005; Atienzar and Jha 2006).

Therefore, the change in the number of bands and the variation in their intensity, in the RAPD-PCR profile, are associated with alterations of genetic material. The genomic template stability (GTS, %) is directly related to the extent of DNA damage and also to the efficiency of DNA repair and replication (Rocco et al. 2011). For example, a high level of DNA damage does not necessarily decrease the genomic template stability (in comparison to a low level of DNA alterations) because DNA repair and replication may be inhibited due to excessive, lethal actions of the pollutant-induced adducts. If the survival of a population is affected, a toxic effect can completely inhibit a biological response; in contrast, the genomic template stability cannot be completely affected because the induction of DNA damage may not increase linearly (plateau effect). Furthermore, since genomic template stability may be related to different kinds of DNA damage, such as DNA adducts, mutations, rearrangements, etc., it would be difficult to anticipate a dose–response relationship.

Conclusions

In conclusion, based on the results obtained, we can state that phenol–chloroform and Salting-out are more suitable methods than Chelex resin for DNA amplification by RAPD-PCR technique in DLEC cells. This condition is due to an increased grade of purity and DNA integrity. The levels of DNA damage can be measured in DLEC cells by using the RAPD-PCR technique, which is rapidly becoming one of the most used methods in genetic toxicology.

In addition the results of the tests that we performed show significant differences in RAPD-PCR patterns in pollutant-treated cells as compared to the control groups, with respect to the variation in band intensity,

disappearance of bands, and appearance of new bands of amplified DNA.

Besides we show that the reproducibility of the RAPD patterns is possible in the DLEC experimental model, confirming that the variation of bands is stable. Moreover, variations in GTS% may be used as a suitable biomarker.

Finally, the DLEC cell line represents a good experimental model to assess the genotoxic effects of chemical compounds by means of RAPD-PCR technique.

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