Ecotoxicology, 13, 303–309, 2004

Molecular Characterization of Contaminant-Indicative RAPD Markers

CHRISTOPHER W. THEODORAKIS[1,*] AND JOHN W. BICKHAM[2]
[1]*Texas Tech University, The Institute of Environmental and Human Health, Lubbock, TX*
[2]*Texas A&M University, Department of Wildlife and Fisheries Sciences, College Station, TX*

Accepted 31 March 2003

Abstract. This paper describes genetic markers which can be used to study selection and genetic adaptation of organisms to radionuclide and other types of contaminant stress. Previous research using the randomly amplified polymorphic DNA (RAPD) technique has identified several markers which revealed genetic differences between contaminated and reference western mosquitofish (*Gambusia affinis*) populations. Experimental evidence suggested that these markers may be associated with loci involved in determining relative fitness in radionuclide-contaminated environments (''contaminant-indicative markers''). In the present study, Southern blot analyses show these markers to be highly conserved in DNA sequence and molecular length in sea urchins, mosquitofish, herring gulls and humans. Such conservation is thought to be rare among RAPD bands. Results of DNA sequencing efforts did not provide definitive evidence as to the identity of these loci, but indicated that short segments (<40 bp) of known DNA sequences were homologous to various regions of the RAPD sequences. Furthermore, the regions of homology seemed to be non-randomly distributed along the length of the RAPD markers. Although the identity of these bands is still unknown, the high degree of conservatism suggests that these loci might play an important role in molecular processes.

Keywords: RAPD; environmental contamination; DNA sequence; *Gambusia affinis*; evolutionary toxicology

Introduction

The randomly amplified polymorphic DNA (RAPD) technique has been used to study such diverse phenomena as population subdivision (Kuusipalo, 1994), genetic diversity and genetic distance (Naish, et al., 1995), species identification (Dinesh et al., 1993, Bardakci and Skibinski, 1994), and construction of genetic linkage maps (Postlethwait et al., 1994). In has been applied in fields such as population genetics, taxonomy, species identification, parentage assessment (see Hadrys et al., 1992 for review), and endangered species management (Hedrick, 1992). In cases where RAPDs were used with other methods of DNA fingerprinting, similar results were obtained (Naish et al., 1995).

As with any molecular genetic assay, the RAPD technique possesses a number of advantages and disadvantages. Perhaps the greatest advantages for this technique are that it can potentially sample a large number of loci, and that no *a priori* DNA sequence information is needed to perform the

*To whom correspondence should be addressed:
Tel.: 806-885-0252; Fax: 806-885-4577
E-mail: chris.theodorakis@tiehh.ttu.edu

assay. The main disadvantages are that the sequences of the amplified DNA fragments are unknown, and some of the amplification products are not reproducible. Because the sequences of the markers are unknown, determination of homology between species and possible functions of these loci is problematic.

Thus, determination of the molecular characteristics (i.e., DNA sequence, homology between species) of RAPD markers may provide useful information as to the identity of these markers, and would also allow development of single-locus markers based upon the DNA sequence of the RAPD bands. Such "sequence characterized amplified regions (SCARs; Paran and Michelmore 1993) could then be amplified by PCR for use in genetic analysis. Although SCARs have been applied in plant pathology, crop science, aquaculture and species identification (Zhou et al., 2001, Jun et al., 2002, Polashock and Vorsa, 2002, Yau et al., 2002), to date, however, this approach has not received much attention in the fields of population genetics and ecotoxicology. Perhaps one reason for this is that the RAPD technique can produce many polymorphic markers, so deciding on which marker to focus efforts of molecular characterization is difficult. Ideally, one could concentrate efforts on markers which were most informative in terms of differentiating among populations. Also, if there were markers which appeared to be correlated with fitness components, then these markers would be good candidates for study.

In this regard, several recent investigations (Theodorakis and Shugart 1997, 1998; Theodorakis et al., 1998, 1999) have identified RAPD markers that may be linked to loci that impart a selective advantage in radionuclide-contaminated habitats. It was found that certain RAPD markers were present at a higher frequency in two contaminated sites than in two reference sites (These bands will hereafter be referred to as "contaminant-indicative bands" or CIBs; Theodorakis and Shugart 1997). Furthermore, fish with CIBs had higher fecundity and fewer strand breaks than the fish that did not (Theodorakis and Shugart 1997, 1998; Theodorakis et al., 1999). In another study (Theodorakis et al., 1999), in which *G. affinis* were caged in a radionuclide-contaminated pond, it was found that fish which displayed the contaminant-indicative bands had greater survival rates than did fish which did not. An additional investigation used the same RAPD primers to compare the population genetic structure of eastern mosquitofish (*G. holbrooki*) living in radionuclide-contaminated and reference ponds on the DOE Savannah River Site (SRS) facility near Aiken, SC. It was found that some of the contaminant-indicative bands identified in *G. affinis* at ORNL were also present in the SRS *G. holbrooki* at a higher frequency in the contaminated population than in the reference populations. Southern blotting confirmed that the *G. affinis* contaminant-indicative bands were homologous to *G. holbrooki* contaminant-indicative bands. All of the above findings are consistent with the hypothesis that the contaminant-indicative bands are markers of loci which influence relative fitness and radioresistance in mosquitofish from radionuclide-contaminated populations. Consequently, it would be of interest to determine the molecular characteristics of these bands.

Thus, the objectives of this study are to (1) determine the DNA sequence of three contaminant-indicative bands, (2) compare the nucleotide sequences of these bands to the GenBank database to search for homology with any previously described loci, (3) investigate conservatism among RAPD products amplified from a diversity of species using the same primers and (4) develop PCR primers to amplify the contaminant-indicative bands, rather than the entire RAPD fingerprint.

Methods and materials

DNA sequencing

RAPD products from *G. affinis* were amplified according to Theodorakis et al., (1998) using primers OPD2, OPD7 (Operon Technologies, Alameda, CA) and UBC16 (University of British Columbia). They were separated by electrophoresis in 3% agarose for 4 h at 10 V/cm. The bands $OPD2_{1560}$, $OPD7_{1390}$, and $UBC16_{1020}$ (Theodorakis and Shugart, 1997) were excised from the gel and purified with Commercially available DNA purification kits (Quiagen Corp., Santa Clarita, CA). Bands were inserted into a plasmid vector (pNoTA/T7; 5'-3' Corp., Boulder, co.) and transfected into *Escherichia coli* according to

manufacturer's directions. This plasmid contains PCR primer binding sites derived from M13 phage DNA sequences flanking the insertion site. Insertion of the RAPD band into the plasmid was verified by reamplifying the band using the original RAPD primers. The size of the reamplified bands was then compared to the size of the original bands. The RAPD insert was then sequenced by PCR using cycle sequencing kits (Perkin-Elmer ABI) using commercially available M13 sequencing primers (Sigma Chemical Co., St. Louis, MO). Sequencing reaction products were analysed on a Perkin-Elmer ABI 373 or 377 automated sequencer (Perkin-Elmer Corp., Norwalk, CT). Sequences of RAPD products were compared to known sequences in the GenBank database (National Institutes of Health, National Center for Biotechnology Information). Determination of relative nucleotide content (%AT) and searches for open reading frames were performed using Sequencher® software (Gene Codes Corp., Ann Arbor, MI).

Blotting and hybridization

Contaminant-indicative RAPD bands were isolated as above. The bands were then used to make hybridization probes by adducting them with biotin using commercially available kits (Rad Free® Labeling and Detection Kit, Schliecher and Schuell, Keene, NH).

Genomic DNA was isolated from green sea urchin (*Strongylocentrotus droebachiensis*), herring gull (*Larus argentatus*), and western mosquitofish (*G. affinis*), and was obtained from commercially prepared human placental DNA (Sigma Chemical Co., St. Louis, MO). RAPD amplifications were conducted using primers OPD2, OPD7 and UBC16. The amplification products were separated using agarose gel electrophoresis as described above. The RAPD products were then transferred to nylon membranes using Southern blotting techniques (Sambrook et al., 1989). The *G. affinis* probes were then hybridized to DNA bound to the membranes at 42 °C for 16 h. The hybridization cocktail consisted of 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50% deionized formamide, 5% blocking powder (Schliecher and Schuell) and 1% SDS. The membranes were then washed twice with 2× SSC/SDS (0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS) for 5 min. at room temperature and twice with 0.1× SSC/SDS (15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS) for 5 min at 60 °C. Hybridization of the probe was assayed with chemiluminescent detection on X-ray film (Kodak X-Omat; Sigma Chemical, St. Louis, MO, USA) using strepavidin-alkaline phosphatase conjugate and Lumiphos® substrate, as per manufacturer's directions (Schliecher and Schuell).

Results

DNA sequencing

The sequences of each band were deposited in GenBank, with the accession numbers and molecular lengths of each of these bands reported in Table 1. A search of GenBank revealed that short stretches (19–40 bp) of the RAPD bands were homologous to other known sequences (Fig. 1). All RAPD bands sequenced were at least 64% AT (Table 1). Several possible open reading frames were identified (i.e. a sequence bounded by a start Codon [ATG] and a stop Codon [TGA, TAG]), but the transcription products of all of them would be < 75 amino acids in length.

Blotting and hybridization

Probes made from *G. affins* bands $OPD2_{1590}$, $OPD7_{1390}$, and $UBC16_{1020}$ hybridized to RAPD

Table 1. Genbank accession numbers, molecular lengths and relative nucleotide composition (%AT) of three RAPD markers

RAPD marker[a]	Accession #	Molecular length (bp)	%AT
$OPD2_{1560}$	AF136525	1677	66.0
$OPD7_{1390}$	AF136526	1032	65.3
$UBC16_{1020}$	AF136527	1049	64.7

[a]Nomencalture of the RAPD markers is as described in Theodorakis and Shugart (1997).

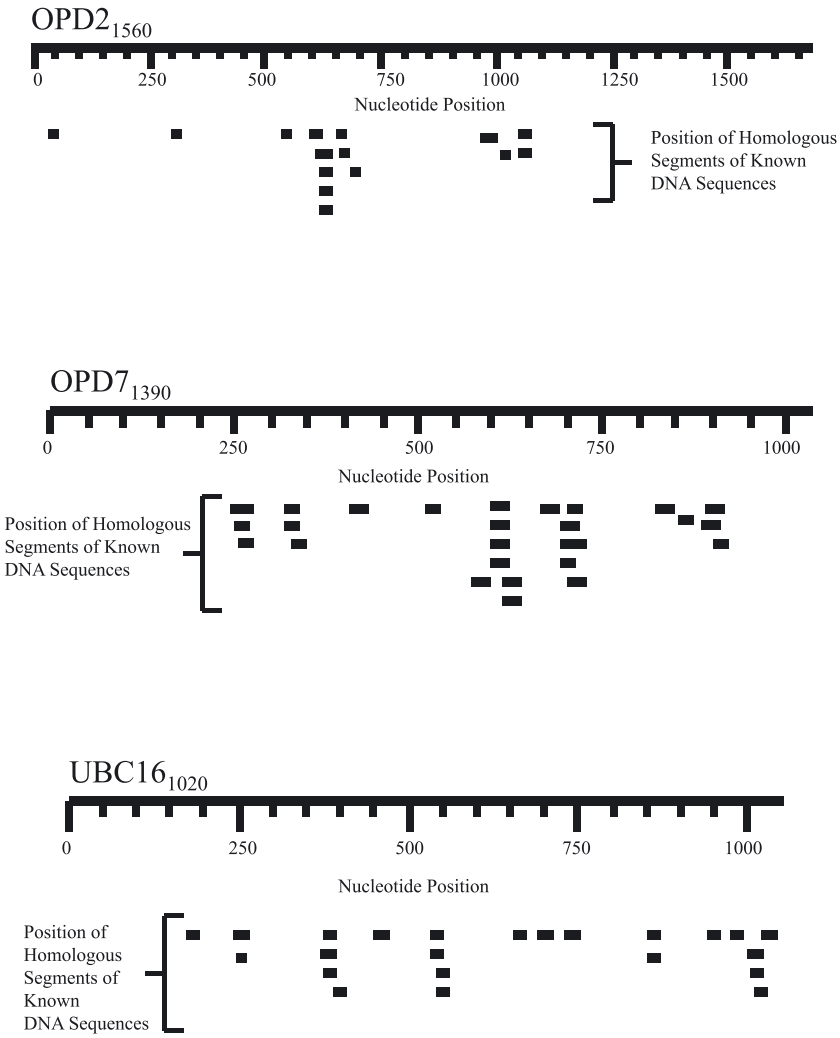

Figure 1. Schematic diagram of three RAPD markers and the relative position and length of homologous segments of known DNA sequences (determined from GenBank). The long line with tick marks represents the RAPD band, with the tick marks representing nucleotide position along the RAPD band. The shorter bands beneath represent segments of known genes corresponding to homologous segments on the RAPD band.

products from sea urchins, herring gulls and humans, with the exception that probe OPD2$_{1590}$ did not hybridize to herring gull RAPD products. In all cases, the RAPD bands were the same length in the four species (Fig. 2).

Discussion

The most significant finding of this study is the high degree of conservation between bands from species over a variety of taxa. These bands are not only homologous in sequence (as indicated by Southern blotting) but also of very similar (if not the same) size. This suggests that there may be an important physiological or molecular function associated with these loci. Although the exact nature of this function is not known, the studies by Theodorakis et al. discussed above suggest that these markers may be linked to loci that contribute to relative fitness and radioresistance in contaminated habitats. The high degree of conservation

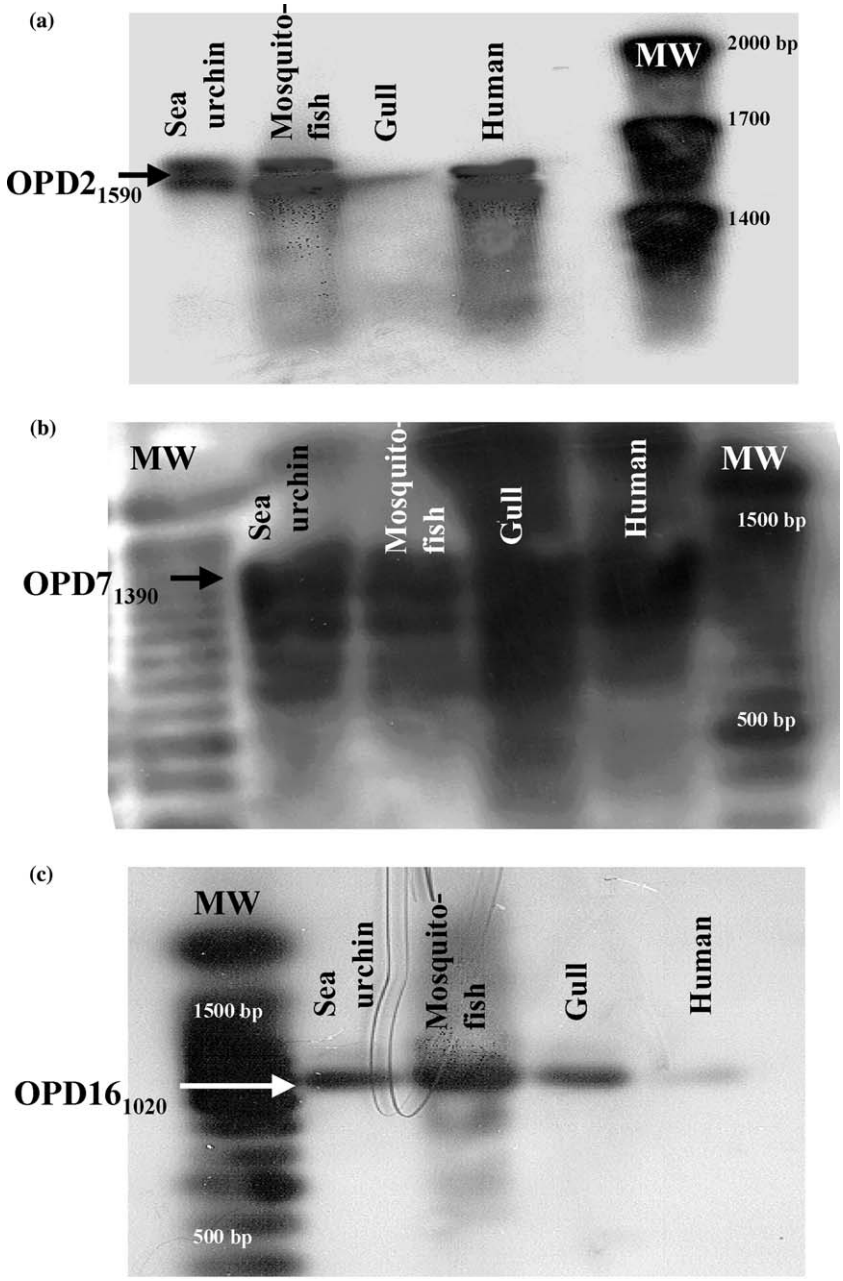

Figure 2. Hybridization of *Gambusia affinis* RAPD bands $OPD2_{1590}$ (a), $OPD7_{1390}$ (b), and $UBC16_{1020}$ probes to green sea urchin (*Strongylocentrotus droebachiensis*), mosquitofish (*G. affinis*), herring gull (*Larus argentatus*) and human RAPD products amplified with primers OPD2 and OPD7, respectively. The hybridization signal was detected using X-ray film and Chemoluminescence. MW = 100 bp molecular length standard.

between species also indicates that these markers would be widely applicable to studying effects of contaminants on population genetics and adaptation to xenobiotic stress.

These findings may have implications for systematic study as well. Other studies have used RAPD markers for studying systematic relationships between various taxa, but have only included

data on presence/absence of certain bands (Espinasa and Borowsky, 1998). However, that approach only gives limited amounts of information. A more definitive analysis would be to sequence particular RAPD bands that are found in a variety of taxa, such as the bands presented in the present study.

It was noted that these data reveal a discrepancy between the molecular length as determined by electrophoresis and by DNA sequencing. Theodorakis and Shugart (1997) estimated the size of the contaminant-indicative bands amplified with primers OPD2, OPD7 and UBC16 to be 1560, 1390 and 1020 bp, respectively. Sequencing analysis revealed the true molecular lengths for $OPD2_{1560}$ and $UBC16_{1020}$ (1677 and 1049 bp, respectively) to be fairly close to these estimates. However, the exact size of band $OPD7_{1390}$ (1032 bp) fell far short of that estimated by electrophoresis. It is unlikely that there was a deletion during cloning because this band was reamplified from the cloned insert using the original RAPD primer, and subjected to agarose gel electrophoresis, which indicated the size of the product to be approximately 1390 bp. Hence, it may be that this band assumes a configuration that impedes its migration through the gel, making it appear longer than it actually is. Nonetheless, this band will continue to be referred to as $OPD7_{1390}$, in order to be consistent with previously published work, and to facilitate its identification in an agarose gel.

Although the identity of these markers remains a mystery, the fact that the RAPD bands are not homologous to any known sequences implies that they may be novel markers of contaminant-induced selection and radioresistance. Such markers would have promise for studies dealing with population genetic alterations in response to contaminant stress. One such application of these markers would be the development of hybridization probes and PCR primers that could be used in a wide variety of species. The results of the present study indicate that such primers could amplify PCR products from multiple taxa, and thus have wide applicability in toxicological study.

Perhaps one of the most efficient methods of doing this would be to use these RAPD markers as hybridization probes or to design band-specific primers that amplify only the contaminant-indicative markers, rather than the entire RAPD fingerprint (i.e., SCARs). Markers produced by these probes would be amenable to techniques which provide more DNA sequence information than does the RAPD technique: e.g., DNA sequencing, restriction enzyme digestion, or single-strand conformational polymorphism assays. The present study provides preliminary results which indicate that such an approach would be feasible, given the fact that at least some of the band-specific primers described herein amplify products from multiple species. However, more work needs to be done to develop other primers that could amplify products from multiple species.

Therefore, work is needed in order to gain additional insight as to the possible function of these loci. This would include northern blotting, cloning and sequencing of regions flanking the amplification sites, and mapping of the contaminant-indicative loci to specific regions of the chromosomes. Also, it would be informative to examine the population genetic structure of a wide variety of species exposed to multiple contaminants, using these markers. It is fully anticipated that such an approach will lead to the development of loci that are useful in the study of the effects of contaminants on the population genetics of native organisms.

Acknowledgements

This paper represents contribution number 91 of the center for Biosystematics and Biodiversity at Texas A&M University. This work was funded, in part, by a grant from the US Department of Energy, Cooperative Agreement No. DE-FC04-95AL85832 and by NIEHS grant ES04917. The opinions and conclusions presented herein are solely those of the authors and do not represent the official position of the US Department of Energy or NIEHS. Preliminary results of this study were presented at the 19th annual meeting of the Society of Environmental Toxicology and Chemistry, Charlotte, NC, November 1998.

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