

Chapter 2

A Novel Method to Archive Plant Material for DNA Analysis

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Introduction

The idea for this laboratory exercise originated in the molecular systematics laboratory of Rob DeSalle at the American Museum of Natural History in New York. Rob, his students, and colleagues work on phylogenetic problems in many organisms using the polymerase chain reaction (PCR) to amplify selected segments of DNA. Then they study the DNA sequences of those segments (the PCR products) to identify the organisms and to describe evolutionary relationships between them. We thought it would be a good idea to try to develop a lab exercise in which undergraduates use similar methods, without the regulatory complications of using tissue from humans or other vertebrates. Instead, we isolate and analyze DNA from food plants in a supermarket or common plants in the yard. This might be the first in a series of experiments entitled "The Phylogeny of a Picnic."

One of us (KN) noticed two recent articles pertinent to this project. In the first article, Lin *et al.* (2000) crushed young leaves of several types of plants onto specially treated paper cards designed for collecting human DNA from blood. This article by Lin *et al.* is the first adaptation (we know of) of the card technology to plants. Lin *et al.* found that DNA in dried crushed plants on the cards stored at room temperature was stable for at least a month. Dried pieces of the paper card with plant material on them were then treated with "FTA reagent," a proprietary solution used to eliminate PCR inhibitors and to leave DNA bound to the paper. The paper pieces were then rinsed, dried again, and placed directly in polymerase chain reactions. PCR removed some DNA from the paper and then amplified it. The second article (Iovannisc 2000) described a similar product, called "Guthrie cards," also designed for archiving blood samples. We compared the two commercial products to develop an undergraduate lab exercise for studying plant DNA. In our hands, the best combination of materials for the plant project was 1) Guthrie cards sold as "IsoCode cards" by Schleicher and Schuell, Inc. and 2) FTA liquid reagent sold by Life Technologies, Inc. The rest of the materials are more likely to be stocked in an undergraduate lab.

The novel aspect of this approach is the method of DNA extraction. Extracting plant DNA is often difficult using conventional means because undesirable material including PCR inhibitors often co-purifies with the DNA. The card method is simple and quick. It also avoids using the dangerous organic reagents phenol and chloroform.

There are several possible applications of this technique in general biology, botany, genetics, and ecology courses. For example, students could collect a fresh leaf in the field (or in a supermarket), squash the leaf onto a card, dry the card, and store crude but stabilized DNA until it was convenient to do the rest of the lab work. Only a small piece of a leaf would be needed, allowing study of rare or endangered plants.

The plants we have used so far in this project are common plants, *Arabidopsis thaliana*, (a weedy mustard-family plant), Dandelion (*Taraxacum officinale*), Poppy (*Papaver somniferum*), Violet (*Viola spp.*), Petunia (*Petunia spp.*), Spinach (*Spinacia oleracea*), and Basil (*Ocimum basilicum*). The genome of *A. thaliana* has been completely sequenced recently.

The PCR primers we have used so far are from: (1) plant 18 S ribosomal RNA, a very well-conserved sequence, (2) ITS II, a highly divergent sequence coding for ribosomal RNA (ITS RNA is degraded in RNA processing) (White *et al.* 1990), and (3) *rbcL*, part of the famous ribulose biphosphate carboxylase ("rubisco") chloroplast gene which codes for an essential photosynthesis enzyme. Rubisco sequences are of intermediate variability, less variable than ITS II but more variable than 18 S.

In this exercise, we will squash leaves onto IsoCard paper, dry the paper, cut it up into tiny pieces, soak and rinse the tiny pieces with two reagents, and dry it again. While waiting during the soaking and drying steps we will prepare and run PCR's using some tiny pieces of card paper containing plant DNA which we have prepared already using the same methods. Then we will analyze some plant DNA sequence data obtained from a sequencing facility. We will download graphics and text data, reformat the text, and send reformatted data to two web sites, BLASTN and MultAlin. BLASTN looks for matches of our sequence with database sequences. MultAlin compares our sequences with each other, telling us which ones are the most similar, and how they differ from each other.

For full detailed descriptions of gel electrophoresis of PCR products, recovery of PCR products from gels, and DNA sequencing of PCR products, see Gurney *et al.* (2000), Hershberger (2000), and Nolan *et al.* (2000) in the proceedings of the 21st ABLE workshops, plus Gurney *et al.* (2001) in the proceedings of the 22nd ABLE workshops. We include outlines of these steps in the Instructions for Students below.

Materials

- PCR SUPERMIX (Life Technologies)
- FTA reagent* (Whatman Biosciences)
(*Ted Gurney tried the experiment once while omitting the FTA wash step, and he obtained positive results. However, he has not repeated that step yet.)
- Isocode cards (Schleicher & Schuell) 104-95-000 (pkg. of 25)
- Agarose
- Ethidium bromide
- 10 X TBE (tris-boric acid buffer)
- Disposable tips (500- μ l, 20- μ l and 10- μ l)
- Pipettors---10- μ l, 20-200- μ l, and 1000- μ l
- Gel boxes, trays, combs, Power supplies
- Distilled water (sterile preferable)
- TE buffer: 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 10 ml per student
- Forceps
- Small, sharp scissors
- Microfuge tubes
- Microfuges
- PCR tubes
- PCR machine
- Stop load
- 1 kb pair ladder (or other DNA ladder)
- UV light box
- Polaroid camera, or other gel photography system
- Kim wipes
- Hair dryers
- Foil, and saran wrap
- UV face shields
- Fresh spinach (or other green leaves)
- Fresh plant leaf, at least 300 mg, not wilted, one per student
- Top-loading balance able to read to ± 10 mg, one per 5-10 students
- Leaf crusher: heavy walled glass test tube, *e. g.*, 15-ml Corex centrifuge tube, 1 per student
- Square of Parafilm, 5 x 5 cm, one per student
- Stainless steel screen for drying paper (Can be the bottom of a stainless steel mesh dishwashing basket)

- Pre-prepared card pieces with DNA already on them, three per student (preparation described below)
- Pre-prepared PCR mixture(s) containing primers and Taq polymerase
- Here is the recipe for one 25 μ l PCR, but of course you will make a bigger batch for all of the students. Make it fresh just before class and keep it on ice. You might want to try more than one set of primers.
 - 17.4 μ l distilled or deionized water
 - 2.5 μ l of 10x PCR buffer containing magnesium
 - 2.5 μ l of dNTP mixture (10 mM each of all four)
 - 1.25 μ l of primer 1 (10 μ M)
 - 1.25 μ l of primer 2 (10 μ M)
 - 0.1 μ l of Taq polymerase, *e.g.*, Klentaq1
- Pre-prepared stop mixture to terminate PCR
 - 100 mM Tris-HCl pH 8
- 10 mM EDTA
 - 30% glycerol
 - 0.1% bromphenol blue
- Vortex mixer, one per 4 students
- Pipetmen and tips, one Pipetman of each size and its box of tips per 4 students
- Forceps, one per student
- Kimwipes, one box per 4-5 students
- Primers for 18rRNA gene: 5'-AACGGCTACCACATCCAAGG- 3'' and
: 5''-CCGAAGGCCAACACAATAGG- 3''.
- Primers for the rbcL gene: 5'' -TTGGCAGCATTCCGAGTAAC- 3'' and
5' -AAGTCCACCGCGAAGACATT- 3'
- Primers for ITS 3 and 4 5' -GCATCGATGAAGAACGCAGC- 3'
5' -TCCTCCGCTTATTGATATGC- 3'

Primers can be ordered from Operon Technologies (1-800-688-2248). There is what is called a \$5 (US) set-up fee and the cost is 60 cents per base. The primers are reconstituted upon arrival with RNase-free water (they come dehydrated in pico to nanomole quantities and should be diluted to 10mM). Each primer will vary slightly in concentration when it is shipped to you. The primer is further diluted to nanomole quantities when it is used in the PCR.

Notes for the Instructor

There is no particular background required for this lab but students experienced in micropipette use (Pipetman or equivalent) will have an easier time.

To begin the project with live plants and carry it through to sequence analysis, the project should take three labs of about 4 hours each assuming that there will be one lab per week. There would be some saving in TA work if the first and second labs could be held on successive days, but we realize that the scheduling would be difficult. The interval between the second and third labs must be at least a week to allow enough time for sending material to the sequencing facility and getting the data back.

The first lab consists of extracting plant DNA using the S&S IsoCode cards, running some PCR's, and storing the PCR products in the refrigerator. On the evening before the second lab, TA's will load the PCR products on a gel and start it running. If the two labs could be on successive days, students could load and run the gels themselves.

The second lab consists of observing PCR products on fluorescent ethidium bromide-agarose gels, cutting the products out of the gel, extracting DNA from the gel slices, mixing the extracted DNA with one of the PCR primers, and sending the mixture to a DNA sequencing facility.

The third lab is a computer lab, not a wet lab, so it could be done as homework if students have access to on-line computers and if students are computer-literate. Students will download sequence data from the sequencing facility over the web or from a class computer, observe the graphics form of the data, reformat the text form of the data, and analyze it using the web sites BLAST and MultAlin. If the students are computer neophytes, this lab must be done with close supervision and help. As an alternative, the third lab could be a stand-alone virtual lab done with supplied data; then the first and second labs (the "wet" labs) could be skipped altogether.

We think that background and relevance to other things studied in the course are very important but we are concentrating on methods here so in the interests of space we leave it to the instructor to give the background, to instill some "cosmic awareness" in the students.

First lab

Suggested outline and schedule

Short lecture (30 minutes)

Crush leaves onto cards (40 minutes)

Dry cards with hair dryer and start PCR using prepared card material (30 minutes)

Cut up cards, soak and wash (30 min)

Start drying card pieces overnight (20 min)

Add stop mixture to PCR's and mix (20 min)

Write up lab notes in the time remaining.

- The "pre-prepared" material is not available commercially so instructors or TAs have to make it up. If there is more than one section, students from one section could make card material for the next section. We are fairly sure that pre-prepared card material can also be stored at -20°C indefinitely, from one year to the next.
- The plant stems should be kept in water until the last minute to prevent wilting. We need only 300 mg of leaf for each student or team of students. Zip-lock bags of fresh leaves such as spinach and basil would also be appropriate.
- The slightly yellowish part of the S&S IsoCard is the active region, the part of the card able to bind DNA. Keep it clean and do not touch it. It is not necessary to use a whole IsoCard for each leaf. One card cut into three 1.5 x 1.5 cm squares is plenty for three different leaves.
- A crude card-drying chamber can be made from a hair dryer blowing through a box. Put a meat thermometer through the cardboard to read the temperature inside the box. Aim for lots of airflow and an air temperature below 90°C.
- After squashing a leaf onto IsoCard paper and drying it students will cut it up into smaller pieces, each of which can fit into a PCR tube. Make sure the smaller pieces are small enough to fit into PCR tubes and not stick out above the liquid in the tubes. The article from which this workshop was adapted was written by Life Technologies personnel, and therefore used Life Technologies products, including a special (expensive; about \$100 US) punch to make uniform 2-mm circles of card paper. It seems unnecessary to us to buy several special expensive Life Technologies punches for a teaching lab. Some stationery stores sell 2 mm punches for a couple of dollars each! Uniform round paper pieces are nice but they are not necessary; paper pieces of any shape made with ordinary scissors will work too.
- During class, the TA's will estimate the number of PCR's to be done, and make up batches of PCR reagents, on ice, for each of the three primer pairs. To save expensive reagents you might want to assign

different primers to different students. Somebody should do the no-paper controls too. Then all of the class will share the data. If the lab becomes a class project, with maybe 2-3 fold redundancy, there are fewer chances that some students will have no data.

Here is a PCR program that works well for MJ Research PCR machines with heated lids:

- | | |
|----------------|--|
| 1. 94° 1 min. | 5. Go to step 2 for 29 additional cycles |
| 2. 94° 10 sec. | 6. 72° 1 min. |
| 3. 55° 10 sec. | 7. 30° 10 sec. |
| 4. 72° 50 sec. | 8. end |

- This PCR program is different from the program we used last year; it has a higher annealing temperature (step 3). If the annealing temperature is lower than 55°C, there will be lots of background. Even so, there still is a little background of smaller PCR products; see Fig. 1 below.
- The dry card paper put in the PCR tubes will soak up a good deal of PCR liquid and that liquid will be lost. In a PCR of 25 µL, the paper might soak up 10-15 µL but even so, the remaining 10-15 µL should be enough for the rest of the procedure. Perhaps some of that lost liquid could be recovered from wet paper by high-speed microfuging but we have not yet tried that idea.
- The night before the second lab, TA's will prepare an agarose gel / ethidium bromide gel, load it with the students' PCR products, and run it slowly overnight, terminating the electrophoresis at the beginning of class. Materials needed for this part are:
 - Pre-prepared 0.7% agarose gel containing ethidium bromide
 - Electrophoresis buffer
 - Power supply for electrophoresis
 - UV light box, scalpel, forceps, and microfuge tubes for recovering gel slices
 - Protective gear to prevent UV exposure to skin and eyes
 - Photo equipment for recording gel data, digital camera or Polaroid camera, with red filter.
 More details were given in last year's workshop (Gurney *et al.* 2001).
- We recommend a 0.7% agarose gel for electrophoresis instead of a higher percentage because agarose must be eliminated later and agarose elimination will be more certain if there is less agarose to begin with. We use regular agarose, not special low temperature melting agarose, because the DNA extraction method uses sodium perchlorate, which melts regular agarose. A 0.7% gel is not hard to handle with a little experience, so maybe practice a little to prevent ripping. Running the gel overnight can be convenient but the PCR products do diffuse more during an overnight run than in a rapidly run gel. Figure 1 (in the Third Lab's Sample Data section) is from a gel that was run overnight.

Second lab

Suggested outline and schedule

- Short lecture (30 minutes)
- Observe PCR gel on UV light box and photograph it (30 minutes)
- Some students cut PCR products out of gel, put in microfuge tubes, and determine net weight (30 min)
- Simultaneously other students compute sizes of PCR products by comparing them to markers.
- Some students add binding buffer to tubes with gel slices, melt agarose (30 min)
- Simultaneously other students label the tubes that will receive diatom-purified DNA.
- Add diatoms to melted agarose and bind DNA to diatoms (30 min)
- Wash DNA bound to diatoms, cooperative effort (30 min)
- Some students add elution buffer to diatoms and elute DNA (30 min)
- Simultaneously other students add primer to tubes.
- Mix eluted DNA with primer (30 min)
- TA's will send tubes to sequencing facility.
- Write up lab notes in time remaining.

- The lab will proceed much faster if there is some division of labor to avoid bottlenecks. To avoid a bad bottleneck at the UV light box step, photograph the gels before class and put digital images of the fluorescent gels on all of the lab computers so the students can copy them or observe them at leisure. Observing images of the gels on the computer is easier and safer (by avoiding UV exposure) than seeing the real thing.

Third lab

The materials and methods for the third lab were also in last year's workshop (Gurney *et al.* 2001).

Suggested outline and schedule

Short lecture (30 min)

Download graphics data and look at them. You will need Editview software (30 min)

Download text data and look at them. You will need any word processing software (30 min)

Resolve ambiguities, N's (30 min)

Make single sequence files for BLAST and multiple FASTA files for MultAlin (30 min)

Do BLAST searches of databases. Quantify matches (30 min)

Do Multalin comparisons (30 min)

The searches and comparisons might reveal data flaws. Compare with graphics and correct text.

Repeat searches and comparisons (30 min)

Student Outline

First lab: Plant DNA extraction and PCR of plant DNA

1. Put the IsoCode card on the Parafilm and then put the Parafilm on three layers of tissue paper. Weigh plant leaves to get 300 ± 50 mg of leaf material. Cut it up in small pieces, about 4 x 4 mm. Crush the leaf pieces onto the IsoCode card with round bottom of a glass test tube. Keep smashing leaf pieces onto the paper until the paper saturates, until green juice begins to run off the paper. The paper will look messy with fragments of leaf still on it.
2. With forceps peel the paper off the Parafilm and put it on the wire screen. Dry the paper (with smashed leaf on it) using a hair dryer set on warm, not hot. The temperature should not go over 90° C because high heat can ruin DNA, especially as the water evaporates. It should dry within 30 minutes.
3. With scissors, cut up the dried paper into tiny pieces, about 1 x 2 mm. The paper will still look messy with leaf material stuck to it and that is OK, but if leaf material falls off, toss the leaf material and save the paper.
4. Place all of the paper pieces into a 1.7-ml microfuge tube.
5. Add 500 μ l of FTA reagent to the paper pieces in the tube. Vortex or shake gently by hand occasionally for 5 minutes at room temperature. The paper pieces must get wet. Do not shake too hard or the FTA reagent will all turn to suds.
6. Discard the used FTA reagent by pipetting it off into a waste container using a P1000 Pipetman. If there are suds, you can get rid of them by microfuging. Microfuge the tube (with balance tube) for a second or two and then try to get more liquid off. Try to remove as much liquid as possible. If leaf material comes off the paper, discard the leaf material but keep the paper. The used FTA reagent should become quite green.
7. Add 500 μ l more reagent FTA reagent to the tube and re-suspend the paper pieces by vortexing, but not too hard. Maybe you will have to pick the papers apart with forceps to make sure they are independent. Shake repeatedly for 5 minutes or longer and discard the used FTA reagent. Try to get as much of the FTA reagent off as possible by microfuging and more pipetting, as above.
8. Add 1 ml TE buffer to the tube and vortex to suspend the paper pieces. After the pieces are suspended, let them sit for about half a minute and then shake again. Remove and discard as much liquid as possible using the same methods as above. Repeat twice (for a total of three TE washes), discarding as much of the wash solution as possible each time. The FTA reagent contains detergent(s) that might inhibit the PCR so it is important to rinse the paper thoroughly with TE to get rid of detergent.
9. Dump the rinsed pieces of IsoCode card onto three layers of Kimwipe and spread them out with forceps. The Kimwipe will blot up most of the remaining liquid. Dry them with a hair dryer as above, or, if you

have the time, just let them air dry at room temperature overnight. We will have pre-prepared dry IsoCode card pieces for you if you cannot get yours ready in time to do PCR with them.

10. Place three tiny pieces of rinsed and dried IsoCode card into a PCR tube. The pieces should be small enough to stay submerged in 25 μ l of liquid in the PCR tube.
11. Add 25 μ l of PCR reaction mix (on ice) that has been prepared by the TA's. Keep everything on ice until PCR begins. The first step of PCR (94°C for one minute) will detach the DNA from the card and then the DNA will be amplified.
12. Somebody in the class should do negative controls, a PCR tube without papers for each of the different reaction mixes, the ones with different primer pairs.
13. After adding the PCR reaction mix to the tubes containing papers, spin the tubes very briefly, one second or less, in microfuge to put all liquid in the bottoms of the tubes. It is not desirable to get the centrifuge up to full speed.
14. Place the PCR tubes on ice until the rest of the students are ready to run their PCR's. Wait for everybody to get ready (unless you have more than one PCR machine). Then put all of the tubes in the PCR machine and start the program running.
15. When the program has finished running, take tubes out of the machine and add 4 μ l of stop mix to each tube, vortex, and place the tubes at 4°C until you are ready to do the electrophoresis.
16. The TA's will load and run the gel for you the night before the second lab. If you can do this part yourself pipette as much liquid as possible out of the PCR tube and load it on the pre-prepared gel. The papers in the tube will soak up liquid and that liquid will be lost, or you might be able to recover some of it by microfuging the tube. Be sure to include a gel lane with DNA size standards. Begin running the gel overnight.

Second lab: Electrophoresis of PCR products and gel excision in preparation for DNA sequencing [See last year's workshop (Gurney *et al.* 2001). This is a condensed version of that.]

1. Put on protection from UV light. Cover your eyes with a face shield and your skin with a lab coat. Then observe the gel on the UV light box. Somebody should take a picture of the gel and circulate copies to everybody. Minimize UV exposure to the gel and the students; taking a picture accomplishes that.
2. Using a small knife cut out your fluorescent PCR products from the gel in little blocks of agarose. Using forceps put one slice in a pre-weighed microfuge tube and weigh the tube again. The maximum amount of agarose allowed per microfuge tube is 350 mg net weight. To keep from exceeding this limit be sure to cut as closely as possible to the fluorescent DNA.
3. Add BioRad Prep-a-Gene binding buffer to the agarose slice in the tube. Binding buffer melts the agarose and then facilitates binding of DNA to diatoms. The amount of binding buffer to add in μ l is 3.5 times the net weight of the gel slice in mg.
4. Heat at 50°C until the gel slice is completely melted, about 15 minutes, maybe longer for large slices.
5. Add 5 μ l of BioRad Prep-a-Gene diatomaceous earth matrix and mix the tube for 10 minutes or longer at room temperature.
6. Microfuge 15 seconds at room temperature and wash the matrix by centrifugation once with binding buffer and twice with BioRad Prep-a-Gene washing buffer, 100 μ l per wash, 15 seconds per spin. Remove all dregs at each wash.
7. Elute DNA from matrix in 20 μ l 10 mM Tris-HCl pH 8, 0.1 mM EDTA by heating the suspension at 50° C for 10-20 minutes. Microfuge the tube to get the matrix in the bottom.
8. Mix 6.7 μ l of the supernatant with 0.3 μ l of one PCR primer (10 μ M) and send to sequencing facility.
9. During the waiting periods, compute the size of the PCR product relative to the DNA size standards.

Third lab: Computer analysis of DNA sequences [Again, see last year's workshop, (Gurney *et al.* 2001) for more detail.]

1. Download the graphics and text data from the sequencing facility. Graphics are the primary data, the four color peaks, and the text version (a string of letters, ACGT) is a computer interpretation of the graphics.
2. Check to be sure the data look OK. Make the text version of each sequence as one continuous string and in an unformatted text file. Resolve any ambiguities (such as N's) by consulting the primary data and correcting the text.
- 3) Go to <http://www.ncbi.nlm.nih.gov/blast/> and open BLASTN. Paste in a sequence (unformatted text file) and start a database search for similarities. Each search should take only a few minutes and is very easy. Try another sequence or two. Copy and save the results.
- 4) Put all of the sequences from a particular sequencing primer together in a single unformatted text file in FASTA format (Gurney *et al.* 2001). Go to <http://www.toulouse.inra.fr/multalin.html> and paste in the collection of sequences you just made. Click "start MultAlin." Within one minute, you should receive the sequence comparisons; the most similar sequences will be placed next to each other and differences between sequences will be highlighted in color.

Sample data

Sample results are given in Figures 1 and 2 below. All fragments range from 400-500 base pairs (bp) in length. Note that the 18 S rRNA and *rbcL* chloroplast DNA fragments are the same size for each sample tested. However, the ITS segments are different sizes, depending on the species of plant sampled, as we might expect. This is also evident in the gels that have been run on various species to date. The 18 S, *rbcL* and ITS 3 & 4 for Arabidopsis and dandelions are given here. You may conduct a BLAST search to look for similarities in sequences among the different species.

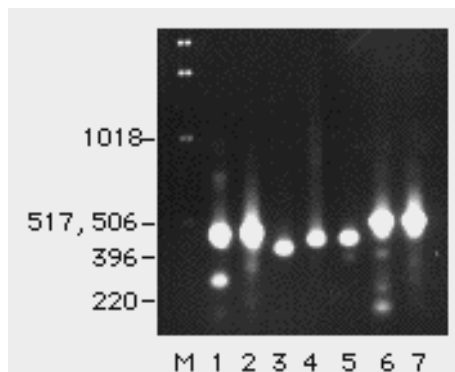


Figure 1. PCR products of Arabidopsis and Dandelion with ITS and *rbcL* primers. Lane markings: **M**—Life Sciences “1 kb” marker (hard to see some bands on this scan); **1**--Arabidopsis 18S; **2**--Dandelion 18 S; **3**--Arabidopsis ITS 3 & 4; **4**--Dandelion ITS 3 & 4; **5**—Dandelion ITS 3 & 4; **6**—Arabidopsis *rbcL*; **7**—Dandelion *rbcL*. (Ted Gurney, *U. of Utah*)

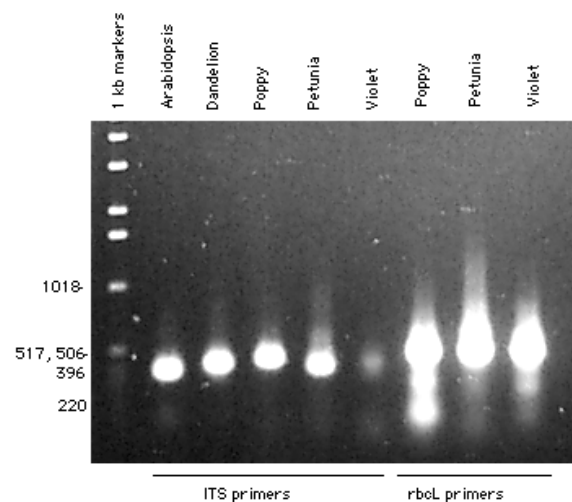


Figure 2. PCR products of Arabidopsis, Dandelion, Poppy, Petunia with the use of ITS and *rbcL* primers. (Ted Gurney, *U. of Utah*)

You can compare the sequences with the database using blastn or with each other using MultAlin or BLAST2. See <http://www.ncbi.nlm.nih.gov/blast/> or <http://www.toulouse.inra.fr/multalin.html>. The searches should take less than a minute for each. You have to remove the little titles (the lines beginning with > before the sequence) for the blast web sites but you have to leave them on for MultAlin.

D = Dandelion A = Arabidopsis

<<<<< Attached TEXT file named "18S1.txt" follows >>>>>

>A18S

GAGCAGGGCGCGCAAATTACCCATCCTGACACGGGGAGGAGTGACAATAAATAACAATACCGGGCTCTTTTCGAGTCTGGTAA
 TTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC
 TCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGGGATGGGTTCGGCCGGTCCGCCTTTGG
 TGTGCATTGGTCGGCTTGTCCCTTCGGTCGGCGATACGCTCCTGGTCTTAATTGGCCGGGTCGTGCCTCCGGCGCTGTTAC
 TTTGAAGAAATTAGAGTGCTCAAAGCAAGCCTACGCTCTGGATACATTAGCATGGGATAACATCATAGGATTTTCGATCCTA
 TTGTGTGCC

>D18S

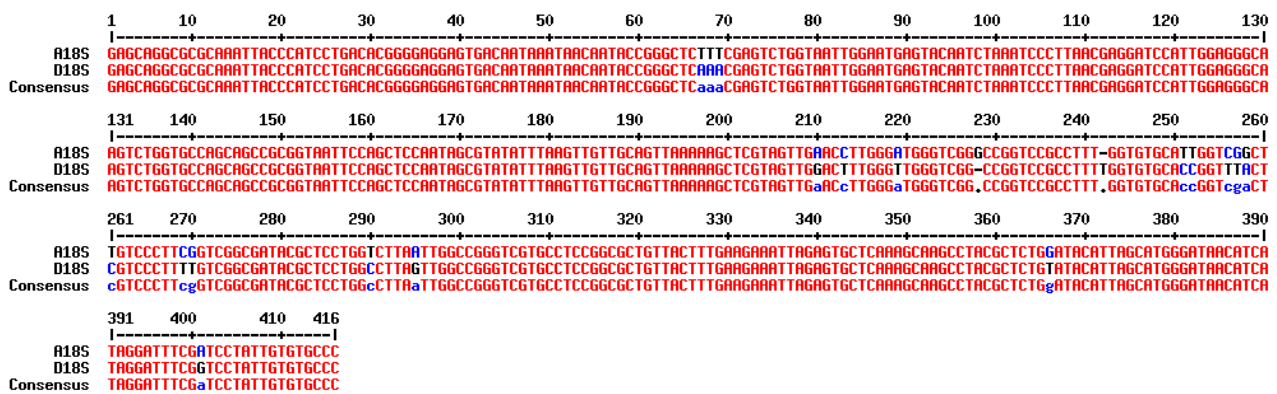
GAGCAGGGCGCGCAAATTACCCATCCTGACACGGGGAGGAGTGACAATAAATAACAATACCGGGCTCAAACGAGTCTGGTAA
 TTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC
 TCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGACTTTGGGTTGGGTTCGGCCGGTCCGCCTTTTGG
 TGTGCACCGGTTTACTCGTCCCTTTTGTCCGGGATACGCTCCTGGCCTTAGTTGGCCGGGTCGTGCCTCCGGCGCTGTTAC
 TTTGAAGAAATTAGAGTGCTCAAAGCAAGCCTACGCTCTGTATACATTAGCATGGGATAACATCATAGGATTTTCGATCCTA
 TTGTGTGCC

BLAST program on Arabidopsis thaliana 18 S rRNA

Sequences producing significant alignments:		(bits)	Value
gi 16506 emb X16077.1	ATRRN18 Arabidopsis thaliana 18S rRNA...	769	0.0
gi 16472 emb X52322.1	ATRDNAF A.thaliana rRNA repeat unit, ...	769	0.0
gi 6598619 gb AC006837.15	AC006837 Arabidopsis thaliana chr...	761	0.0
gi 21219 emb X17062.1	SINARRNA S.alba 18S and 25S rRNA genes	674	0.0
gi 21157 emb X66325.1	SARIBRNA S.alba 18S, 5.8S & 25S rRNA ...	674	0.0

[NOTE: The "Color Key for Alignment Scores" is omitted from the results shown here because this publication is printed in black & white only.]

MultAlin results for Arabidopsis and Dandelion 18 S rRNA:



There is a very high agreement between base pairs---only 12 of 416 are different.

<<<<<< Attached TEXT file named "ITS3.txt" follows >>>>>>

>DITS3

```
GCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTTTTTTGAACGCAAGTTGCGCCCCGAAGCCATCCGGTTCAG
GGCACGCCTGCCTGGGCGTCACGCATCGCGTCGCCCCCATCATACTTCCCTTAAGGGTAGTCGTGGTATTGGGAGCGGA
GATTGGCTTCCCCTGCTTGTGTGCGGTTGGTCAAATAGGAGTCCCCTTCGGTGGACACACGGCTAGTGGTGGTTGTAAA
GACCCTTTTCTTCTGCTGTGTGTTGTGAGCTGCTAGGGAAACCCTCAAAAAAGAACCCAATGTATCGTTCTAGGACGATGC
TTCGACCGCGACCCAGGTGAGCGGGACTACCCGCTGAGTTTAAGCATATCAATA
```

<<<<<< Attached TEXT file named "rbcL3.txt" follows >>>>>>

>ArbcL3

```
TTCTCACCCCTGGAGTTCCTGGAAGAAGCAGGGGCTGCGGTAGCTGCTGAATCTTCTACTGGTACATGGACAACCTGTGTGG
ACCGATGGGCTTACCAGCCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCCCGTTCCAGGAGAAGAACTCAATTT
ATTGCGTATGTAGCTTATCCCTTAGACCTTTTTGAAGAAGGTTTCGGTTACTAACATGTTTACCTCGATTGTGGGTAATGTA
TTTGGGTTCAAAGCCCTGGCTGCTCTACGTCTAGAGGATCTGCGAATCCCTCCTGCTTATACTAAAACCTTTCCAAGGACCA
CCTCATGGTATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGACGTCCCCTATTAGGATGTACTATTAACCAAATTTG
GGTTATCCCGCGAAAACTATGGTAGAGCAGTTTATGAATGTCTTCCGGGGG
```

>DrbcL3

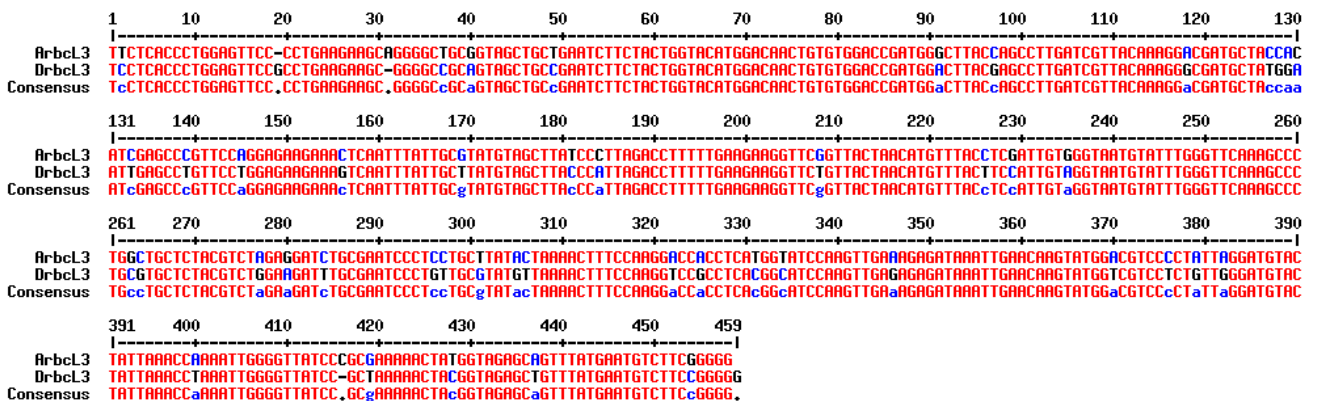
```
TCCTCACCCCTGGAGTTCCTGGAAGAAGCAGGGGCGCAGTAGCTGCCGAATCTTCTACTGGTACATGGACAACCTGTGTGG
ACCGATGGACTTACGAGCCTTGATCGTTACAAAGGGCGATGCTATGGAATTGAGCCTGTTCCAGGAGAAGAAAGTCAATTT
ATTGCTTATGTAGCTTACCATTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTAGGTAATGTA
TTTGGGTTCAAAGCCCTGCGTGTCTACGTCTGGAAGATTTGCGAATCCCTGTTGCGTATGTTAAAACCTTTCCAAGGTCCG
CCTCACGGCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCTCTCTGTTGGGATGTACTATTAACCTAAATTTG
GGTTATCCGCTAAAACTACGGTAGAGCTGTTTATGAATGTCTTCCGGGGG
```

BLAST results for Arabidopsis rbcL3

Sequences producing significant alignments: (bits) Value

gi	Accession	Species	bits	Value
gi	7525012	ref NC_000932.1 Arabidopsis thaliana chloroplas...	850	0.0
gi	5881673	dbj AP000423.1 AP000423 Arabidopsis thaliana chl...	850	0.0
gi	1944431	dbj D88901.1 D88901 Arabidopsis thaliana DNA for...	850	0.0
gi	1928871	gb U91966.1 ATU91966 Arabidopsis thaliana ribulo...	842	0.0
gi	1944433	dbj D88902.1 D88902 Arabidopsis himalaica DNA fo...	827	0.0
gi	2443766	gb AF020325.1 AF020325 Nasturtium officinale rib...	811	0.0
gi	1944439	dbj D88905.1 D88905 Cardamine flexuosa DNA for r...	811	0.0
gi	2443764	gb AF020324.1 AF020324 Cardamine pensylvanica ri...	803	0.0
gi	1944441	dbj D88906.1 D88906 Lepidium virginicum DNA for ...	803	0.0
gi	2443768	gb AF020326.1 AF020326 Neobeckia aquatica ribulo...	799	0.0

MultAlin results of Arabidopsis and Dandelion rbcL



Again, a high percentage of agreement --- 43 of 459 bp -- but not quite as conserved as the 18 S rRNA genes.

BLAST results for Sequences for Dandelion ITS3

gi		1032471		gb		L48338.1		TXMITS2A	Taraxacum officinale Weber ...	402	e-110
gi		308864		gb		L13957.1		LAURGBEHA	Lactuca sativa partial 18S ...	295	1e-77
gi		10440945		gb		AF091204.1		AF091204	Pholistoma auritum inter...	230	5e-58
gi		1572655		gb		U69709.1		MAU69709	Microseris acuminata nuclea...	220	5e-55
gi		1572657		gb		U69708.1		MCU69708	Microseris campestris nucle...	220	5e-55
gi		1572659		gb		U69707.1		MEU69707	Microseris elegans nuclear ...	220	5e-55
gi		1572658		gb		U69706.1		MDU69706	Microseris douglasii nuclea...	220	5e-55
gi		1572661		gb		U69705.1		MPU69705	Microseris pygmaea nuclear ...	220	5e-55
gi		1572660		gb		U69704.1		MLU69704	Microseris lindleyi nuclear...	220	5e-55
gi		1572656		gb		U69703.1		MBU69703	Microseris bigelovii nuclea...	220	5e-55

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