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# Effective Population Size: Biological Duality, Field & Molecular Approaches

Sarah A. Woodin   Michael Grove   Daniel D. Heath

Biology is a field with numerous subdisciplines, each with apparently different questions and goals. Courses often emphasize the complexity and diversity of the subdisciplines rather than the common intellectual links. As a result, students can fail to make the connections between questions asked by molecular biologists and those asked by organismal and population biologists. One emphasizes the organisms as packages of DNA and other biochemical constituents, while the other stresses population structure, functional morphology, and behavior. The following laboratory exercise was developed to promote students' understanding of the commonality of certain basic questions to all of biology and to demonstrate the power of using different approaches with very different assumptions. The exercise was specifically designed to force the students to experience simultaneously the same species both as packages of DNA and as distinct individuals with behavior. The core of the exercise can be done as a class over a five-week period in a setting where students can work for short periods outside of class as well as during class time.

The specific goal of the laboratory exercises described here is to estimate effective population size ( $N_e$ ) using two approaches. Effective population size is defined as the number of breeding individuals in a population. Effective population size is a major determinant of the relative heterogeneity of the population through its effect on inbreeding. As such,  $N_e$  affects the frequencies of phenotypes displayed at both the organismal (e.g. coat color,

wing length) and the molecular (e.g. enzyme alleles and their respective activities) levels as well as the probable response of the population to alterations in the selective regime. Effective population size is often much smaller than the actual population size, particularly in populations with large differences in numbers of males and females, large fluctuations in size, or low dispersal so that parents mate with offspring (Futuyma 1986). The implications of low  $N_e$  can be serious; for example, if only 25% of a population actively breeds [i.e.  $N_e = (0.25) \times (\text{actual population number})$ ], then the genetic material available for future generations is limited to 25% of what was present in the original population. If  $N_e$  were to remain at 25% of the actual population size over a number of generations, the loss of genetic material would eventually lead to a reduction in genetic diversity (due to inbreeding and genetic drift). If the actual population were very large, this loss of genetic diversity would probably be negligible; however, if the population were small, the consequences of low  $N_e$  could be serious. Low genetic diversity has been shown to be associated with reduced vigor and survival in organisms (Hunter 1996). An even more important consequence of low genetic diversity is the probable reduced evolutionary potential of the population (or species). If a population has a very low  $N_e$ , and thus low genetic diversity, it will have little genetic (or phenotypic) variation and will be incapable of adapting to changes in the environment via natural selection (Avisé 1994; Hunter 1996), clearly important for endangered or heavily exploited populations (Dobson et al. 1992). A recent review suggests that  $N_e/N$  ratios are much smaller than previously thought (Frankham 1995).

The object of the exercise is to determine the effective population size of a field population of the fruit fly *Drosophila*, using mark-recapture techniques, and to compare it to values obtained from molecular genetic mark-

ers (Random Amplified Polymorphic DNA or RAPD). *Drosophila* is uniquely suited for questions of this type because it can be easily collected in the field and raised in the laboratory on simple media, and it has easily extractable DNA.

This class project is designed to accomplish four main goals. First, the question asked can be addressed by both field (Begon et al. 1980) and molecular laboratory approaches (Hadrys et al. 1992), allowing the students to see directly the strength of applying two approaches with very different assumptions to the same question. Second, the students experience the biotic duality of organisms both as packages of DNA and as individuals. Third, the field portion forces the students to use spreadsheets and a computer network to exchange data effectively with the group of students collecting data the next day and to analyze and interpret a large dataset. Finally, the use of RAPDs requires skills in basic laboratory techniques, such as pipetting and diluting solutions, as well as in more advanced molecular techniques. The students learn these techniques in a setting where they are investigating a question the answer to which is not known; thus, there is a strong sense of discovery and excitement, even for the instructor.

## Capturing Fruit Flies

The easiest way to capture wild fruit flies is to place rotting fruit, such as bananas or peaches, in a container that has an internal diameter less than the opening of the insect net to be used. The bait needs to be protected from rain, but the flies must be able to enter. Any cover will suffice if it can be suspended over the container with an opening between the cover and the container. Typically we mush the rotting fruit to increase the surface area of the bait, sprinkle some yeast on

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top, and place the baits in the shade to prevent thermal stress. After 24 hours the bait will have attracted a number of *Drosophila*. To capture them, the insect net is held over the container while the cover is removed. Because the flies are photopositive, holding the closed end of the net up and tapping on the container makes them fly up into the net. The flies can then be transferred into a jar, taken to the laboratory, anesthetized, examined, and cultured (see Flagg 1988).

## Mark-Recapture Techniques

Mark-recapture techniques are often used to estimate population size for species that are mobile and thus cannot be counted directly (Krebs 1989). A subsample of the population is caught and labeled so that they can be identified; then they are released back into the population. These are the marked and released individuals (R). After a period of time during which the marked individuals are assumed to mix within the population, another subsample of the population is captured and examined for marks. The marked individuals represent the number of marked recaptures (M). The number of unmarked individuals caught in the second sample is symbolized by U. Assuming that the flies have 100% survival between the time of release and the time of recapture (24 hours), the size of the population (N) is estimated from these values:

$$N = (U + M) (R/M)$$

The errors in this calculation stem from the survivorship assumption of 100% and from the sizes of the samples. With very small samples the estimate of population size has a very large variance that can be estimated directly if mark-recapture experiments are done on several days with a different mark for each day. With several days of data a mean and a standard deviation can be calculated for population size.

In all mark-recapture approaches the problem is to label individuals with marks that are easily recognized by the investigator but do not affect the survivorship of the individual. Fruit flies can be easily marked with fine fluorescent paint chips (Crumpacker 1974). Anesthetized flies are gently rolled in a petri plate with a very small amount of paint chips. Very small amounts of dye can be detected in the dark under ultraviolet light. Do not use too much dye or the flies will die because of spiracle occlusion. The

flies are then transferred to another petri plate and allowed to recover. After recovery, they are released into the original population. It is important to check the flies after release to record handling deaths. After 24 hours, a second sample of flies from the population is captured. One can obtain several measurements of population size by using different colors on different days, and estimate survivorship from the time period over which marked flies from previous days are recaptured (Begon et al. 1980).

The next step is to convert this data into densities. Unfortunately, one cannot merely take the value for N and divide by the area within which the traps were set because flies, as mobile organisms, are attracted to the traps from outside that area as well as from within it. The trap area used in our class is 10 m by 20 m and Begon et al. (1980) have shown that flies are attracted to traps from 30 m away, so the area actually trapped is estimated to be 40 m by 50 m.

We have one complete set of student data for *Drosophila melanogaster* populations in front of the Biological Sciences building in Columbia, South Carolina. Flies were trapped on three successive days and marked with a different fluorescent paint on each day. For the analysis presented here, we combined the data from all three days. We marked and successfully released 94 male flies and 112 females. On subsequent days, we captured 93 unmarked males, 72 unmarked females, 6 marked males, and 3 marked females. Based on these numbers, we estimated the densities of males and females to be 0.78/m<sup>2</sup> and 1.4/m<sup>2</sup> respectively. These density estimates correspond surprisingly well to those for populations of *D. pseudoobscura* from Mount Parnes, Greece, where the estimates were 0.75 males/m<sup>2</sup> and 1.15 females/m<sup>2</sup> (Begon et al. 1980).

## Calculation of Effective Population Size (N<sub>e</sub>) from Mark-Recapture Data

To calculate N<sub>e</sub>, one needs to know two values: first, the density of the flies, calculated from the mark-recapture data; and second, the area of the panmictic circle, the area within which flies breed randomly. The formula for N<sub>e</sub> is the area of the panmictic circle times density. The formula for the area of the panmictic circle is:

$$Area = \pi \frac{4}{3} s^2 t$$

where s<sup>2</sup> is the symbol for the variance of the distance dispersed by flies during one activity period. Using a central release point with traps at different distances, Begon et al. (1980) have estimated s to be 33.4 to 43.3 for males and 22.1 to 34.1 for females of *D. pseudoobscura*. We used these estimates. The number of activity periods within which breeding might occur, t, is more problematic. *Drosophila* has two activity periods per day and reproduces within hours of hatching, so the number of activity periods within which breeding might occur is the length of the adult lifespan times two. Survivorship in the field appears to be short. Using mark-recapture techniques, Rosewell and Shorrocks (1987) estimated lifespans of *D. melanogaster* to be 2.4 days for females and 3.0 days for males in Leeds, Great Britain, yielding values for t of 4.8 and 6.0. These values are consistent with those of Sevenster and Van Alphen (1993) who showed that *D. melanogaster* adults live 21 days in the presence of food but die within 2 days in the absence of food. From our mark-recapture data, we estimated t to be 10. We used a range of values for t, representing high and low survivorship conditions.

## Extraction of DNA

All of the molecular methods for estimating heterogeneity of the genetic material of a population require extraction of DNA. DNA extraction is usually done using very toxic substances such as phenol and chloroform (Sambrook et al. 1989). We have found that in *Drosophila* high quality DNA is easily obtained using a simpler and less toxic method. The protocol is given in Table 1.

## PCR & RAPD Protocols

Given extracted DNA, genetic heterogeneity, the value necessary for calculation of N<sub>e</sub>, can be estimated by a variety of techniques. The DNA extraction protocol in Table 1 yields genomic DNA of 20,000 or more base pairs in length. This *Drosophila* genomic DNA performs well as a template for PCR amplification. For the purpose of this exercise, we explored the use of RAPDs in *Drosophila*. The advantage of using RAPDs vs. other DNA visualization techniques to estimate genetic heterogeneity is that we do not need to know which genes are polymorphic within the population, nor do we need knowledge of specific DNA sequences, or large amounts of DNA. Screening

Table 1. Single Fly DNA Extraction Protocol for Sodium Acetate Method

1. Freeze individual flies in 1.5-ml microcentrifuge tubes.
2. Put microcentrifuge tube with fly on bed of crushed dry ice along with a micropestle; allow to cool for 3 to 5 minutes.
3. Grind frozen fly with micropestle. The fly should be reduced to powder. If it starts to get sticky, put the tube with the fly and the micropestle back onto dry ice.
4. Make a master mix of Proteinase K (Pk) and Pk buffer: approximately 0.4 mg/ml of the Pk enzyme in the Pk buffer. The buffer is 10 mM Tris (pH 8.0) with 10 mM EdTA (pH 8.0) and 1% sodium dodecyl sulfate (SDS) (Sambrook et al. 1989, p. B.13).
5. Add approximately 100  $\mu$ L of the Pk in Pk buffer to the ground fly and mix with micropestle. Try to get all of the fly off of the micropestle. Remove the micropestle and close the top of the microcentrifuge tube.
6. Incubate the ground fly in Pk and buffer at 64° C for 1 hour. Do not vortex or you may shear the DNA!
7. Centrifuge at 14,000 rpm for 3 minutes. Do not vortex!
8. Label new 0.5-ml microcentrifuge tubes.
9. Move 90  $\mu$ L of supernatant from the original tube into a new 0.5-ml microcentrifuge tube.
10. Add 0.1 volumes of 3M sodium acetate (NaOAc), mix by inversion and then add 0.6 volumes of isopropanol (e.g. for 90  $\mu$ L of supernatant this would be 9  $\mu$ L of NaOAc and 60  $\mu$ L of isopropanol). At this point the mixture should look faintly cloudy. Mix by inversion for 1 minute.
11. Centrifuge at 14,000 rpm for 15 minutes.
12. Carefully dump the liquid, leaving the tiny pellet of DNA in the tube. Do not dump the pellet of DNA; it is easy to do! If you do not see a pellet, do not give up. There is probably some precipitated DNA that you cannot see. Gently tap the tube upside down on a paper towel to get rid of as much liquid as possible.
13. Add 300  $\mu$ L of 70% ethanol. 'Snap' the tube several times to try to dislodge the pellet; you should be able to see a small pellet floating around. Let stand 15 minutes to overnight. Actually, the DNA can be stored like this for years.
14. Centrifuge at 14,000 rpm for 10 minutes.
15. Carefully aspirate the 70% ethanol with a pipet. Do not suck up the pellet!
16. Dry the DNA. You can let it air dry by leaving the caps of the microcentrifuge tubes open with a lint-free cloth such as a Kimwipe® over the tops to prevent dust from entering.
17. Dissolve the DNA in approximately 100  $\mu$ L of double-distilled water overnight at room temperature, gently mixing now and then by inversion. Store at 4° C or at -20° C.

Note: All of the microcentrifuge tubes, micropestles, pipet tips and solutions need to be sterile and thus should be autoclaved and then stored in closed sterile containers. All of the solutions and their preparation are described in more detail in Sambrook et al. (1989).

for primers can be done within two days as detailed below, or can be done by the instructor outside of class time.

The PCR protocol that we used to generate our RAPDs is of very low stringency, i.e. the match between the primer and the *Drosophila* DNA did not have to be 100%, so we were able to successfully amplify DNA fragments using primers that probably only match the *Drosophila* DNA exactly at the 3' end (Table 2). If one were using primers specific for particular sections of DNA where the match was expected to be perfect, the PCR protocol used would be considerably different. The slow temperature ramp (Table 2, Step 4) is critical to producing repeatable RAPDs, and thus a thermal cycler capable of fast cool-down speeds and controlled temperature increases is necessary, i.e. air-cooled, water-cooled, and manual units will not work. Any contaminants introduced into the microcentrifuge tubes will lead to meaningless RAPD patterns because PCR will amplify any DNA present, especially when using

low stringency reactions (Kocher & Wilson 1991). Use gloves, keep the tubes sealed as much as possible, keep the enzymes, DNA and primers on ice, and autoclave all tubes, pipet tips and solutions. We expected to have serious contamination problems using RAPDs with students; however, we have experienced none.

### Examination of Results of PCR on Agarose Gels

Once the PCR reactions are complete, the DNA fragments are visualized on agarose gels (Sambrook et al. 1989). We have found that using special high concentration agarose gels (High Resolution Blend, Agarose 3:1, Biotechnology Grade; AMRESCO, Solon, OH, 44139) greatly increases the resolution of the multiple bands that are characteristic of RAPDs and reduces autofluorescence of the gels. Typically we use 1.5 to 3.0% gels, depending on the expected size of the DNA (higher percentage for resolution of shorter lengths of DNA). With normal agarose we use a concentration of 1.0 to 1.5%. Using such gels, each fly is scored for the presence or absence of specific bands.

### Selection of Primers for RAPDs

RAPD primers are single strands of artificially manufactured DNA, generally 8 to 12 base pairs in length. We

Table 2. PCR Protocols (A) and Recipes (B) for RAPDs.

#### A. PCR Protocols

Step	Temp (°C)	Duration
1	94°	1 min (denaturing)
2	92°	40 s (denaturing)
3	35°	1 min (annealing)
4	+40°	0.3°C s <sup>-1</sup> (slow thermal ramp)
5	75°	1 min 30 s (polymerase extension)
6	Steps 2-5	35 times (repetition)
7	75°	5 min (final extension)
8	4°	End (storage)

#### B. PCR Recipes for RAPDs

Substance	Quantity	Concentration in Final Mixture
Template DNA	1 $\mu$ L	5-50 ng
Primer	1 $\mu$ L	100 ng
dNTPs	0.5 $\mu$ L	0.2 mM each
MgCl <sub>2</sub>	2.0 $\mu$ L	2.5 mM
Taq Buffer	2.5 $\mu$ L	1X conc.
Taq DNA Polymerase	0.2 $\mu$ L	1.0 unit
ddH <sub>2</sub> O	17.8 $\mu$ L	
TOTAL	25.0 $\mu$ L	

obtained a set of 100 different RAPD primers from The University of British Columbia, Nucleic Acid—Protein Service Unit, c/o Biotechnology Laboratory, Rm. 237—Westbrook Bldg., 6174 University Blvd., Vancouver, BC, Canada V6T 1Z3, although other sources exist. The primers that we found to work well can be used, or the process of selecting appropriate primers can be included as part of the class project. The students enjoyed the “search for the best primer” and it was instructive as well. However, primers that are known to work should be included in the “unknown” group of primers since only about 50% of the RAPD primers we tested gave reliable amplification.

Once primers are identified, they are screened for useful characteristics. RAPDs using different primers can yield a wide range of variation, from fixed patterns to “fingerprint” patterns that are different for almost every fly. For the purpose of our exercise we wanted primers that produced a manageable number of variable bands. Figure 1 shows RAPDs for six primers, each with the same five flies from a wild population. Table 3 gives the specific DNA sequence for all of the UBC primers mentioned here. We have chosen a variety of results to illustrate the range of possible RAPD outcomes using *Drosophila* DNA as template. Approximately half of the 56 primers tested produced either no amplification, or “smears,” indicative of non-specific priming. UBC-18 illustrates a relatively simple banding pattern (Figure 1); such a pattern is easy for students to interpret, but often yields low variation. UBC-30, UBC-12 and UBC-13 produced multi-band variable RAPD banding patterns (Figure 1); these are all good choices for most experiments. UBC-25 and UBC-3 are both questionable primers, since UBC-25 produced many tightly spaced bands that varied widely in intensity and UBC-3 has one very bright band and a few weak signal bands (Figure 1).

Once useful primers are identified, they must be verified. Again, this is an optional portion of the exercise, although by doing it, the students will gain confidence in the technique. One of the main criticisms of RAPDs is their tendency to be unrepeatabile. Therefore the class must test the primers for repeatability by running a single fly (or perhaps two) a number of times, and then checking to see if the bands always show up in the same place. Two examples of such repeatability tests are in Figure 2. One of the advantages of using RAPDs with *Drosophila*

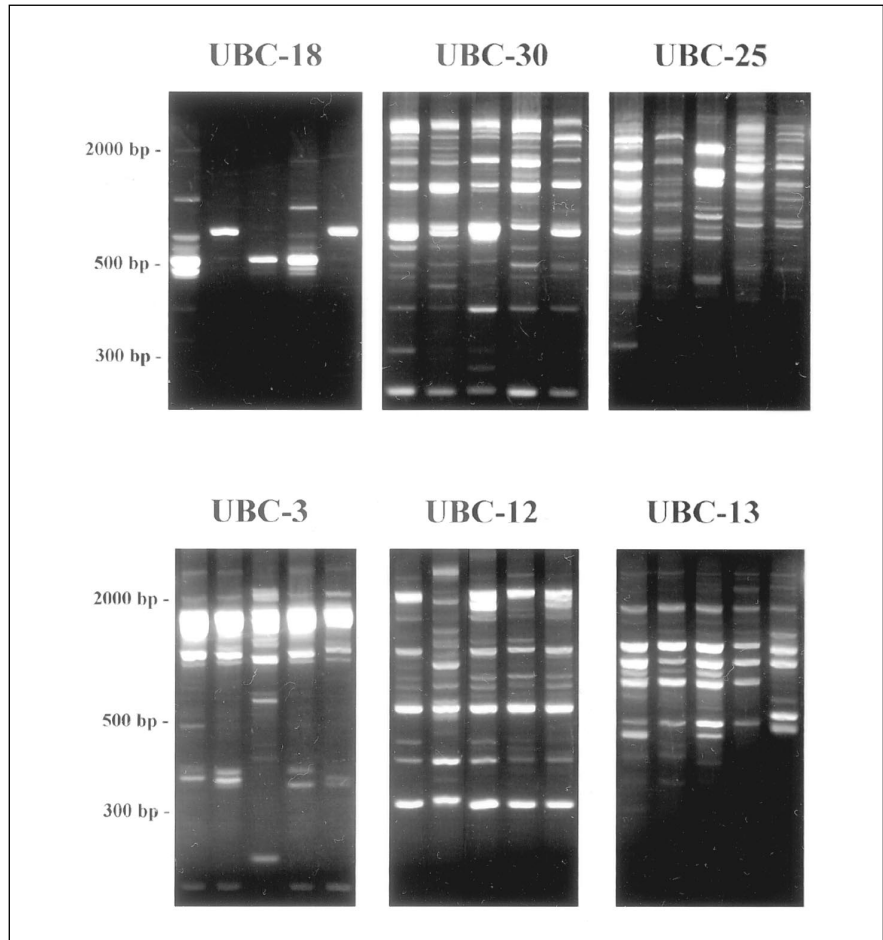


Figure 1. Photographs of 2.5% agarose gels showing RAPD band patterns using six RAPD primers and DNA from five wild-caught *Drosophila*. Note the wide range in banding pattern complexity, clarity and variability. Primer sequences are given in Table 3.

Table 3. DNA sequences of primers from the University of British Columbia Biotechnology Laboratory.

Primer Number	DNA Sequence
UBC-3	CCTGGGCTTA
UBC-12	CCTGGGTCCA
UBC-13	CCTGGGTGGA
UBC-18	GGGCCGTTTA
UBC-25	ACAGGGCTCA
UBC-30	CCGGCCTTAG

is the extremely high repeatability of the technique. Even in the hands of inexperienced students, the banding pattern was remarkably repeatable. There are usually weak bands that are not repeatable in RAPDs (see Figure 2; Arrow A, Fly A). Even repeatable very faint bands (Arrow C, Fly A) should not be used. The concept and importance of repeatability in science is well demonstrated by this verification step. It also serves to emphasize the importance of band selection.

Once primers are identified that are repeatable and have variation, the class can proceed to the actual screening of the wild-caught flies. With a higher-level class a further step is possible and relatively easy. It is theoretically possible (but very unlikely) that two flies which appear to share a RAPD band may in fact have two different DNA fragments that happen to be the same size or very close in size. This can lead to errors in population analyses, and very serious errors for parental analyses (especially for human paternity cases!). To test for this, we removed a piece of the agarose gel that contained a specific band at 600 bp and “re-amplified” the single band (Figure 3). A simple method for removing such a piece of agarose is to use a 1000 mL pipet tip to poke a hole in the center of the band, twist the tip in the gel, and then gently pull out the tip from the gel. A small core of the gel should remain in the tip and can be expelled into a microcentrifuge tube. This gel core is then used as the

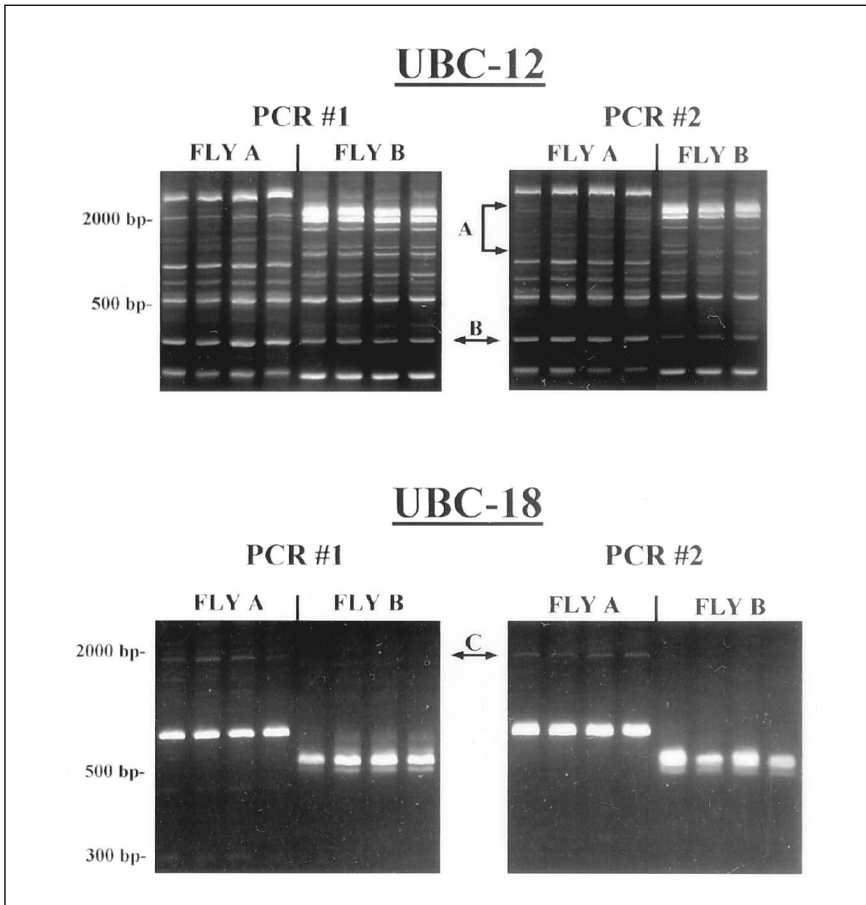


Figure 2. Photographs of 2.5% agarose gels showing the repeatability of the RAPD technique in *Drosophila* for two primers chosen to represent both complex and simple banding patterns (UBC-12 and UBC-18). DNA from each of two flies was used as a template for four replicated reactions. Two separate PCR runs using the same two flies and primers were also performed (PCR #1 and PCR #2). Some bands are faint in one gel and absent in the second; thus they are clearly not repeatable and should not be used in experiments (marked by the A arrow). There are many bands that are obviously repeatable (for example, the B arrow). Some faint bands are repeatable, but are very faint and probably should not be used (C arrow).

template for a PCR using the same RAPD primer as for the original. However, since the DNA fragment that we excised from the gel has the entire RAPD primer at each end, we can run the reaction at high stringency. We used a 45°C annealing temperature, no slow temperature ramp, and only 25 cycles to produce the single band shown in Figure 3, Arrow A. We then used six different restriction enzymes to “cut” the re-amplified band. If the three DNA fragments were not homologous, we would expect them to have different restriction enzyme sites, and hence different patterns in Figure 3. Clearly there is no evidence that these bands are nonhomologous. At this point, it should be made clear to the students that a test for homology such as we have done can only conclusively show nonhomology, that is, we could always test more restriction enzymes.

A more rigorous test of homology would be by direct sequencing of the DNA fragments.

### Calculations of Effective Population Size from Band Positions on Gels

DNA should be extracted from 15 to 20 flies from each population and PCRs run using at least two different primers. Bands on photographs of the resulting gels are then scored for each individual fly. Thus each fly will have a “present” or “absent” score for a number of bands for each primer.

The calculation of effective population size using RAPD data is as follows (Avisé 1994, pp. 28–33):

$$N_e = \frac{1}{8\mu} \left[ \frac{1}{(1 - H)^2} - 1 \right]$$

where  $\mu$  is the mutation rate for the marker and  $H$  is the observed population heterozygosity. Since flies cannot be directly identified as heterozygotes using RAPD markers (heterozygotes and homozygote dominants will both have a band),  $H$  must be calculated using the Hardy-Weinberg principle. Flies whose bands are scored as “absent” are homozygotes for the recessive state; thus the incidence of the “band-absent” flies is equal to  $q^2$ , the frequency of the recessive homozygote genotype. We can then use  $q$  (the square root of  $q^2$ ) to calculate  $H$ :

$$H = 2q(1 - q) = 2pq$$

For example, the value of  $q^2$  for Band A on Figure 4 is 0.5. The band above Band A has no variation and cannot be scored.

There are no precise estimates of the mutation rate for RAPD alleles; however, the absence of a RAPD band is thought to be due to the loss or gain of a primer site through a point mutation. Rate estimates for such point mutations are typically  $10^{-6}$ .

### Actual Data Obtained in an Undergraduate Class Setting

We have values for  $N_e$  from mark-recapture data and RAPDs for the population of flies in front of the Biological Sciences building in Columbia, South Carolina. Utilizing survivorship rates consistent with literature values, 2.4 to 3.0 days, effective population sizes estimated from mark-recapture data range from 50,968 to 63,710 flies. These data agree to a surprising extent with estimates from RAPDs. Using the standard mutation rate for point mutations of  $10^{-6}$ , our estimate of effective population size is 65,005 flies. As the values in Table 4 demonstrate quite clearly, calculations of  $N_e$  depend very heavily upon the values used for lifespan and mutation rate and thus one’s assumptions about the parameters of either the population or the mutation rate of its genetic material. The dependence of the answer on the assumptions of the analysis is an important part of this exercise. For example, if survivorship is high, yielding a lifespan of 11.5 days, then  $N_e$  increases by a factor of four over the values for shorter lifespans (Table 4). The power of using two approaches with different assumptions is obvious to the students. Actually, the dependence of the answer on these assumptions about lifespan caused one class to design a second group project on fecundity values for

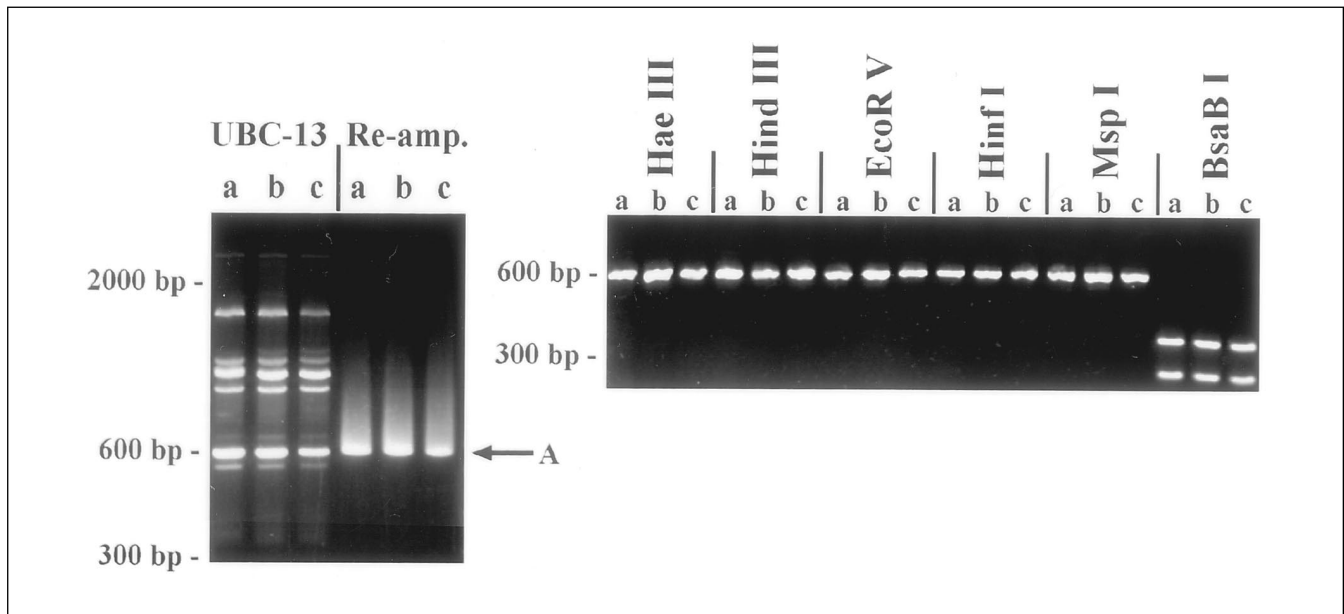


Figure 3. Photographs of two 2.5% agarose gels that illustrate the testing of homology of bands that appear to be at the same position. The left-hand photo shows the original RAPD for three flies with the UBC-13 primer and the result of a moderate stringency re-amplification of one of the bands from the original gel (A arrow). The right-hand photo shows the results of six restriction enzymes used to restriction cut the re-amplified fragment. Note that the three flies all show the same results for all six restriction enzymes, indicating that the bands at position A are very likely homologous DNA fragments.

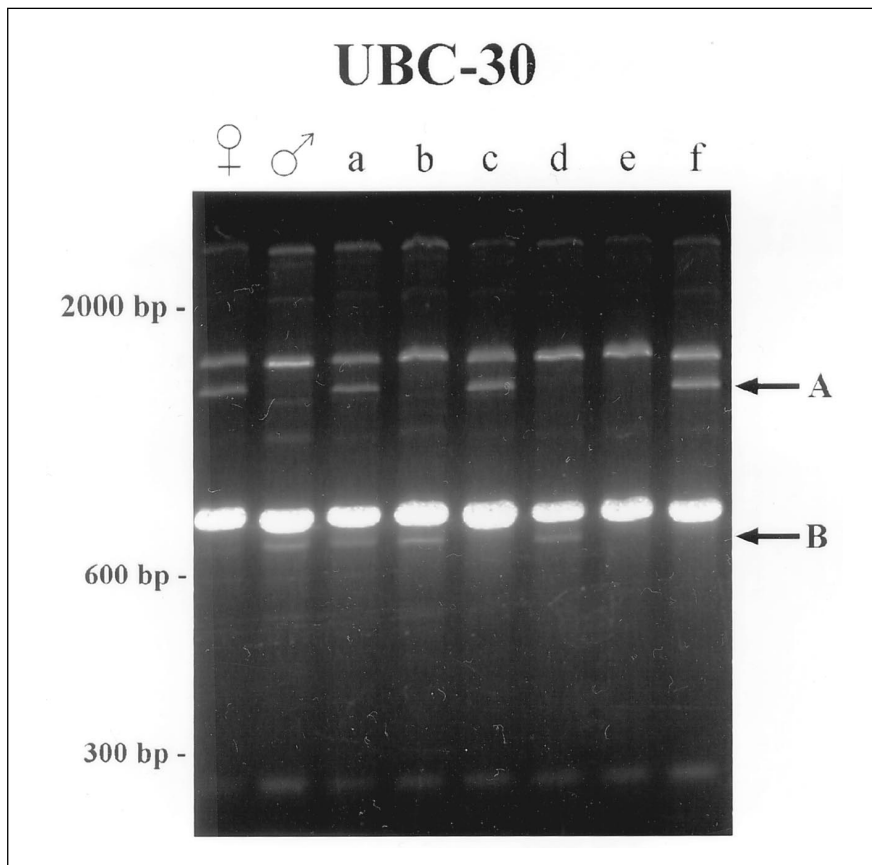


Figure 4. Photograph of a 2.5% agarose gel showing the RAPD banding pattern for a family of *Drosophila*. Arrows A and B show bands that occurred in only one of the parents, labeled ♀ and ♂. The segregation of these variable bands can be clearly seen to be inherited by only half the offspring, labeled a to f.

females of different ages to address the question of relative contributions to future populations of individuals of different ages.

### Other Possible Applications

RAPD markers are currently widely used in evolutionary, ecological and genetic studies. Although we outlined a specific application of RAPDs to the population genetics of *Drosophila*, these markers could also be used for behavioral or evolutionary experiments. For example, Figure 4 shows the results of a RAPD analysis of a family of *Drosophila*. Note that two bands (Figure 4: A and B) which were variable in the parents appear to be inherited in a Mendelian fashion in the offspring. These markers could be used to identify parentage in behavior experiments, or alternatively in sexual selection experiments (e.g. multiple males with one female, which male sired the most offspring).

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Table 4. Values of effective population size ( $N_e$ ) derived from field mark-recapture data or laboratory genetic analysis using RAPDs.

Field Data		Laboratory Data	
Lifespan (Days)	$N_e$	Mutation Rate	$N_e$
2.4	50,968	$10^{-6}$	65,005
3.0	63,710	$10^{-7}$	650,050
5.0	106,183		
11.5	244,221		
60.0	637,100		

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