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Choice of Methodology for Assessing Genetic Impacts of Environmental Stressors: Polymorphism and Reproducibility of RAPD and AFLP Fingerprints

MARK J. BAGLEY[1,*], SUSAN L. ANDERSON[2] AND BERNIE MAY[1]
[1]*University of California Davis, One Shields Avenue, Davis, CA 95616, USA*
[2]*University of California Davis, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923, USA*

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Abstract. PCR-based multi-locus DNA fingerprints represent one of the most informative and cost-effective measures of genetic diversity and are useful population-level biomarkers of toxicologic and other anthropogenic impacts. However, concerns about reproducibility of DNA fingerprints have limited their wider use in environmental biology. We assessed polymorphism and reproducibility of two common fingerprinting techniques, RAPD (randomly amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism), in pedigreed populations of rainbow trout (*Oncorhynchus mykiss*) to derive general rules for selective removal of problematic fingerprint bands. We found that by excluding bands that comprised less than 1% of total intensity, and by excluding the largest and smallest 10% of the bands, we could achieve nearly 100% reproducibility of AFLP fingerprints. Similar application of band exclusion criteria to RAPD fingerprints did not significantly enhance their reproducibility, and at least 15% of RAPD bands were not fully repeatable, heritable, or transmittable. The RAPD technique produced more polymorphic fingerprints than AFLP; however, considering that a substantial proportion of RAPD markers did not demonstrate Mendelian inheritance patterns, the AFLP methodology is to be preferred for future research.

Keywords: population genetics; fingerprint; RAPD; AFLP; reproducibility

Introduction

An increasingly diverse array of molecular marker approaches is available to researchers interested in assessing the genetic and evolutionary responses of populations to anthropogenic stressors (D'Surney et al. this volume). These strategies can loosely be classified into those that reveal detailed information about individual, well-characterized loci and those that assess polymorphism at large numbers of anonymous or poorly characterized loci simultaneously. The former class includes both protein markers (e.g. allozymes) and DNA markers such as microsatellites or restriction-fragment length polymorphisms (RFLPs) of mitochondrial DNA or targeted nuclear genes. The latter class is represented by the so-called multi-locus "DNA fingerprints", in which complex, highly polymorphic DNA marker profiles are generated.

One of the most important applications of molecular markers for population assessments is the measurement of within-population genetic diversity, since this trait

*To whom correspondence should be addressed: Molecular Ecology Research Branch, National Exposure Research Laboratory, US Environmental Protection Agency. 26 W Martin Luther King Drive, Cincinnati, OH 45268. Tel.: 513 569 7455; Fax: 513 569 7609; E-mail: Bagley.mark@epa.gov

is influenced by exposures to past stressors and may contribute to evaluations of toxicologic impacts at the population level (Bickham and Smolen, 1994), including assessments of future extinction risk (Evenden and Depledge, 1997). Simulation modeling indicates that an accurate assessment of within-population genetic diversity requires the analysis of approximately 200 polymorphic markers from a multi-locus DNA fingerprint or 50 polymorphic microsatellite markers (Mariette et al., 1999). This result is important since DNA fingerprints that incorporate hundreds of polymorphic markers can be produced relatively easily with little or no prior information about the genetics of the organism. In contrast, microsatellite panels containing in excess of a dozen or so markers exist for only a few commercially or scientifically important species, and development of new microsatellite markers for an organism entails significant time, expense, and expertise (see Armour et al., 1994). Thus, the allure of DNA fingerprinting methods is that they offer an expedient approach to measuring genetic diversity in natural populations and therefore, offer a more practical method for assessing population-level impacts of contaminants.

The most common DNA fingerprinting strategy currently used for genetic analyses of natural populations is randomly amplified polymorphic DNA analysis (RAPD; Williams et al., 1990; Welsh and McClelland, 1990). In the last couple of years, the technique has been used extensively to assess the impacts of known stressors on genetic diversity (e.g. Theodorakis and Shugart, 1997; Nadig et al., 1998; Krane et al., 1999). Its popularity stems from its relatively simple and straightforward extension of the polymerase chain reaction (PCR) process, as well as its high discriminatory power. Nonetheless, the technique has been criticized as poorly reproducible, incorporating both Mendelian genetic and nongenetic, artifactual information in the fingerprints (Ellsworth et al., 1993; Perez et al., 1998). More recently, amplified fragment length polymorphism analysis (AFLP; Vos et al., 1995) has become popular, because the fingerprints are perceived to be more repeatable (Vos et al., 1995; Mueller et al., 1996; Janssen et al., 1996) and polymorphic (Sharma et al., 1996; Paran et al., 1998). Both methods were developed by researchers studying inheritance and linkage among markers in pedigreed populations of domesticated plant species, and both have achieved their greatest success as markers for molecular breeding studies. It is often not appreciated by researchers studying the genetics of natural populations that a subset of markers (or "fingerprint bands") is commonly discarded in these molecular breeding studies, because they show questionable or ambiguous inheritance patterns. Since researchers studying natural populations by RAPD or AFLP typically do not have access to pedigreed individuals, the delineation of general rules for distinguishing reliable from unreliable markers would be of considerable value. Methods for eliminating artifactual bands are particularly important for studies of genetic toxicological effects, in which differences in the distribution of rare alleles between populations may implicate differences in mutation rates, selection, or past population sizes.

Here, we report the results of RAPD and AFLP analyses performed on mated pairs of rainbow trout (*Oncorhynchus mykiss*) and their offspring to assess the degree of polymorphism and reproducibility of the two methodologies. Characteristics of reproducible and anomalous DNA fingerprint bands were compared in order to determine those factors that most strongly influenced reproducibility. This information will be invaluable for selecting a highly informative subset of bands for population genetic analysis. Our focus was primarily on electrophoretic patterns on gels; other studies have addressed the influences of PCR conditions on the reproducibility of DNA fingerprints (Meunier and Grimont, 1993; Ellsworth et al., 1993; Park and Kohel, 1994; Schweder et al., 1995).

Methods

Three full-sib rainbow trout families were constructed by crossing the F_1 progeny of an anadromous strain (Oxbow Hatchery, Idaho) and a freshwater-resident strain (Clear Springs Foods, Idaho) back to the freshwater-resident strain. DNA was extracted from pelvic fins of each of the six parents twice, once using a standard organic extraction procedure (Taggart et al., 1992) and once using a slightly modified procedure that included proteinase K digestion in a strong urea buffer (Asahida et al., 1996). DNA was extracted from caudal fins of six larval progeny per family once using the method of Asahida et al.

The PCR protocol for RAPD analysis was as originally described (Williams et al., 1990; Welsh and McClelland, 1990). The AFLP protocol was modified slightly from the original description (Vos et al., 1995) so that fluorescein-labeled PCR primers could be substituted for ^{32}P-labeled primers. RAPD fingerprints are

typically detected on relatively low-resolution agarose gels, whereas AFLP fingerprints are usually analyzed by more resolute polyacrylamide gel electrophoresis (PAGE). To facilitate comparison between the two methods, AFLP fingerprints were resolved by 5% denaturing PAGE while RAPD fingerprints, which tend to produce higher molecular weight DNA fragments (bands), were resolved by 3% denaturing PAGE. The unlabelled RAPD fragments were visualized by post-electrophoresis staining with the intercalating dye Vistra Green (Amersham Pharmacia Biotech) using an agarose overlay (see Rodzen et al., 1998). A total of eight 10-mer RAPD primers and eight AFLP primer combinations were evaluated (Appendix 1).

All gels were scanned with a Fluorimager 595 (Molecular Dynamics) laser scanner. DNA bands were detected and fluorescence-quantified using the default settings for FragmeNT (Version 1.1, Molecular Dynamics), followed by manual addition and subtraction of bands where the software algorithm made errors that were visibly obvious. We attempted to identify all possible bands, including many that probably would not normally be scored for various reasons, to best characterize the features of reproducible bands. The bands were sized with FragmeNT by comparison to DNA standards (BioRad Low-Range Fluorescent Ladder) loaded in three of the 33 lanes for each gel. Bands were classified as segregating (polymorphic) or monomorphic (found in all individuals). Segregating bands were further classified as "repeatable", if the band was amplified from both genomic DNA extractions for all parental samples that expressed the band; "heritable", if, for each progeny sample that expressed the band, at least one of its parents also expressed the band; and "transmittable", if, for each parental sample that expressed the band, at least one of its six progeny also expressed the band. A band that failed any of these three criteria in any of the samples was scored "not reproducible". Repeatability, heritability, and transmittability of fingerprint bands were assessed in relation to the intensity, size, and isolation of the bands.

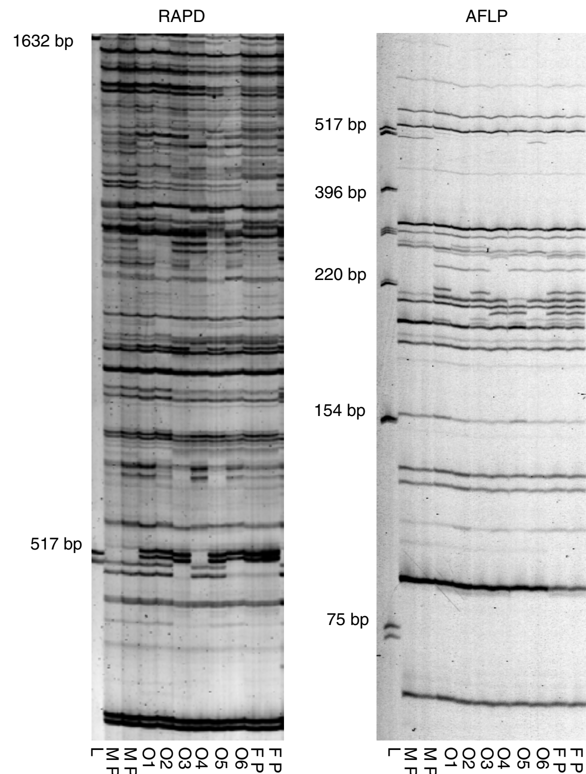

Figure 1. Partial RAPD and AFLP fingerprints for one of the three family groups analyzed showing both monomorphic and polymorphic DNA markers (bands). Primer OPG5 was used for this RAPD fingerprint and primers Eco GGA and MseCTT were used for the AFLP fingerprint. L: DNA size standard; MP: fingerprints from two DNA samples for the male parent; FP: fingerprints from two DNA samples for the female parent; O1–O6: fingerprints for the six offspring.

Results and discussion

Partial fingerprints for one family group are shown in Fig. 1 and are representative of the differences observed between the RAPD and AFLP techniques. A total of 261 distinct RAPD bands and 238 distinct AFLP bands were identified in the study. Approximately 74% of the RAPD bands and 53% of the AFLP bands were scored as segregating (polymorphic) within at least one of the three rainbow trout families. A total of 20.7% of segregating RAPD bands and 11.3% of segregating AFLP bands were scored "not reproducible" (Table 1). Lack of reproducibility of RAPD bands was influenced primarily by problems with heritability, but also by problems with transmittability and repeatability. Reproducibility problems for AFLP bands were primarily due to a lack of repeatability (Fig. 2).

Adequate band intensity is believed to be important for reproducible scoring of RAPD fingerprints (Hadrys et al., 1992), and exclusion of low-intensity bands is probably the most common adjustment applied in any DNA fingerprint analysis. To examine the effect of band intensity on reproducibility, bands that had an

Table 1. Average number of segregating (polymorphic) bands and reproducibility problems of RAPD and AFLP fingerprints for pedigreed populations of rainbow trout.

	RAPD	AFLP
A. All bands		
Number of segregating and monomorphic bands	38.9 ± 20.7	31.6 ± 11.8
Number of segregating bands	28.9 ± 15.1	16.6 ± 9.3
Percent of segregating bands not reproducible	20.7% (48/231)	11.3% (15/133)
B. Exclude bands less than 1% of total lane intensity		
Number of segregating bands	17.1 ± 10.5	6.8 ± 2.8
Percent not reproducible	16.1% (22/137)	1.9% (1/54)*
C. Exclude largest 10% and smallest 10% of bands		
Number of segregating bands	23.8 ± 13.5	13.6 ± 8.0
Percent not reproducible	20.0% (38/190)	8.3% (9/109)
D. Exclude bands with poor separation		
Number of segregating bands	11.9 ± 5.2	11.6 ± 5.5
Percent not reproducible	27.4% (26/95)	10.8% (10/93)
E. Combine criteria B and C		
Number of segregating bands	14.1 ± 9.0	6.3 ± 2.7
Percent not reproducible	15.0% (17/113)	0% (0/50)*

Data are provided for all bands scored (A) as well as for the remaining bands following different band exclusion criteria (B–E). The number of segregating bands represents the average (± s.d.) per primer (RAPD) or primer combination (AFLP). The percentage of segregating bands that is not reproducible is based on the total for all eight primers or primer combinations.
*Band exclusion criteria that resulted in significantly improved reproducibility relative to the unfiltered fingerprints are marked with an asterisk ($p < 0.05$, one-tailed Fisher's exact test).

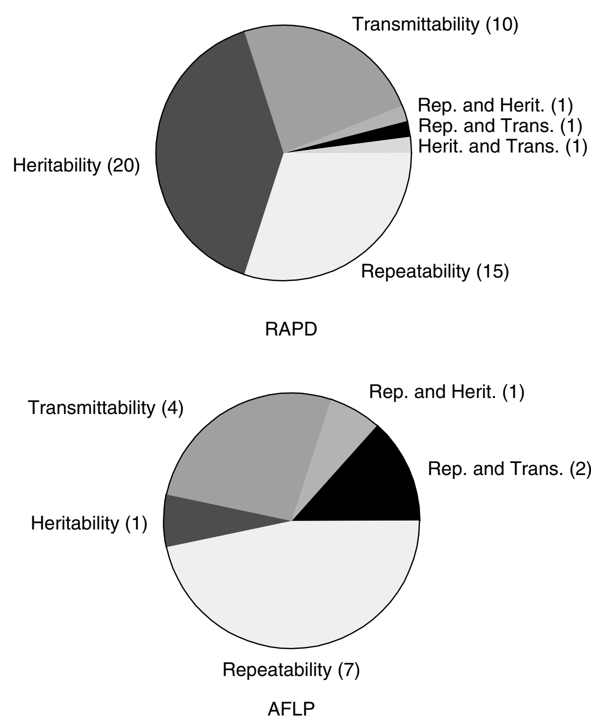

Figure 2. Reproducibility problems for RAPD and AFLP bands categorized as problems with heritability, transmittability, repeatability, or combinations of the three. Data are presented for RAPD and AFLP fingerprints prior to imposing band exclusion criteria. Parenthetic values are absolute counts summed over eight primer sets.

average intensity less than 1% of the total lane intensity were excluded. For this calculation, band intensity was measured as the average intensity of the band across all lanes in which it was scored, and total lane intensity was calculated as the sum of the average intensities for all bands. This resulted in the exclusion of 56.0% of all RAPD bands and 59.0% of all AFLP bands. Following the exclusion of weak bands, 16.1% of RAPD bands (22 bands) still had reproducibility problems but only 1.9% of AFLP bands (1 band) had reproducibility problems (Table 1). The improvement in reproducibility was not significant for RAPD bands ($p = 0.22$, Fisher's Exact Test) but was significant for AFLP bands ($p = 0.04$). Increasing the selectivity to exclude RAPD bands less than 2% of total lane intensity removed 69.6% of all RAPD bands but 15.7% (11/70 bands) were still not reproducible.

A second possible band exclusion criterion is to remove bands of either very high or very low molecular weight. Amplification of high molecular weight bands may be more strongly influenced by differences in DNA quality or PCR reaction conditions, causing them to be less reproducible. Additionally, very low molecular weight bands may include so-called "primer dimers", or artifactual amplification products produced from the interactions of primers alone. Exclusion of the largest and smallest 10% of bands had little measurable effect on RAPD band reproducibility, as 20.0% of the remaining bands still had reproducibility problems (Table 1). Exclusion of the largest and smallest 10% of AFLP bands left 8.3% of the bands scored with reproducibility problems, but the increase in reproducibility was not significant ($p = 0.31$, Fisher's Exact test).

Finally, bands that are too close together might result in confusion during band scoring, so bands that were very close in size were removed from the data set. In this case, bands were excluded if for any two bands x and y, with x larger than y, $\log(\text{size of band } x) - \log(\text{size of band } y) < 0.01$. While this procedure reduced the number of segregating bands by 58.9% for RAPDs and 30.1% for AFLPs, it did not significantly

increase reproducibility for either data set ($p = 0.88$ and $p = 0.55$, respectively; Fisher's exact test).

The above results suggested that it might be useful to combine the band size and intensity criteria, at least for AFLP gels. Imposing both the 1% of total lane intensity criterion together with exclusion of the smallest and largest 10% of bands excluded 51.2% of segregating RAPD bands. The estimate of the number of bands with reproducibility problems was 15.0%, which was not significantly better than the estimate for all RAPD bands ($p = 0.18$, Fisher's Exact Test). Applying the same criteria to AFLP bands resulted in the exclusion of 62.0% of segregating bands, but reproducibility was 100% and significantly improved over the unfiltered data set ($p = 0.01$).

We found no evidence to suggest that AFLP fingerprints are more polymorphic than RAPD fingerprints for rainbow trout. This may be partly due to the fact that we analyzed RAPD fingerprints with acrylamide gels rather than agarose gels, which provided us with increased resolution and possibly allowed us to distinguish more RAPD bands than is usually possible.

To summarize, by systematically excluding bands of low intensity and bands that are very large or very small, the AFLP methodology proved to be extremely reproducible. Therefore, we recommend that researchers investigating the genetics of natural populations with the AFLP method should incorporate a band size and intensity filter prior to band matching analysis. Unfortunately, none of the band exclusion criteria that we investigated significantly increased RAPD band reproducibility. This is not to say that RAPD fingerprints would not be made more reproducible by excluding bands based on intensity and size, but it does suggest that other, unidentified factors strongly influence RAPD band reproducibility.

It is worth reiterating that this study was designed to produce a large number of bands with imperfect reproducibility in order to have sufficient power to test the utility of different band exclusion algorithms. PCR templates were produced with two distinct DNA extraction methods and bands were considered to be problematic if a reproducibility problem was identified in any one of the three families. Thus, the magnitude of the reproducibility problems reported here is probably exaggerated relative to actual population genetic surveys (but see Perez et al., 1998). Nonetheless, the differences in reproducibility between AFLP and RAPD methods are real and of sufficient magnitude that we recommend future DNA fingerprinting studies incorporate the AFLP methodology.

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Appendix 1. RAPD primers and AFLP selective amplification primers used in the study. All sequences are written 5′–3′

Name	Sequence
RAPD primers	
OPA2	TGCCGAGCTG
OPA3	AGTCAGCCAC
OPA5	AGGGGTCTTG
OPA10	GTGATCGCAG
OPA12	TCGGCGATAG
OPG3	GAGCCCTCCA
OPG5	CTGAGACGGA
OPG7	GAACCTGCGG
AFLP primer pairs	
EcoGCA	GACTGCGTACCAATTCGCA
MseCAT	GATGAGTCCTGAGTAACAT
EcoGAA	GACTGCGTACCAATTCGAA
MseCAC	GATGAGTCCTGAGTAACAC
EcoGTA	GACTGCGTACCAATTCGTA
MseCGA	GATGAGTCCTGAGTAACGA
EcoGTA	GACTGCGTACCAATTCGTA
MseCCA	GATGAGTCCTGAGTAACCA
EcoGCA	GACTGCGTACCAATTCGCA
MseCAA	GATGAGTCCTGAGTAACAA
EcoGGA	GACTGCGTACCAATTCGGA
MseCAG	GATGAGTCCTGAGTAACAG
EcoGAA	GACTGCGTACCAATTCGAA
MseCCT	GATGAGTCCTGAGTAACCT
EcoGGA	GACTGCGTACCAATTCGGA
MseCTT	GATGAGTCCTGAGTAACTT

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