

RAPD Analysis of Genetic Diversity - an Inquiry-Based Laboratory for Undergraduates

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Abstract

Darwin observed that there is variation within populations and this insight was critical to his formulation of the theory of natural selection. Later work defined the physical basis of heredity and the role of mutation in generating differences between individuals. Today the genetic diversity of organisms within a single species or within closely related species can readily be investigated at the molecular level by comparison of their DNA. While DNA sequencing, microsatellite analysis and the use of hybridization methods have been employed to characterize DNA, these procedures require knowledge of the DNA sequence and/or relatively large amounts of DNA. Recently a procedure known as random amplification of polymorphic DNA (RAPD) has been developed which can be applied to all organisms using nanogram amounts of DNA and without prior knowledge of their sequences or the use of radioactivity. An investigative undergraduate laboratory has been developed using RAPD analysis to characterize the genetic diversity and genetic structure of a rare tree, *Lyonothamnus floribundus*. RAPD analysis of genetic diversity is suitable for undergraduate research, an honors laboratory in introductory biology or an upper division laboratory in molecular biology, genetics, ecology or evolution and the organism of choice could be any local plant, fungi, or animal (such as an insect). In this investigation of *L. floribundus*, a species endemic to the California Channel Islands, plant material is

marked and samples collected in the field where the ecology and evolution of the plants are discussed. DNA is isolated in the laboratory from young leaves using a DNeasy Plant kit and quantified using a UV spectrophotometer or a fluorometer. PCR amplification of this template DNA is performed on a thermal cycler in Ready-to-go RAPD Analysis Beads and one of a set of random oligonucleotide primers. The PCR products are analyzed by agarose gel electrophoresis, photographed to provide a permanent record, and the genetic differences calculated from the banding patterns obtained. One of several computer programs such as Paup (Phylogenetic analysis using parsimony) are then used to analyze the relatedness of the trees.

I. The Research System:

Genetic diversity within rare plant species and the genetic structure of surviving populations are of considerable interest because of their important evolutionary implications and their relevance to conservation efforts. Careful study of the fossil record, morphology, ecology, and isozymes have been important in efforts to illuminate the evolutionary history of a species. Methods which directly survey the total plant genome now promise to provide new information which will significantly enhance our understanding of the genetic status and the genetic history of rare species. In 1990 Williams and coworkers (Williams *et al.*, 1990) and Welch and McClelland (Welsh and McClelland, 1990) developed a method of genome analysis which relies on a comparison of fragments amplified by the polymerase chain reaction (PCR) using as short oligonucleotide primer of random sequence. The random amplification of DNA polymorphisms (RAPD) provides a unbiased method of quantifying genomic diversity and has several distinct advantages for the analysis of rare plants: no prior knowledge of the DNA sequence is necessary, the amount of DNA needed for PCR amplification is very small, and the procedure is simple, rapid, inexpensive, and reproducible.

In the present study, undergraduates have used RAPD analysis to investigate genetic structure and genetic diversity in *Lyonothamnus*, a genus known from the fossil record but now surviving in the wild as a single species, *L. floribundus*, on four islands off the California coast. The growth pattern of the trees in which trunks are spaced close together in isolated groves, the presence of extensive root-

sprouting, visible root connections between trunks in some groves, together with the near-absence of successful propagation from seed in nature suggest that many or all of the existing groves of *L. floribundus* may be clones, each derived from a single root-stock (Junak, 1987). This study represents the first molecular genetic analysis of the relictual endemic *L. floribundus* and has implications regarding the history, dispersal, reproduction and the conservation of this rare species.

2. Research Techniques

RAPD analysis includes the following procedures and techniques:

sample collection, DNA extraction and quantification, the polymerase chain reaction (PCR), electrophoresis and data analysis.

Sample collection:

Field trips to the University of California Natural Reserve System Field Station on Santa Cruz Island took place in February and March when the ironwood trees have new growth. Groves were selected that were adjacent to roads or other landmarks, and individual trees marked with aluminum tags. Young leaves (4-6 leaves about 2-3 cm long) were collected and placed in labeled baggies in the field. These were then refrigerated for one to several days and then weighed and frozen in liquid nitrogen for long term storage at -70°C.

DNA extraction and quantification.

Genomic DNA was extracted from the frozen samples using a DNeasy Plant kit according to instructions provided by the manufacturer. Briefly, frozen tissue was ground in liquid nitrogen and mixed with a lysis buffer containing RNase. After centrifugation to remove insoluble material, the supernatant was passed through a "Qiasredder" minicolumn. The DNA in the flowthrough was then rendered hydrophobic by the addition of a chaotropic agent and ethanol and purified by hydrophobic chromatography on a mini-spin column. The DNA concentration of the eluate was determined using a UV spectrophotometer or a TKO 100 Fluorometer (Hoefler Scientific Instruments, San Francisco, CA).

Polymerase Chain Reaction (PCR)

Random 10-mer oligonucleotide primers were used singly for RAPD analysis. The PCR reactions were performed using Ready-To-Go RAPD Analysis beads which contain the thermostable DNA polymerase, nucleotides and buffer optimized for RAPD reactions.

Electrophoresis

The PCR products were fractionated by electrophoresis and the sizes of the amplified fragments determined by comparison with DNA standards electrophoresed on the same gel. The gels were stained with ethidium bromide and photographed. The images were also recorded electronically with a video camera connected to a macintosh computer using the program NIH image.

Data Analysis

The banding patterns were determined by visual analysis of the photographs. All bands amplified by a single primer were numbered and each tree or grove of trees, if all were identical for all primers, was scored for the presence or absence of these markers. The polymorphic bands were use for further data analysis. A distance matrix was calculated and parsimony and neighbor joining clustering was carried out using the program Phylogenetic Analysis Using Parsimony (PAUP) Version 4.0 (Swofford).

3. Experiments:

Introduction

RAPD analysis is suitable for use for undergraduate research projects and/or in laboratories in ecology, genetics and population genetics, molecular biology and diversity or for honors students at any level beginning with introductory biology.

The advantages of RAPD analyses for undergraduate investigations include the following:

- 1) The project can begin with collection in the field and a discussion of the ecology and history of the organism.
- 2) The procedures do not involve the use of radioactivity, pathogens, human subjects or vertebrates.
- 3) The materials can be stored frozen for an indefinite period of time after each segment of the analysis. Students can work every day, once or twice a week or occasionally interrupt the analysis for a holiday or quarter break at any point without adversely effecting the results.
- 4) The analyses require many samples at all stages so that many students can participate equally in the project.

5) The method is new, can be applied to all organisms, and illustrates principles of taxonomy, population biology, ecology, evolution and molecular biology. It demonstrates to students the integration of disciplines. **The selection of a suitable organism.**

Perennial plants have several advantages for student research projects: the location of the organism is fixed which facilitates marking and resampling, only a very small sample is required (200mg) which means that in some case even rare or endangered species can be examined, mating can be manipulated to enable analysis of the progeny of controlled crosses. The projects in progress at UCSB are being carried out at the UC Natural Reserve System Field Station on Santa Cruz Island and in the undergraduate teaching laboratories at the UCSB campus. Islands are natural laboratories for the study of biodiversity. In some respects, however, botanic gardens, National Parks, forest preserves, nature preserves, streams, and ponds are also "islands." Questions regarding diversity can arise, for example, from investigations of local species or varieties, analyses of rare or endangered species, or interest in the relationships between local cultivated plants and the wild varieties from which they came.

Safety considerations

1. Liquid nitrogen should be used with supervision. Heavy gloves, eye protection and sufficient clothing to prevent contact with legs and feet in case of a spill are required. Student need to be instructed on the importance of keeping the sample frozen until it is added to the lysis buffer.

2. Ethidium bromide should be respected as a potential carcinogen and handled only with gloved hands.

Instructor should prepare the stock solution and provide this to students in small quantities. Once the substance is added to the agarose or buffer, all further work with the system should also completed with gloved hands.

Activated charcoal can be used to remove ethidium bromide from dilute buffer solution.

Suggested experiment:

Questions: Are ironwood trees within a grove genetically identical, i.e. are they a clone of a single individual? Are individual groves genetically distinct? Are groves located close together more closely related than distant groves?

Equipment and chemicals required:

microfuge

P-1000 and P-200 pipetmen

mortar and pestle

liquid nitrogen

spatulas

electrophoresis chamber and power supply

UV transilluminator and photo documentation equipment for gels

agarose

DNeasy plant kit

random primers

Ready-To-Go RAPD Analysis Beads

thermal cycler

aluminum tags for trees

pGEM DNA markers

ethidium bromide

balance

Procedures:

1. Groves are selected for analysis, trees are permanently marked with tags, and located on hand drawn maps. Samples of young leaves are collected in labeled baggies and transported to the laboratory.
2. The leaves are weighed and 200mg samples are wrapped in aluminum foil, labeled and frozen in liquid nitrogen for storage at -70°C. Alternatively, the frozen samples may be processed immediately.
3. DNA is extracted using a DNeasy Plant Kit. With the exception of ethanol, all disposable materials described in the procedure are provided as part of the kit. Time required: 2-3 hours
 - a. The frozen samples are ground in a mortar and pestle in a small quantity of liquid nitrogen.
 - b. Using a precooled spatula, the powder is collected and transferred in approximately equal amounts to two microfuge tubes, each containing 400µl of the lysis buffer (AP1). (AP1 contains NaCl to maintain osmotic strength, EDTA to bind divalent cations, polyvinylpyrrolidone to bind polysaccharides and sodium dodecyl sulfate to solubilize cell membranes.)

- c. 20 μ l of a 20mg/ml RNase A solution is added to each tube and the tubes are mixed well then incubated at 65°C for 10 minutes. During the 10 minutes the tubes are mixed occasionally. When preparing several samples, the microfuge tubes containing the lysis buffer, sample and RNase are mixed well and kept at room temperature until all tubes were prepared. Then all are incubated simultaneously for 10 minutes. While RNA contamination of the DNA does not interfere with subsequent PCR amplification, it can interfere with quantification.
 - d. 130 μ L of AP2 solution (potassium acetate to precipitate the sodium dodecyl sulfate) is added to each tube and the mixtures placed on ice for 5 minutes. The precipitates and any cell debris are then removed by centrifugation in a microfuge set at the maximum speed for 5 minutes.
 - e. The supernatant is transferred in several portions with a P 200 Pipetman set for 200 μ L to a labeled Qiashredder minicolumn and centrifuged through the column in 2 minutes at maximum speed in a microfuge. This column is designed to trap cell wall polysaccharides bound to polyvinylpyrrolidone. Polysaccharides can interfere with PCR reactions.
 - f. The volume of the DNA containing flowthrough from the Qiashredder is measured by transferring 100 μ l aliquots to a labelled microfuge tube. 0.5vol of Buffer AP3 (guanidine hydrochloride - to increase the ionic strength of the solution) and 1 vol of 100% ethanol are added to the tube and the contents mixed gently. The increase in ionic strength and the presence of ethanol increase the hydrophobicity of DNA.
 - g. The solution is then passed through a mini-spin column where the DNA binds to the matrix by hydrophobic interactions. The column is washed with two 500 μ L aliquots of wash buffer containing NaCl and ethanol and then eluted with 100 μ l AE solution (dilute Tris buffer) or water.
4. The quality and quantity of the DNA extracted from each sample can be evaluated by measuring the OD of the solution at 260 and 280 nm in a UV spectrophotometer. A 260/280 ratio of 1.7-1.8 indicates relatively pure DNA. The OD₂₆₀ of 50 μ g/ml pure DNA is 1 and his formula can be used to calculate the quantity extracted. A typical yield was 6 μ g from 200mg tissue.

An alternative method of DNA quantification is by fluorometric analysis. This method relies on the fluorescence of Hoechst dye #33258 intercalated into DNA and requires only 2 μ l samples of the extracted DNA. The fluorometer is calibrated with a standard containing 100ng/ μ l of DNA.

5. PCR Analysis: Ready To Go RAPD Analysis Beads were used for the amplification procedure.

Oligonucleotide primers were purchased from Operon Technologies. Primer (45 ng), DNA (4.5 ng) and glass distilled water are added to each 0.5ml tube containing a bead to a final volume of 25 μ L.

Amplification was carried out in a thermal cycler programmed for denaturation at 95° C for 5 min, followed by 45 cycles of 1 min at 95° C, 1 min at 36° C, and 2 min at 72° C. The use of these beads greatly facilitated this study. They are supplied in individual thin-walled PCR tubes and are stable at room temperature. These beads are ideal for student use because they eliminate the need to prepare master mixes and thus eliminate many potential pipetting errors. Reaction conditions are consistent and the results are highly reproducible.

6. The PCR products were analyzed by electrophoresis on 1.7% agarose gels. The gels contained ethidium bromide (7 μ l of a 10mg/ml stock per 100ml gel solution). pGEM DNA markers were purchased from Promega and applied to two of the wells on each gel to provide accurate size data and to facilitate comparison of results from different gels. PCR products using a single primer from trees within a grove were run on the same gel (Figure 1).

Controls:

The pattern of PCR products obtained should be template specific. If human or other DNA has sufficiently contaminated the pipettors or reagents, this DNA will be amplified to produce artefacts.

1. Prepare a sample of DNA from a different, unrelated species of plant and amplify this in parallel, using the same primer, with the template DNA extracted from the experimental organism. The patterns should differ considerably.

2. Primer controls are reactions which include a bead, water and primer but no template. This controls for DNA contamination of the primers.

Data analysis:

Preparation of a presence/absence table

All the amplified bands for each primer are numbered and each genetic unit (individual tree or grove of trees if all are identical) is scored for the presence or absence of each band. This is done for each of the primers used and the total data for the experiment are compiled as a single table. The bands which are polymorphic

(do not appear in all trees) are then selected for further analysis. The table is imported into one of several possible programs for phylogenetic analysis, such as PAUP (Phylogenetic analysis using parsimony). The program calculates a distance matrix, and can perform parsimony analysis and neighbor-joining clustering to generate phylogenetic trees

Sources of Material:

DNeasy Plant Kit - Qiagen Inc. Tel 800-426-8157

Ready-To-Go RAPD Analysis Beads - Pharmacia Biotech Tel 800-526-3593

Random Primers - Operon Technologies Inc. Tel 800-688-2248

pGEM DNA markers - Promega Corp. 800-356-9526

Figure Legend: RAPD analysis of two groves of ironwood trees (V and J) on Santa Cruz Island. In each case, DNA from 3 trees has been amplified with primer C13 (AAGCCTCGTC). THE PCR products are identical for the 3 trees within each grove, but two bands are present in grove V that are absent in grove J (marked with astericks). These polymorphisms reflect genetic differences. Lane S on the left of each grove are pGEM DNA Markers, 2,645, 1605, 1198, 676, 517, 460, 386, and 350 bp.

References

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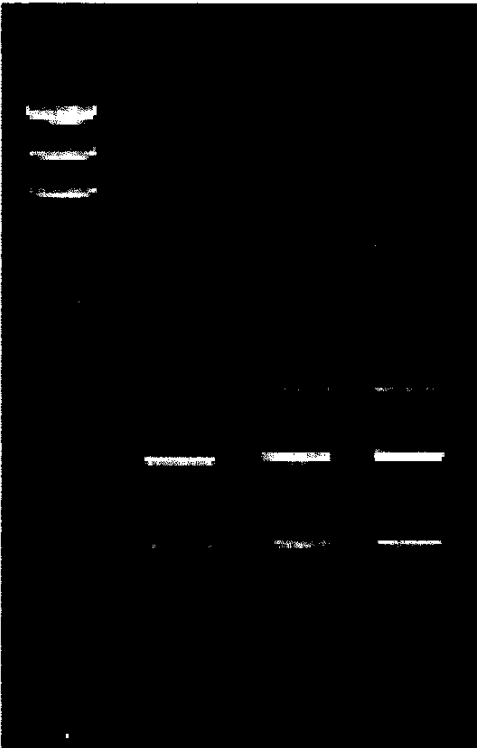
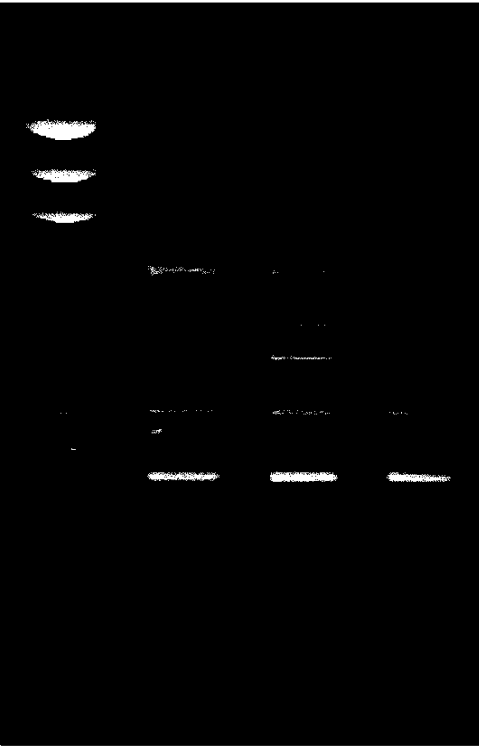
Primer C13

S

V grove

S

J grove



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