Randomly Amplified Polymorphic DNA (RAPD) Analysis of Ambystomatid Salamanders

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The polymerase chain reaction (PCR) has revolutionized many technical approaches to molecular biology. PCR-based techniques provide fast and efficient alternatives to traditional methods underlying gene cloning, library construction, and DNA sequencing. More recently, the PCR-based RAPD (Randomly Amplified Polymorphic DNA) technique has been used to rapidly generate polymorphic markers for linkage analysis, fingerprinting, and population studies in a variety of different systems (Williams et al. 1990, Welsh et al. 1990, Arnold 1991, Marten et al. 1991). Here I report on a preliminary investigation of RAPD in a family of hybrid ambystomatids.

Background. The RAPD technique makes use of a single-stranded, ten-base oligonucleotide primer in the PCR reaction. The primer is allowed to anneal to complementary nucleotide sites in a sample of denatured genomic DNA. Taq polymerase then utilizes the genomic DNA as a template and synthesizes a complementary sequence by extension from the 3' end of each primer. If two primer annealing sites occur facing one another on opposite strands of a segment of genomic DNA, the sequence flanked by these sites will be specifically amplified during multiple PCR cycles. Generally, only sequences < 3 kb are amplified.

Amplified sequences are size-fractionated in agarose gels, stained in ethidium bromide, and visualized under UV light.

Each primer, in combination with the DNA template, yields a specific pattern of size-fractioned sequences, or banding pattern. When a primer is used in the separate amplification of DNA from genetically similar individuals, the banding patterns will probably be similar. The individuals will share bands, corresponding to DNA sequences of the same size, that are presumably amplified from characteristic loci in the genome. In contrast, genetically dissimilar individuals will have fewer bands in common because of differences in the number and location of primer annealing sites. Individual RAPD loci are defined by the presence or absence of size-specific bands. Although examples of codominant RAPD's are known, for the majority of RAPD's, presence of the band is assumed to be the manifestation of a dominant allele and its absence (the null pattern) to signify a recessive allele. Segregation analysis can be used subsequently to verify that size-specific bands are inherited as hypothesized.

Methods. DNA was isolated from individuals of a backcross family by the procedure of Sambrook et al. (1989, pp. 9.17-9.19; See box, p. xx). The P1 adults were a male *A. mexicanum* and a female *A. tigrinum* and the P2 adults were an F1 male hybrid produced from the P1 adults and a female *A. mexicanum*. The *A. mexicanum* were obtained from the Indiana University Axolotl Colony, and the *A. tigrinum* was obtained from the Charles Sullivan Co. (Nashville, TN).

Although the RAPD protocol is relatively simple to perform, the precision and quality of each amplification is highly dependent upon differing concentrations of the reaction components. Problems associated with precision can generally be corrected, but the quality of RAPD amplification varies with each primer. In most cases, the PCR products of each primer can be optimized by adjusting concentrations of the reaction components. However, this is an expensive and time consuming process. For this reason, I screened several primers and chose PCR conditions that reproducibly yielded the highest quality amplifications from the majority. These conditions were 22 ng DNA for *A. mexicanum* and 30 ng DNA for *A. tigrinum*, 2 mM primer, 2.5 mM $MgCl_2$, 0.06 U Taq polymerase (Perkin Elmer Cetus), 180 mM each of dATP, dTTP, dCTP, cGTP (Pharmacia), and 1x PCR buffer (100mM Tris-HCl, pH 8.3, 500 mM KCl) at a total reaction volume of 15 ml. The PCR cycling parameters were: 5 min initial melt at 94°C; 45 cycles of 1 min 94°C, 1 min 35°C, 2 min 72°C; 5 min extension at 72°C. PCR amplifications were analyzed on 1.2% agarose gels (SeaKem LE, FMC Corp) in 1x TAE buffer, stained with ethidium bromide.

Ten different primers were used in the RAPD analysis. Each size-specific band that was generated from a PCR amplification was considered a potential RAPD locus. Bands from each of the P1 adults were scored if they

were observed in the F1 hybrid. Bands specific to the P2 A. *mexicanum* were scored if they were observed in F2 offspring. These data allowed for a comparison of the level of polymorphism between the P1 adults (interspecific polymorphism) and between the P1 and P2 *A. mexicanum* (intraspecific polymorphism).

Results. Randomly amplifed polymorphic DNA loci are characterized by the presence or absence of bands. Therefore, the segregation of bands is easily analyzed in the offspring. Figure 1 shows an example of segregating bands amplified by primer X (5'-GGACCCTTAC-3'). This primer detects both inter- and intraspecific polymorphisms.

Scorable RAPD bands were classified by their presence or absence in the P1 adults and the P2 *A. mexicanum*. Among these individuals, there were 7 possible relationships for each band:

a band could be unique to each of the individuals;

a band could be shared by two of the individuals;

or a band could be shared by all individuals.

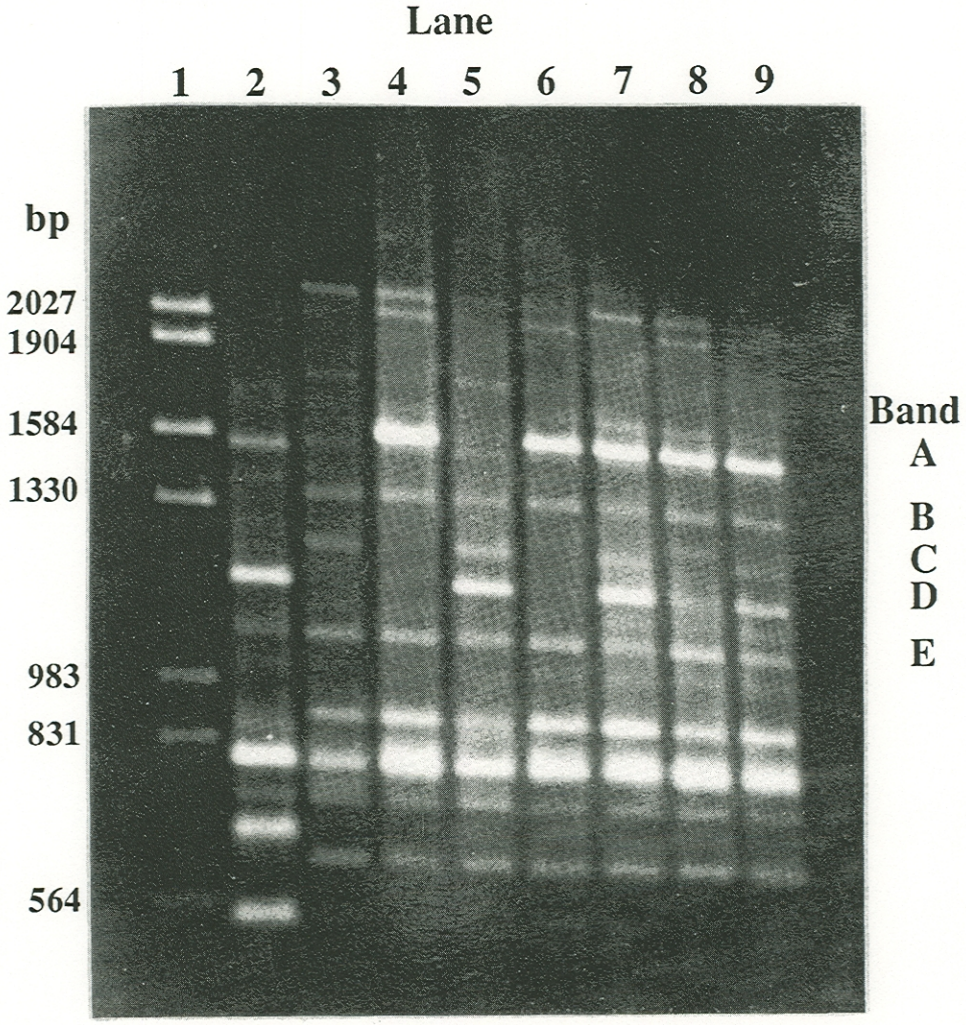

Figure 1. Inheritance and segregation of bands among individuals of a hybrid ambystomatid family. Lane 1 = molecular size markers (bp), Lane 2 = P1 *A. tigrinum*, Lane 3 = P1 *A. mexicanum*, Lane 4 = P2 *A. mexicanum*, Lane 5 = F1 Hybrid, Lane 6-9 = F2 offspring. Band A is diagnostic for the P2 *A. mexicanum* and is inherited by all of the F2 offspring. Band B is commonly shared by the P1 and P2 *A. mexicanum* and is inherited by the F1 hybrid and all of the F2 offspring. Band C is diagnostic for the P1 *A. mexicanum* and is inherited by the F1 hybrid and F2 offspring in lanes 7 and 9. Band D is diagnostic for *A. tigrinum* and is inherited by the F1 hybrid and F2 offspring in lanes 7 and 9. Band E is commonly shared by all individuals.

These data were used to construct a matrix showing the number of shared bands between the P1 adults and P2 *A. mexicanum* (Table 1), and a matrix showing the number of diagnostic bands (present in one individual, absent in the other) between the P1 adults and P2 *A. mexicanum* (Table 2).

Table 1. Matrix showing the number of shared bands between the P1 *A. tigrinum*, P1 *A. mexicanum*, and P2 *A. mexicanum*. Bands are not necessarily shared uniquely.

	P1 *A.tigrinum*	P1 *A.mexicanum*	P2 *A.mexicanum*
P1 *A.t.*	—	77	81
P1 *A.m.*		—	150
P2 *A.m.*			—

A total of 198 segregating bands were scored in this study. More bands were scored for the P1 and P2 *A. mexicanum* (155 and 163, respectively) than in the *A. tigrinum* (112). Fewer than half (76) of the total number of scored bands were shared by all three individuals. Most of the bands present in the P1 and P2 *A. mexicanum* were commonly shared (Table 1); only 18 bands specific to *A. mexicanum* were diagnostic between these two individuals (Table 2). Similarily, these individuals shared about the same number of bands with the P1 *A. tigrinum* (Table 1). These results suggest a low level of polymorphism among the *A. mexicanum* individuals in this family. In contrast, the level of interspecific polymorphism was considerably higher. Thirty-five bands were diagnostic for *A. tigrinum* and 78 bands were diagnostic for *A. mexicanum* in the F1 hybrid (Table 2).

Discussion. In theory, genome size and composition probably set the bounds on the number of RAPD loci that are amplified for a given set of PCR conditions. In this study, more segregating bands were generated for *A. mexicanum* than *A. tigrinum*. This suggests that *A. mexicanum* may have more potential RAPD loci than *A. tigrinum*. *A. mexicanum* has a slightly larger genome size than *A. tigrinum* and may have higher levels of repetitive DNA. It would be premature, however, to suggest a causal relationship between these genome characteristics and species-specific differences in the number of amplified RAPD loci. It is possible that differences in the number of amplified RAPDs between these species were generated by the way RAPD loci were scored. For example, if many of the RAPD loci in *A. tigrinum* are heterozygous, approximately half of these loci would not have been scored because null alleles would not have been observed in the F1 hybrid.

The incidence of RAPD polymorphism appears to depend upon degree of genetic similarity. The level of interspecific polymorphism detected by RAPD's in this study was much higher than the level of intraspecific polymorphism.

According to pedigree records from the IU Axolotl Colony, the P1 and P2 axolotls have one common grandparent and share additional ancestors further back in the lineage. This may explain the low incidence of polymorphism observed between these two individuals. A more thorough investigation of polymorphism among different strains is needed to assess the potential utility of RAPD's in studies of *A. mexicanum*.

Table 2. Matrix showing the number of diagnostic bands (present in one individual, absent in the other) between the P1 *A. tigrinum*, P1 *A. mexicanum*, and P2 *A. mexicanum*.

		Absent		
		A.tigrinum P1	*A.mexicanum* P1	*A.mexicanum* P2
Present	P1 *A.t.*	—	35	31
	P1 *A.m.*	78	—	5
	P2 *A.m.*	82	13	—

This study indicates that the RAPD technique may be an efficient method for generating linkage data among hybrid ambystomatids. Approximately 59% of the total number of loci scored between the P1 adults were diagnostic. I am in the process of determining

DNA Isolation Technique Salamanders

Modified from Sambrook et al., 1989. Molecular Cloning, pp. 9.17-p.19 by Randal Voss.

Blood is diluted in Acid Citrate Dextrose solution B (ACD) (1 volume blood to 1-2 volumes ACD) and stored at –80°C in 1.5 ml eppendorf tubes.

1. Thaw tube in water bath at room temperature.
2. Take 100-200 µl blood/ACD mixture and put in 1.5 ml eppendorf tube. (I generally work up duplicate isolations and vary the amount of blood/ACD mixture for each, 100 µl in one and 200 µl in the other. I generally work up about 6-16 tubes at a time.)
3. Spin the sample at 5000 rpm (setting 5 x 1000 on Eppendorf model 5415) in a microfuge for 15 minutes (room temperature).
4. Pour off supernatant. Resuspend the pellet in 1 ml PBS.
5. Repeat step 3.
6. Pour off supernatant. Resuspend the pellet in 400 µl of a solution consisting of 10 mM Tris-HCl (pH 8.0), 0.1 M EDTA, 20 µg/ml RNAse (A). After the pellet is resuspended, add SDS to a final concentration of 0.5%. (Adding the SDS after the pellet is resuspended seems to improve the isolation procedure.
7. Incubate with gentle shaking at 37°C for 1 hr. (I float my samples in a water bath and turn on the shaker to gently agitate the water).
8. Add proteinase K to a final concentration of 100 µg/ml. Gently mix.
9. Place in 50°C water bath for 2-3 hrs. Invert 1 time each hour.
10. Phenol Extract 2 times. I do all extractions at room temperature.
11. Phenol/Chloroform extract.
12. Chloroform extract.
13. I add 100 µl of 5 m Ammonium Acetate and 1 ml of 100% EtOH.
14. I invert the tube several times to precipitate the DNA. Usually a floating mass of DNA will be visible in the tube. Fish this mass out of the tube and put it in a fresh 1.5 ml eppendorf tube. (I don't pellet the DNA if I can see the large floating mass. Sometimes it takes a minute or two for the DNA to become visible. The DNA yield is not as high when you do not pellet the DNA, but the yield is sufficient for most applications. More important, you avoid the potential nightmare of resuspending the tightly packed pellet.)
15. Add 1 ml of ice cold 70% EtOH. Invert the tube a couple of times.
16. Spin down the pellet in a microfuge for 4 minutes at 10,000 rpm (at 4°C).
17. Repeat steps 15 and 16, but only spin for 2 minutes.
18. Resuspend the pellet before it completely dries in water or TE. (I start with 100 µl and add more if necessary, in 25-50 µl increments, until the pellet is totally resuspended.)
19. If all goes well, you should obtain 20-60 µl of DNA.
20. I quantify my DNA samples using the Hoefer TKO 100 Fluorometer.

what proportion of the RAPD loci are targeting single or moderately repetitive sequences. These results should indicate whether RAPD linkage analysis is feasible. Alternatively, the closely allied technique of bulked segregant analysis (Michelmore et al. 1991) may allow for the rapid detection of markers for genes that segregate in hybrid crosses. For example, the axolotl genes for dark/white and melanoid segregate in crosses according to expected mendelian ratios (unpublished data). In the future I plan to use both of these PCR-based strategies in molecular studies of ambystomatids.

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