

*Ecology*, 79(2), 1998, pp. 361–382  
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## WHAT MOLECULES CAN TELL US ABOUT POPULATIONS: CHOOSING AND USING A MOLECULAR MARKER

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**Abstract.** The rapid development of molecular techniques offers a palette of technical approaches for population biologists interested in a wide range of questions. For example, these tools can be used to determine individual reproductive success or to measure rates of genetic divergence among populations. Which technique is most appropriate for a particular question depends upon (1) the extent of genetic polymorphism required to best answer the question, (2) the analytical or statistical approaches available for the technique's application, and (3) the pragmatics of time and costs of materials. Here we evaluate the application of several major techniques (protein electrophoresis, nuclear and mitochondrial RFLPs [restriction fragment length polymorphisms], minisatellite and microsatellite VNTRs [variable number tandem repeats], RAPDs [random amplified polymorphic DNA], and DNA sequencing) to an array of questions regarding individual identification, exclusion and assignment of parentage, and various levels of population structure. In our evaluation, we briefly explain the technical components of each molecular approach and assess whether the typical outcomes expected from each approach will provide useful information as applied to each level of inquiry. For studies of population genetic structure, protein electrophoresis remains a powerful tool for most taxa, although techniques based on nucleic acids (particularly DNA sequencing and mitochondrial DNA RFLPs) are useful here as well. Recently developed nucleic acid techniques (e.g., VNTRs) can often identify enough genetic variability to address questions of self-identification or parentage. Some of the newest techniques (RAPDs and microsatellites) are potentially useful across a number of levels of inquiry, although procedures for adopting them are still developing.

**Key words:** *allozyme; DNA; DNA fingerprinting; molecular markers; population structure; restriction fragment length polymorphism (RFLP).*

### INTRODUCTION

Population biologists seek to understand how variations in survivorship, fertility, and gene flow contribute to changes in allele frequencies within and among populations. Events such as mate acquisition, reproduction, immigration, and hybridization are of particular interest in efforts to understand the process of adaptation to particular ecological circumstances. To determine ecological correlates of fitness within populations and to determine the extent of divergence among populations, one often needs access to independently segregating genetic markers that do not influence the organism's phenotype. Empirical studies that employ allozyme polymorphisms as genetic markers have contributed a great deal to our understanding of population processes. However, due to a lack of sufficient allozyme variation, many enticing research

directions have been impractical to date, especially those requiring information about genetic relatedness of individuals within populations.

In the last decade, a diverse array of new molecular genetic tools has become available for high-resolution genetic studies of population-level processes. The hope of obtaining highly informative genetic markers for tracking individuals and/or their genes under field conditions has led many population biologists to consider switching to DNA-based techniques. At present, though, the rate at which new molecular techniques are being developed far outstrips their efficient and reasoned incorporation into studies of population ecology. In many cases, statistical methods for analyzing novel genetic data have yet to be formulated. This has led to confusion among population biologists about various new techniques, often causing people to prematurely abandon standard techniques (such as electrophoresis of allozymes) that provide readily interpretable data. The allure of exploring newly accessible variation in DNA sequences needs to be tempered by a thorough

Manuscript received 28 July 1994; accepted 20 December 1994; final version received 19 June 1997. For reprints of this Special Feature, see footnote 1, p. 359.

consideration of the trade-offs associated with different techniques and the types of data obtained from them.

Our aim is to provide this perspective. First we define the basic categories of genetic techniques available for use in population ecological studies, including explanations of fundamental procedures common to all techniques as well as features unique to particular classes of techniques. We present the basic categories of techniques in order of increasing resolution of resulting information, and we evaluate how these techniques can be applied to a standard range of questions in population ecology. Features such as cost, technical difficulty, appropriateness of resulting data, and sophistication of existing statistical methods are all considered. Technological innovations are proceeding so quickly that "new" molecular techniques may soon be viewed as outdated, while seemingly impossible approaches become more feasible. In this climate of technical change, this review is meant to serve as a foundation for those now in need of high-resolution genetic markers.

Another goal of this paper is to make molecular techniques more accessible to a general audience. With the recent proliferation of ecological and evolutionary studies that employ molecular markers, population biologists must have some understanding of these techniques in order to comprehend and evaluate current literature. This review is intended to provide more information than recent essays on minisatellite, microsatellite, and RAPD [random amplified polymorphic DNA] markers (Burke 1989, Queller et al. 1993, and Hadrys et al. 1992, respectively), and less detail than texts by Hoelzel and Dover (1991), Hoelzel (1992), Avise (1994), and Hillis et al. (1996).

#### BASIC TECHNIQUES

In this section, we begin with a brief review of allozyme variation, followed by a more detailed explanation of genetic markers obtained from DNA itself (e.g., RFLPs [restriction fragment length polymorphisms], RAPDs, mini- and microsatellite DNA). All of these techniques involve careful extraction of molecules, either proteins or DNA, and electrophoretic separation of the molecules on a gel so that polymorphisms can be detected. The type of genetic variation that is sampled by these methods includes allelic variation in gene products (allozymes) and length variation in specific DNA fragments. For the greatest possible resolution, divergence in nucleotide base sequences can also be examined using DNA sequencing techniques.

#### *Allozymes*

*Background and technical considerations.*—Prior to the development of electrophoretic techniques in the 1950s (Smithies 1955, Hunter and Markert 1957), few single-locus genetic markers were available to population biologists. Early markers included genes for

Mendelian traits such as flower or fruit color and serological incompatibility reactions. Protein electrophoresis provided a new source of marker genes, and allowed individuals to be identified as homozygotes or heterozygotes at a given locus. The term "allozyme" refers to different allelic forms of nuclear-encoded enzymes, whereas "isozyme" is a more general term referring to different biochemical forms of an enzyme identified by electrophoresis.

Beginning with a series of papers in 1966, allozyme frequencies in multilocus surveys were used to quantify genetic variation in populations of humans and *Drosophila* (Harris 1966, Johnson et al. 1966, Hubby and Lewontin 1966). These surveys revealed a surprising amount of variation in natural populations and stimulated an era of rapid exploration. Now, some 30 years later, we have a rich and varied literature in which allozyme data have been used to address questions dealing with local mating patterns, fine-scale structure within populations, and broadscale variation across species' ranges (e.g., Nei 1972, 1973, Selander and Johnson 1973, Clegg 1980, Nevo et al. 1983, Weir and Cockerham 1984, Slatkin 1985, Loveless and Hamrick 1984, Hamrick 1989, Brown 1990).

Below we review basic electrophoretic procedures to give the reader a sense of the labor and expense required per sample (for further details, see Richardson et al. 1986, Soltis and Soltis 1989, and Acquaah 1992). At the start of an electrophoretic study, one must determine the appropriate methods for extracting active, nondenatured enzymes. Preparing samples for electrophoresis is relatively simple, but special care must be taken to prevent the enzymes from losing activity. Depending on the organism, tissue samples can be stored cold, frozen, or freeze-dried prior to extraction. Specific buffers are used to extract the enzymes, sometimes including ingredients to counteract protein-binding substances such as plant tannins. Tissue samples are individually ground or homogenized in the buffer, applied to a gel (usually starch or polyacrylamide), and enzymes are separated by size, shape, and/or charge along an electrical gradient (~30 samples are run on each gel, each in a different "lane"). Enzyme-specific stains are applied to the gel so that the positions of different allozymes can be visualized as colored bands.

Interpreting stained electromorph bands on a gel requires a thorough understanding of the genetic basis of allelic variation for each enzyme (reviewed by Richardson et al. 1986, Wendel and Weeden 1989). Homozygotes at a given locus typically yield one band, while heterozygotes typically yield two, three, or five bands, depending on the quaternary structure of the enzyme. If one has some familiarity with common banding patterns, an initial screening of 20–30 individuals is often sufficient to estimate the number of common alleles per locus for a given enzyme. It is often important to know whether particular allozymes are

TABLE 1. Summary of the percentage of allozyme loci that are polymorphic in different organisms.

Category	Number of species studied	Percentage polymorphic loci
Vertebrates	551	23%
Mammals	184	19%
Birds	46	30%
Fish	183	21%
Invertebrates	361	38%
Gymnosperms	56	58%
Monocots	80	40%
Dicots	338	29%

*Notes:* Animal studies were summarized by Nevo et al. (1983), and were based on surveys that averaged 23 loci per species. Plant summaries are from Hamrick and Godt (1990) and averaged 16 loci per species.

genetically linked to each other. To ascertain whether this is the case, formal analysis of progeny genotypes is needed.

*Advantages and disadvantages of allozymes.*—Obtaining allozyme data is relatively inexpensive and straightforward once the basic procedures have been perfected for a given species. Large numbers of samples can be processed with far less training and time per sample than the DNA methods described below. Furthermore, comparable data from previous studies and a wealth of standard statistical procedures make allozymes appealing for studies of both fine- and broad-scale genetic variation (Weir 1990). Most allozymes represent codominant Mendelian loci. In general, if it is feasible to use variation in allozymes rather than nucleic acids for a particular question, one should do so.

To provide minimal statistical confidence, many applications of allozymes require at least 10–20 independently segregating, polymorphic loci (each having  $\geq 2$  alleles with a minimum allele frequency of 0.05). Unfortunately, some species are monomorphic for most allozymes, and the number of allozymes that can be screened by standard procedures is limited. Different taxonomic groups exhibit tremendous variation in the amount of allozyme variation they possess (Table 1). On average across taxa, less than half of all loci are polymorphic, and loci with  $>3$  alleles are uncommon. Narrow endemic species and others that have experienced genetic bottlenecks often lack polymorphic loci (e.g., Barrett and Kohn 1991). In some cases, even widespread species with apparent genetic variation in morphological traits can be monomorphic at all or most allozyme loci (e.g., Mashburn et al. 1978). Thus, population biologists often encounter species for which allozymes cannot be used as genetic markers. Moreover, even species that are highly polymorphic typically lack sufficient variation for applications such as genealogical analysis in natural populations (Devlin and

Ellstrand 1990, Devlin et al. 1992, Snow and Lewis 1993).

A further limitation of using allozymes rather than noncoding DNA as genetic markers is that allozymes may differ in metabolic function (e.g., Mitton 1989). Many statistical models in population genetics assume that phenotypic differences among allozymes are minimal and selectively neutral, but exceptions are known (DiMichele and Powers 1982, Patarnello and Battaglia 1992). For example, Watt and his coworkers (1983) found that allozymes of phosphoglucosomerase (PGI) influence temperature tolerance in *Colias* butterflies. Clinal geographic variation in allozyme frequencies, particularly along climatic gradients, may be due to natural selection (e.g., Bergmann 1975, 1978, Powers et al. 1986). Also, microhabitat specialization by electrophoretically distinct genotypes has been reported (e.g., Hamrick and Allard 1972, Heywood and Levin 1985). These correlations suggest that selection can act directly on allozymes or on traits to which they are genetically linked. Finally, selection can maintain genetic variation itself, both within individuals due to heterosis, and within populations affected by balancing selection for polymorphisms (e.g., Mitton 1989, Quattro and Vrijenhoek 1989, Karl and Avise 1992). Most multilocus allozyme studies are probably robust with regard to concerns about microevolution and/or ongoing selection, and there is substantial evidence that the majority of observed variation in enzymes is neutral (Kimura 1983). Nonetheless, it is clear that the ideal genetic marker is one that represents noncoding DNA rather than a gene product that is exposed to selective processes.

#### *Methods for DNA analysis*

*Overview.*—With the development of new molecular techniques, population biologists now have the option of examining variation in nucleic acid sequences. Several of the methods described below are capable of detecting single nucleotide mutations. Highly variable regions of DNA can sometimes provide a unique “fingerprint” for each individual, and access to such fine-scale genetic variation is one of the most compelling reasons for choosing to work with DNA markers.

The physical aspects of DNA also offer several advantages over allozymes. DNA is found in nearly all cells of all organisms and it can be recovered from both living and dead tissue. Furthermore, tissues can be easily stored under field conditions, and in many cases only nanograms are needed for analysis (when amplified by PCR [polymerase chain reaction]). The molecule is so stable that recognizable sequences can remain intact for hundreds of millions of years (e.g., Cano et al. 1993). For most applications, however, fresh or recently preserved DNA is needed for analysis. One must also carefully control for contamination when the DNA fragment of interest is amplified by PCR.

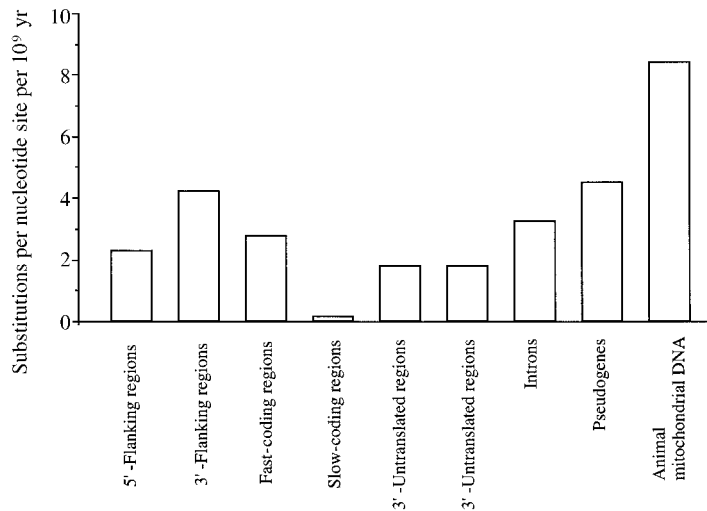


FIG. 1. Average rates of nucleotide substitutions in different parts of the nuclear genome. Data are from Li et al. (1985).

Different regions of the genome experience different selective pressures, depending on the genetic product and/or the tendency of the DNA to withstand changes in nucleotide sequences (Li et al. 1985, dePamphilis and Palmer 1990). For example, strong selection on a DNA sequence such as that coding for Histone HI (a highly conserved DNA binding protein) presumably prevents the accumulation of much variation arising by mutation. On the other hand, noncoding regions can accumulate mutational change in a neutral manner, such that only genetic drift influences the level of polymorphism in a population. Therefore, although the *rate* of mutation may be equal in all parts of the genome, *observed* mutation rates and subsequent population divergence vary due to different selective pressures (Fig. 1). Thus, the optimal DNA segment to use for any particular study depends not only on the degree of relationship among individuals sampled, but on the level of selection imposed on the different regions of the genome.

For readers who may be unfamiliar with molecular techniques, we first review genome organization and discuss the most common methods used to isolate and characterize DNA polymorphisms.

#### *Types of DNA.*—

1. *Nuclear DNA.*—DNA can be classified in two broad categories: nuclear and organellar. Nuclear genomes are much larger than organellar, ranging from  $<10^6$  nucleotide bases (=1000 kb [kilobase]) in some bacteria to  $>10^{11}$  in some plants (Cavalier-Smith 1985, Li et al. 1985). Diploid organisms have two copies of each genetic region (locus) on homologous pairs of chromosomes. These two copies are called alleles, regardless of whether they represent coding or noncoding regions of the genome. Coding regions (exons) are often interspersed with more variable noncoding regions (introns or intergenic regions).

Nuclear DNA (nDNA) contains both unique single-

copy and nonunique, duplicated or repetitive regions. Single-copy regions generally code for a particular gene product. Repetitive DNA consists of core sequences that are repeated in varying degrees. They may be made up of coding segments such as the ribosomal RNA (rRNA) genes, or noncoding tandemly repeated units. The latter include some of the most variable markers identified in the genome of eukaryotes: minisatellite and microsatellite sequences. The repeated units are linked together as consecutive tandem repeats and these repetitive sites may be found at one locus or at many regions scattered throughout the genome. Variation in the number of repeat units is common (Lewin 1990). These variable number tandem repeats (VNTRs) are also called satellite DNA, in reference to the distinct “satellite” peaks seen in a CsCl density gradient centrifugation. Large repetitive units of satellite DNA are often associated with heterochromatin near the centromere. Smaller regions (made up of repeat units  $<65$  bp [base pairs]) are known as minisatellite DNA and can occur throughout the genome (Jeffreys et al. 1985a). Smaller still is microsatellite DNA, which has consecutive repeat units of only 2–6 bp (Tautz 1989).

Another class of repetitive nuclear DNA is ribosomal DNA (rDNA). In eukaryotes, ribosomal DNA occurs in tandem repeats and codes for ribosomal RNA. Much of the sequence of rDNA is conserved across taxa, while other regions are quite variable. Therefore, these molecules are extremely useful in determining phylogenetic relationships, whether ancient or more recently derived (see Hillis and Dixon 1991). Within taxa, however, rDNA sequences are highly conserved so these regions are often less appropriate for most within-population studies (e.g., Learn and Schaal 1987, Capossela et al. 1992; but see Flavell et al. 1986).

2. *Organellar DNA.*—The second major class of DNA is found in chloroplasts and mitochondria. Organellar DNA is inherited in a non-Mendelian, cyto-



plasmic fashion, often with uniparental (usually maternal) transmission. This unique feature of organellar DNA can be used to trace matrilineal and is useful in studies of founder effects, hybridization, and introgression (e.g., Avise et al. 1987, Moritz et al. 1987, Cruzan et al. 1993).

Chloroplast and mitochondrial DNA molecules are small, ~120–220 kb and 15–17 kb, respectively, and circular (Brown et al. 1979, Palmer 1985, 1987). Neither chloroplast DNA (cpDNA) nor plant mitochondrial DNA (mtDNA) are commonly used by population biologists because the former are highly conserved and the latter undergo frequent structural mutations, making it difficult to identify homologous regions (Dowling et al. 1990). In contrast, regions of animal mtDNA may exhibit considerable variation within and among populations. Various studies have shown that the rate of nucleotide substitution is greater in mtDNA than in coding regions of nuclear DNA (Brown et al. 1979). The mitochondrial genome also includes a small non-coding region known as the Displacement Loop (D-Loop), which serves as the origin of replication for the mitochondrial genome. In most animals, the D-Loop is much more variable than the rest of the mitochondrial genome and is therefore a very useful marker for the study of very recently divergent populations or species.

#### *Basic techniques.—*

1. *Extracting DNA.*—DNA can be extracted from tissue samples that are fresh, frozen, dried, or stored in alcohol or buffers (Ausubel et al. 1987, Sambrook et al. 1989, Seutin et al. 1991, Milligan 1992). The desire to minimize impact on study populations has motivated the development of DNA extraction procedures that are noninvasive, involving tissues that organisms leave behind, such as hair, feathers, or feces (Morin and Woodruff 1996). The extraction procedure begins with mechanical pulverization to separate cells and destroy cell membranes and/or cell walls, while leaving the nucleus intact (cell cultures and tissues such as blood do not require mechanical pulverization). Tissue is then immersed in a solution containing a detergent that lyses the nuclear membrane, as well as a proteinase that denatures other proteins, especially nucleases, but leaves nucleic acids intact. Proteins are separated from nucleic acids by extraction with organic compounds (usually phenol and chloroform), and the DNA is purified from the reagents in the extraction buffer by alcohol precipitation or dialysis.

Separation of mtDNA and cpDNA from genomic DNA is a more extensive procedure involving layering of the digested tissue solution on a cesium chloride gradient which is subjected to high-speed centrifugation (Landsmann et al. 1981). Separation of organellar and nuclear DNA is not necessary for many procedures that detect a marker of interest, whether nuclear or organellar, through application of the appropriate probes or primers to total genomic DNA.

Detailed extraction protocols have been developed for many different species, tissues, and types of DNA. A quick search through the literature should reveal a protocol appropriate for any particular organism. Most extraction procedures are completed in small volume (1.5 mL) microfuge tubes, facilitating population biological research that requires samples from large numbers of individuals.

2. *Restriction enzymes.*—Restriction enzymes cleave (“restrict”) DNA at specific nucleotide sequence recognition sites and generate DNA fragments that differ in size when mutations have created or destroyed restriction sites. This size variation is seen in markers known as “restriction fragment length polymorphisms” or RFLPs, described further below. A large number of restriction enzymes are commercially available (>100 enzymes). Each recognizes and restricts a unique 4-, 5-, 6-, or 8-base sequence. The smaller the recognition sequence of the enzyme, the smaller the average size of the DNA fragments produced and the greater the number of different fragments generated by the digestion. For example, 4-cutters typically generate smaller DNA fragments than 5-, 6-, or 8-cutters because specific 4-base sequences occur more frequently in the genome. However, the number of fragments may be so large that fragments of similar size may not be resolvable as separate fragments in an analysis. Thus, choice of enzyme for a restriction digest can be made by considering the size and the number of DNA fragments desired, followed by trial and error to find the best enzyme for a particular system.

The most extensive use of restriction enzymes in population studies has been for the survey of allelic diversity and population differentiation in animal mitochondrial DNA. Enzymatic digestion of the small mtDNA molecule results in a small enough number of DNA fragments that resolvable banding patterns are consistently produced after electrophoresis on an agarose gel (Chapman and Brown 1987). Allele frequencies can then be quantified by the presence or absence of restriction sites among individuals.

Larger molecules such as nuclear DNA cannot be analyzed by restriction enzymes directly because there are too many enzymatic cleavage sites for interpretable banding patterns to be observed. Nuclear DNA cleaved with a 4-, 5-, or 6-base cutter restriction enzyme appears as a smear after electrophoresis. In these instances, fragments of particular interest must be identified after electrophoresis by hybridization with a genetic probe (a fragment of DNA containing the sequence of interest). The presence of sequences complementary to the probe DNA is detected using Southern blot hybridization and autoradiography.

3. *Polymerase chain reaction (PCR).*—The isolation of thermally stable DNA polymerase from the hot springs bacteria *Thermus aquaticus* (*Taq* polymerase) led to an efficient means of amplifying short fragments

of DNA using automated thermal cyclers. The polymerase chain reaction involves replicating target regions of DNA, which are flanked by regions of known sequences (Ehrlich 1989). Synthetic oligonucleotide primers (usually 20–30 bases long) that are complementary to each of the flanking regions are needed. These are combined with a small sample (nanograms) of genomic DNA, plus free deoxynucleotides, a reaction buffer, and *Taq* DNA polymerase. During a series of heating and cooling cycles, the DNA is denatured into single-stranded molecules, the two primers anneal to their complementary sequences on either side of the target region, and the DNA polymerase replicates the region downstream from each primer. The amount of target DNA doubles with each cycle, until microgram quantities are present. For any application restricted to very small amounts of template DNA (forensic work, or any occasion when a particular DNA sample is quite limited, such as work involving museum specimens), one should consider using one of the newer heat-stable polymerases isolated from organisms such as *Thermococcus litoralis* and *Pyrococcus* spp. that have lower error rates than *Taq* polymerase (Cha and Thilly 1995). Under ordinary circumstances, however, the large amount of template DNA available during the early cycles of a PCR reaction minimizes the error rate using *Taq* ( $7.2 \times 10^{-5}$  to  $2 \times 10^{-4}$  mistakes per base).

PCR can significantly decrease the amount of time required to isolate a desired segment of the genome (an alternative is to use a probe, as described below). Also, PCR allows DNA analysis to be performed from small tissue samples. However, for most uses of PCR, one must determine the sequences of regions flanking a given locus, and this can entail considerable effort when working with a new species. The use of “randomly chosen primers,” described under RAPDs below, does allow one to identify genetic markers relatively quickly in species for which extensive sequence information is not available.

PCR techniques are advancing rapidly (Wolfe and Liston, *in press*), and one recent development is a process known as “long PCR,” which increases the fragment size that can be amplified without compromising the precision of amplification (Cheng et al. 1994a). Normal PCR conditions permit the analysis of target DNA up to 3–5 kb, whereas newer techniques may allow amplification of templates as large as 42 kb (Barnes 1994, Cheng et al. 1994b). Many previous studies of RFLPs in natural populations have been limited to small target regions due to constraints of both cloning vectors and the PCR process (e.g., Karl and Avise 1993). Now, the entire mitochondrial or viral genomes may be amplified in one step, a technique that will facilitate efficient and extensive analysis of these DNAs. Likewise, long PCR may be used to isolate genetic markers or particular genes from larger regions of the nuclear genome than is possible with normal

PCR. Long PCR also eases concerns about limited or partially degraded samples because even if only a small fraction of the DNA is intact, target segments from unbroken DNA can be amplified completely in one bout. The development of long PCR approaches to questions in population ecology holds much promise.

4. *Southern blots and hybridizations.*—Southern blotting is a common technique for identifying polymorphic fragments of DNA that differ in size (measured in nucleotide base pairs) because of the gain or loss of restriction sites. Usually, a radioactively labeled, single-stranded DNA “probe” of a known genetic region is used to identify the allelic fragments. First, DNA fragments are cleaved with a restriction enzyme, separated on an agarose gel, and treated with sodium hydroxide to separate complementary strands, making the DNA single-stranded. The DNA is then transferred from the gel by blotting onto a nitrocellulose or nylon filter, and is permanently fixed to the filter by baking or exposure to UV radiation. The filter can then be hybridized with a specific fragment of single-stranded DNA (the probe) that has been labeled either radioactively or by a nonradioactive labeling method. Hybridization allows one to determine the size of the fragments (based on their position after electrophoresis) that carry sequences complementary to those in the probe. The specific region of annealing appears as a “band” on X-ray film exposed to the filter, which has been hybridized with a radioactively labeled probe (nonradioactive methods are also available).

The probe can be obtained from various sources, including probes developed for another species. If sequence divergence is sufficiently small (usually less than ~25%), such “heterologous” probes will allow the identification of known genes. Probes can also be obtained by choosing a restriction fragment from a genomic DNA library of the species under study (described below), or by using synthetic oligonucleotides of known sequence, such as (CA)<sub>8</sub>, a common microsatellite marker. As mentioned above, it is not necessary for the sequence of the probe to be identical to the sequence being studied. Hybridization conditions determine the “stringency” or degree of sequence similarity required between probe and substrate for hybridization to occur, and at lower stringency heterologous probes will hybridize with the fragment of interest despite minor sequence differences. This makes it possible to use probes that are isolated from different populations or even different species to survey allelic diversity in DNA fragments. One example of such universal probes are those used to detect multilocus VNTR loci, commonly known as DNA fingerprints (Jeffreys et al. 1985a).

5. *Genomic library construction.*—If probes are not available for a given application, one can often produce them from a sample of genomic DNA by creating a recombinant DNA library (Ausubel et al. 1987, Sam-

TABLE 2. Simplified schematic banding pattern for one locus (two alleles) and approximate levels of polymorphism that can be detected under optimal conditions with different types of genetic markers.

	Lane					Number of alleles per polymorphic locus	Number of polymorphic loci per gel
	1	2	3	4	5		
	Genotype						
	1,1	1,1	1,2	2,2	2,2		
Allozyme	—	—	—	—	—	2–5	1–2†
RFLP‡	—	—	—	—	—	2 (+/- restriction site)	1–8
Multilocus VNTR	(alleles from many different loci  )					many (number not known)	10–30§
Single-locus minisatellite or microsatellite VNTR	—	—	—	—	—	2–>50	1
RAPD¶	—	—	—	—	—	2 (+/- priming site)	1–10

† With starch gels, up to five horizontal slices can be stained per gel, so a maximum of 5–10 polymorphic loci could be screened.

‡ When a restriction site is present and the individual is homozygous at this locus (lanes 4 and 5), the size of the two fragments sums to the size of the larger fragment from an individual lacking the restriction site (lanes 1 and 2).

§ With multilocus VNTRs, which are cut with restriction enzymes, many bands are present in each lane. Although loci are rarely identified, each band can represent an allele from a different locus (fragments from alternate alleles typically run off the gel).

|| Banding pattern shown in Fig. 2.

¶ Note that heterozygotes are not detectable in RAPDs; individuals in lanes 4 and 5 are referred to as homozygous recessive because no band is obtained at this locus.

brook et al. 1989, Watson et al. 1992). This term refers to a collection of bacteria containing plasmids or phage vectors into which DNA fragments from the study organism have been inserted. Usually, DNA fragments are generated by digesting genomic DNA using restriction enzymes. The fragments are inserted into a cloning site of the vector DNA that has been restricted with the same enzymes. Screening these fragments in various ways allows identification of specific regions of DNA representing genes of interest, loci linked to specific genes, or random genetic segments whose coding function is not known.

6. *Sequencing*.—Nucleotide sequence differences exist among all individuals, even in the most homogeneous populations, yet DNA sequencing to identify such differences is often far too labor-intensive for most population-level studies. DNA sequencing has become a routine procedure since the development of the dideoxy chain termination method. In combination with PCR it provides a method of collecting precise data for short DNA sequences, and it has proven to be especially powerful when combined with the analysis of various regions of the animal mitochondrial genome. Reagents are available for sequencing of DNA generated from phage vectors or asymmetric PCR reactions (Erlich 1989), and stretches of 250–800 nucleotide bases can be sequenced in a single sequencing reaction. With the advent of automatic sequencers this technique may become more amenable to applications in popu-

lation biology, but at present DNA sequencing requires too much effort to be practical for most studies involving large sample sizes.

*Classes of nucleic acid markers*.—DNA markers provide a means for powerful, fine-grained analysis of individual genotypes. The challenge for the population biologist is to find the appropriate method that reliably reveals adequate genetic variation for a particular question, with a minimum amount of effort and expense. If allozymes provide enough variation for the question of interest it makes little sense to use a costlier technique. However, in many situations DNA markers are essential. Moreover, the range of questions that can be addressed using DNA markers is expanding rapidly as more efficient techniques are developed.

Since population biologists often need high-resolution genetic markers, the most useful techniques are those that produce a large number of alleles at a single locus and/or many loci with two or more common alleles (see Table 2). The coding function and location of the regions identified by the markers are not of interest unless alleles are in linkage disequilibrium or seem to be correlated with specific traits subject to selection.

1. *Restriction fragment length polymorphisms (RFLPs)*.—Restriction fragment length polymorphisms (RFLPs) were the first DNA markers to be used by population biologists. An RFLP results when variation in restriction enzyme cleavage sites is detected by

Southern blot hybridization. RFLP variation can sometimes be visualized directly by staining with ethidium bromide following electrophoresis of the DNA in an agarose gel (Table 2). This can be done for small molecules, such as mitochondrial DNA, which produce a manageable number of fragments with many restriction enzymes (Landsmann et al. 1981, Tegelström 1992). For the study of nuclear gene segments, Southern blotting is necessary to visualize the specific region of interest. Probes for a specific gene may be available if the region has been studied in another population or related species. Alternatively, the probe may represent a specific sequence that occurs in many regions, such as the repeated segment of minisatellite DNA. Finally, one might be interested in average gene diversity in the genome and choose random probes from a DNA library. Generally, a number of probes are screened for RFLPs with Southern blots representing genomic digests using several enzymes independently (e.g., Karl et al. 1992). Depending on the level of genetic variation in the population, this process can yield enough polymorphic loci to investigate genetic questions within and among populations. Unfortunately, Southern blotting to study RFLPs is relatively time-consuming and expensive.

A more efficient approach to RFLP analysis may be to use PCR to amplify random fragments of the genome. For one or more clones of ~2 kb chosen from a DNA library, sequences are determined for the 100–200 bases at each end of the clone (larger segments can be isolated with “long PCR,” as described above). PCR primers are constructed complementary to some of the flanking region, and these can then be used to amplify the same fragment in other individuals. The amplified product can then be treated with restriction enzymes and the fragments separated on an agarose gel and visualized by ethidium bromide staining to identify RFLPs (Karl et al. 1992). Although the use of PCR renders this method more feasible than traditional RFLP analysis by Southern blot hybridizations, it still requires a great deal of labor, including the construction of a genomic DNA library, some initial DNA sequencing, and fine-tuning of the PCR conditions. It has been reported that many (up to 30%) of the primers do not yield adequate amplification under average conditions (Karl et al. 1992).

2. *Random amplified polymorphic DNA (RAPDs).*—RAPD markers are produced by PCR using short oligonucleotide primers of randomly chosen sequence. Different RAPD patterns arise when genomic regions vary for the presence/absence of complementary primer annealing sites. The primers are typically 10 bp long (Williams et al. 1990, but see Welsh and McClelland 1990) and no specific knowledge of a particular DNA sequence is required to choose or produce a primer. More than 400 different 10-base primers are commercially available to identify RAPD variation. Primers

are used singly, not in combination with a second primer as would be the case for standard PCR. Because of this, amplified fragments are those regions of the genome that are flanked by “inward-oriented” sequences complementary to the primer. Allelic variation consists of the presence or absence of particular amplification products, which can be separated on agarose gels stained with ethidium bromide. The RAPD process typically reveals several polymorphic genetic segments per primer within populations; other segments may appear as monomorphic bands within or across populations (Hadrys et al. 1992). The degree of variability observed for many primers suggests that the technique will be useful for a variety of questions, including individual identification, paternity analysis, strain identification, and phylogenetic analysis.

RAPD markers are rarely inherited as codominant alleles. Loss of a priming site results in complete absence of the enclosed amplified segment, not simply a shift in mobility on the gel (Table 2). In heterozygotes, therefore, differences may appear only as differences in band intensity, which is not usually a reliable phenotype for PCR analysis. As a consequence, information on the parental origin of alleles may be inaccessible for RAPD markers, as compared to codominant markers such as RFLPs or allozymes (Lewis and Snow 1992). Because of their short length, RAPD markers may produce some artifactual amplification products, and careful control of DNA quality and amplification conditions is necessary to ensure reproducible banding patterns (Carlson et al. 1991, Riedy et al. 1992, Scott et al. 1993).

3. *Variable number tandem repeats (VNTRs).*—

a) *Multilocus minisatellite DNA.* Minisatellite DNA occurs at scattered sites in the genome and is thought to be noncoding (Jeffreys et al. 1985a). Composed of short sequences that are repeated in tandem, these regions are hypervariable, primarily due to variation in the number of repeat units at each locus (hence the acronym VNTR). In the conventional multilocus approach, “allelic” products from individual loci cannot usually be identified because the same sequence, which is identified using a genetic probe consisting of a few units of the repetitive sequence, is repeated at different sites in the genome. Mutation to new length alleles most likely occurs by unequal crossing over or strand slippage during replication, resulting in changes in the number of core units between flanking restriction sites (Jeffreys et al. 1988).

Polymorphisms for minisatellite loci are detected by cutting genomic DNA with a 4-cutter restriction enzyme, separating resulting fragments by agarose gel electrophoresis, Southern blotting to nylon, and hybridizing to repeat sequence probes to identify fragment length differences that arise from variation in repeat number. Standard multilocus probes can be used successfully with a wide range of taxa (Burke 1989).



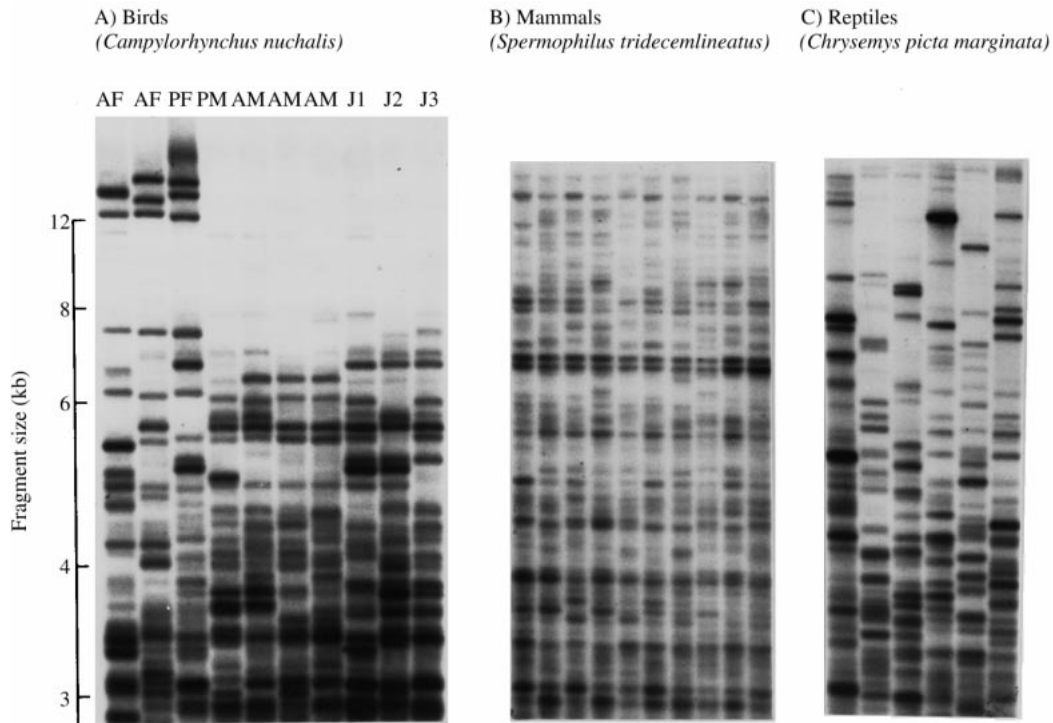


FIG. 2. Examples of multilocus minisatellite "DNA fingerprints" from three vertebrate classes using Jeffreys's multilocus probes. Note the number of "loci" screened and the enormous variability among individuals, especially in birds and reptiles. Panel A represents a nuclear family of a highly social tropical bird, the Stripe-backed Wren *Campylorhynchus nuchalis*. AF = subordinate adult females, AM = subordinate adult males, PF = principal or dominant female, PM = principal or dominant male, J = juvenile. PF is the mother of all Js; PM is the father of J2; the first AM is the father of J1 and J3. Panel B illustrates a thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) mother in the far left lane and nine of her offspring. Panel C represents six painted turtles (*Chrysemys picta marginata*) of unknown relatedness captured at the same site.

When visualized on a Southern blot, DNA fragments appear as a series of 10–30 readable bands per individual (Fig. 2). Allelism between bands is not known without complex segregation analysis, and the pattern is treated as a multicharacter phenotype. Heterozygosities at the individual loci are often high enough that even closely related individuals have an exceptionally low chance of having identical multilocus banding patterns. Minisatellite markers are often well suited to address questions of individual identification and paternity exclusion because a great deal of variation is detectable within populations. However, these markers may pose problems for some applications because of allelic ambiguity.

b) Single-locus minisatellite DNA. The ideal markers to study both individual identification and genetic variability within and among populations are single loci that are highly polymorphic. Development of locus-specific probes for minisatellites provides access to allelic identity for these hypervariable VNTR markers. However, to date this procedure has involved library construction, screening, and sequencing for probe development, followed by sequential gel blot hybridizations until sufficient resolution is achieved. We do not consider this technique further because it is more

labor intensive than using single-locus microsatellites, described below, although "long PCR" technology may facilitate access to these useful markers in the near future.

c) Microsatellite DNA (SSRs). Microsatellite DNA is extremely useful for population studies because single loci with as many as 30–50 alleles can be examined (e.g., Amos et al. 1993). These VNTR markers, also called "simple sequence repeats (SSRs)" are composed of tandemly repeated 2-, 4-, or 6-base repeat units (such as CA, CAAC, or GGAACC) that have been identified by screening genomic libraries with probes made up of tandemly repeated oligonucleotides (Tautz 1989, Hughes and Queller 1993, Queller et al. 1993, Schlötterer and Pemberton 1994). Microsatellite loci are analyzed by amplifying the target region using PCR, followed by electrophoresis through an acrylamide gel to allow resolution of alleles that may differ in size by as few as two base pairs (Table 2).

A disadvantage of microsatellites is that identifying these regions from a genomic library for a new species can be time-consuming. Known primers are not thought to be likely to amplify the same locus across related taxa unless the microsatellite region is flanked by highly conserved sequences where priming sites are located

(Ellegren 1992, FitzSimmons et al. 1995). More recent work suggests that this may occur more often than originally thought; microsatellite primers developed for a species of swallow also detected polymorphic microsatellite markers for 32 of 39 other bird species within the same order, and also detected minimal polymorphism in 6 of 19 bird species of other orders (Primmer et al. 1996). Similar associations between useful microsatellite polymorphism and genetic distance are being found within mammals as well (Deka et al. 1994, Pépin et al. 1995). This good news means that investigators might begin a study on a new species by trying already-available primers developed for other species (as closely related as possible to the study species) before investing in development of primers. Despite the initial difficulty of developing microsatellite markers, they are becoming more widely used than multilocus minisatellite DNA markers because of the great advantages of studying allelic variation at discrete loci.

4. *DNA sequences.*—The most detailed analysis of DNA differentiation can be obtained by sequencing the region of interest from different individuals. Until recently, extensive use of DNA sequencing for population studies has not been practical because the DNA fragments to be sequenced for each individual had to be isolated from subgenomic DNA libraries after identification by Southern blotting and hybridization. Using PCR, however, specific regions can be targeted for amplification if the sequence of the conserved regions flanking the region of interest is available. The amplified fragment can then be sequenced.

The most difficult problem for analyzing genetic markers lies in the selection of the region of the genome that both reveals allelic variation and can be sequenced efficiently. Population biologists are usually interested in DNA sequences that evolve relatively rapidly, such that allelic differences are common within a short segment of DNA. Primer sequences can often be determined from the flanking sequences of the region in the same or closely related species. It is also possible to use known amino acid sequences of the region being studied to construct primers by reverse translation to arrive at the DNA sequence, although problems may be encountered due to redundancy in the genetic code (Erlich 1989). Once primers are synthesized, PCR products can be sequenced to determine the nature and number of substitution events at the nucleotide level or the degree of allelic diversity within a given fragment of DNA. This approach has the advantage of providing the investigator with large amounts of information from a single region of the genome.

*Relative costs.*—We will make rough estimates of the relative costs of the major techniques in terms of three major cost categories: personnel, equipment, and supplies.

Personnel costs associated with running samples can range from salaries for one or more expert technicians,

to graduate student stipends, to free, if graduate students who are supported otherwise run their own samples. While the cost per unit time thus varies enormously, it is simpler to estimate the time itself. Any technique will require set-up and development time, which can be minimized by visiting working laboratories and taking careful notes on everything from equipment to protocol. Certainly several months should be allowed for this. Once a technique is fully operational, a full-time technician running allozyme samples can probably produce genotypes for several thousand individual samples (per locus) per year. A good technician can produce ~1000 multilocus minisatellite DNA fingerprints in a year, or a similar number of RFLP genotypes. Microsatellite genotypes can be produced at a faster pace once the development and optimization of primers are accomplished, which can require as much as a year or as little as a couple of months. Sequencing reactions can be estimated at 50 samples per week for a 300-base sequence using manual sequencing, or for an 800-base sequence using an automated sequencing apparatus.

Equipment costs are similarly variable, depending on what equipment is already available on a shared or communal basis, and whether the facility will need multiples of some items for multiple users. In general, a basic minimal DNA laboratory can be equipped for as little as \$20 000 (assuming availability of a communal spectrophotometer, distilled water apparatus, darkroom supplies, and only one each of microcentrifuge, hybridization oven, thermal cycler), and as much as \$120 000 for a state-of-the-art multiple-project facility.

Operating supplies costs are least for allozyme analyses, at \$1 per sample per locus. Microsatellite markers also cost less than \$1 per sample per locus, once development and optimization of primers are accomplished. Development and optimization of microsatellites can cost from several hundred to several thousand dollars per species, depending upon how quickly suitably polymorphic loci are found. Since this is likely to depend on the history of the population under study (large panmictic populations should harbor more polymorphism than small isolated populations), it is not possible to generalize. Costs of expendable supplies associated with producing markers depending on hybridization of blots from enzyme digests are highest per unit: we estimate ~\$7 per sample for multilocus minisatellite fingerprints and a similar cost for RFLP analysis, which uses basically the same technology. Expendable supplies associated with sequencing reactions for a standard 300 bases should cost slightly less than \$5 per sample.

*Health hazards.*—A number of hazardous materials are involved in these procedures, especially for techniques utilizing DNA markers. Use of some classes of dangerous materials is highly regulated at the institu-

tional level (e.g., radioactive materials for gel blot hybridization procedures or sequencing) to meet federal guidelines. Others are less tightly controlled, and responsibility for their careful use falls to laboratory supervisors. Ethidium bromide is used to visualize DNA at several steps; it is a powerful mutagen that should be handled with extreme caution. Polyacrylamide, used as the matrix through which sequencing and microsatellite products are size separated, is a dangerous neurotoxin. Even the organic solvents used in DNA extraction, phenol and chloroform, can cause significant damage if spilled on the skin or if their vapors are inhaled. A careful researcher can use all of these materials for years without any health consequences if they are handled appropriately. Molecular cloning manuals such as Sambrook et al. (1989) provide excellent guidelines for the handling and disposal of toxic chemicals commonly used in a molecular genetics laboratory.

#### APPLICATION OF MOLECULAR MARKERS

##### *General considerations*

Population ecologists often attempt to provide accurate descriptions of basic demographics of populations by following the histories of individual organisms. Certain demographic parameters are more easily estimated (e.g., death rates among adult age classes in plants), while others are universally problematic. Most critical among these are accurate estimates of individual reproduction, dispersal patterns, and historical rates of movement of individuals among populations. Since estimates of lifetime reproductive success are useful measures of relative success rates of particular genotypes, reliable measures of reproductive success are essential for investigating the process of adaptation to different ecological circumstances. Furthermore, accurate estimates of effective population size depend on accurate estimates of individual fecundity and its variance for both sexes. In these types of studies, assigning paternity has been especially difficult (hence our exclusive focus on females in life tables), leading many researchers to consider using molecular genetic markers.

Matching techniques with questions requires an assessment of the degree of resolution required. There is a direct relationship between the extent of genetic polymorphism required of the technique and the level of relatedness that can be addressed. For example, to assign actual parents from among a group of closely related individuals, high resolution is required to distinguish true differences among individuals. Individual identification of highly mobile animals also requires an approach that reveals enough variation to distinguish each individual by a unique profile. Less resolution is required to distinguish adjacent vegetative clones, or to discern genetic divergence among demes or populations.

In all cases, the appropriate markers must be suitably polymorphic to reveal similarities within units (whether clonally derived individuals, nuclear families, demes, or populations) and differences among units. Of course, the level of polymorphism detected by any technique will depend partly on the genetic history of the population; nonetheless, there should be a consistent relationship between a technique and the variation it assays within a particular population (e.g., VNTRs should always be more variable than allozymes). Weighed into any choice of molecular approach should be its practicality in time and cost of materials relative to other techniques (e.g., Smouse and Chakraborty 1986). One must also evaluate the statistical rigor with which a given approach can be applied. Some techniques have been so recently developed that appropriate statistical tests have yet to be formulated for them.

We discuss three levels at which knowledge of genetic relatedness among individuals becomes important in population studies: (1) individual identity; (2) parentage; and (3) estimating effective dispersal among groups, demes, or populations. Recognizing genotype identity is critical for populations in which organisms propagate clonally or in studies of individual movement patterns, including dispersal or migration (assuming individuals cannot be tagged with permanent coded markers). Patterns of reproductive success or parentage can be described at two levels of resolution: exclusion or assignment. An exclusion approach involves asking whether particular individuals (usually the putative parents in systems in which they are identified a priori based on some objective criteria) could be the parents of the focal individual. Exclusion occurs when an offspring has genetic markers not attributable to either of its putative parents. This approach is also used in studies of self-compatible plants to determine the extent of selfing vs. outcrossing (e.g., Clegg 1980). Assignments involve identification of the two parents of the focal individual by excluding, with high levels of confidence, all other possible parents in the population.

The third level of study within populations involves assessment of population differentiation or the extent to which populations are connected by effective dispersal. The genetic structure of a population reflects the effective movement of individuals or their genes. A highly structured population comprises subunits that have diverged genetically because little effective dispersal occurs among them. We consider studies asking whether significant structure occurs at the level of the neighborhood, social group, or deme. In addition, one might ask about relationships among populations or subunits such as the distribution of various matrilineages or lineages among groups within populations.

In this section we evaluate the application of several categories of molecular techniques (allozymes, nucleic acid RFLPs, VNTRs, and RAPDs) to studies involving individual identification, parentage assessment, and

TABLE 3. Evaluation of appropriate molecular techniques for studies involving parentage exclusion, parentage assignment, and population structure.

Technique	Ease	Parentage: exclusion	Parentage: assignment	Population structure
Allozymes	electrophoresis; analysis straightforward	fair if sufficient polymorphism available	rarely assays sufficient polymorphism	<b>excellent</b> for sufficiently polymorphic taxa
Nuclear RFLPs	extraction, digestion, electrophoresis, library screening, gel blot hybridization; analysis straightforward	fair if sufficient polymorphism available	many polymorphic loci required	good
Mitochondrial RFLPs	extraction, digestion, electrophoresis, gel blot hybridization; analysis straightforward	inappropriate for paternity; low power for maternity	inappropriate	good
VNTRs: multilocus minisatellite†	extraction, digestion, electrophoresis, gel blot hybridization; analysis problematic	<b>excellent</b>	good if number of potential parents is small	good estimator of relative diversity; poor tool for study of divergence
VNTRs: microsatellite (PCR)†	primer development may be long (library screen, sequencing); thereafter extraction, PCR, electrophoresis; analysis straightforward	<b>excellent</b> ; number of primers necessary depends on polymorphism and population size	good for large samples if sufficient number of highly polymorphic loci (primers) available	should be good for study of diversity; less variable loci may be good for study of divergence
RAPDs	extraction, PCR; analysis problematic but developing	possible but may be problematic owing to artifactual bands	good for relative contributions to large broods; more general application developing	may be <b>excellent</b> ; analysis developing
Sequencing	extraction, PCR, prepare DNA template, electrophoresis; analysis straightforward	possible but cumbersome	prohibitively cumbersome	<b>excellent</b> tool for study of divergence; many analytical approaches

† Single-locus minisatellites combine elements of both VNTR categories described here. They are assayed through gel blot hybridization; probe development involves library screening. Application involves power of microsatellites as well as their straightforward analysis.

identifying relationships among groups or subpopulations (Table 3). In most cases, we focus on techniques that are needed for studies of organisms in their native environment; studies of artificially established populations often require less resolution.

#### *Individual identification*

Studies involving the identification of mobile, widely dispersing individuals require methods that reveal the highest level of variation possible. For sessile organisms capable of clonal propagation, however, far fewer markers are needed. In populations of clonal plants or invertebrates, it is often desirable to estimate genetic diversity and the spatial distribution of different genets (i.e., unique genotypes).

*Allozymes and RFLPs.*—Using allozyme or RFLP markers, one could obtain minimum estimates of the distribution of clonal genets in a population (e.g., Ellstrand and Roose 1987, Grosberg 1991, Jelinski and Cheliak 1992). However, these markers possess too few

polymorphisms to be useful for individual identification in most systems. Although numerous RFLP markers can be found in a given species, the cost of developing sufficient numbers of mildly polymorphic markers to specify each individual and of running the numbers of gels required would usually be prohibitive.

*VNTRs.*—For the quickest way to know if individual identification is possible in the study system, multilocus minisatellite VNTRs (conventional “DNA fingerprinting”; Jeffreys et al. 1985a) are probably the best choice. VNTRs are likely to provide unique marker profiles for sessile genets (e.g., Nybom et al. 1990, Rogstad et al. 1991) as well as mobile organisms. We do not discuss the extensive literature on DNA fingerprinting in humans or other model genetic systems (e.g., *Mus*, *Drosophila*, or *Arabidopsis*) because undomesticated species have not been studied at nearly the same level of resolution.

The advantages of using minisatellite VNTRs include the likelihood of assaying individual-specific



polymorphisms in a single hybridization. The polymorphism found among minisatellites in natural populations is usually great, except in island and other extremely isolated populations (Gilbert et al. 1990, Reeve et al. 1990). Since mean allele frequencies across the family of loci screened by each multilocus probe are often estimated to be on the order of  $q = 0.15$ , the likelihood that a fragment present in one individual would be shared by a second unrelated individual is  $2pq + q^2$  (the likelihood that the second individual is either heterozygous or homozygous for the allele, where  $q$  = frequency of average allele and  $p$  = combined frequencies of all other alleles, or  $1 - q$ ) or 0.28. The number of scorable fragments detected per probe/enzyme combination is  $\sim 20$  under typical stringency, so the likelihood of two individuals in the population having identical fingerprints can be calculated as  $0.28^{20}$  or  $8.8 \times 10^{-12}$ . The statistical approaches for individual identification are fairly straightforward (Jeffreys et al. 1985b), but they require the assumption of independent assortment of fragments. This assumption is testable only within large nuclear families (Burke and Bruford 1987); fragments determined to be linked or allelic can be removed from analysis (Bruford et al. 1992). In fact, the proportion of fragments so identified is typically small, making the assumption of independence reasonable in most cases (but see Brock and White 1991). Another statistical difficulty of multilocus VNTR markers stems from the use of pairwise comparisons of individuals within (or between) populations as primary data in applications involving assays of genetic relatedness among individuals within populations or among subpopulations. These band-sharing coefficients (Wetton et al. 1987, Lynch 1990) create problems of nonindependence among data points as individuals are used in multiple pairwise comparisons. Whether this interdependence is best corrected by permutational analyses or by subsampling schemes is not clear (Danforth and Freeman-Gallant 1996).

The disadvantages of VNTRs as conventional multilocus fingerprints include its relatively high cost in materials ( $\sim \$10$ /sample in expendable supplies) and time (close to 10 days from start to finish, although dozens can be run simultaneously). However, the time investment may be worthwhile if one's alternative is to examine multiple single-locus markers to accumulate sufficient polymorphism for individual identity. Scoring of multilocus fingerprints is laborious and often uncomfortably subjective. Allelism among fragments is difficult to ascertain and in fact not often expected, since most fragments have been run off the bottom of the gel to separate and resolve the larger fragments remaining. Therefore, true allele frequencies cannot be calculated. Multilocus minisatellite data appropriate for individual identity or parentage determination (see *Parentage* below) would not typically be

useful for other levels of questions (e.g., interpopulation) that require robust analysis of allelic distributions.

Unique profiles may also be generated through the accumulation of allelic information across several highly polymorphic microsatellite loci (Tautz 1989, Love et al. 1990, Queller et al. 1993). For most studies, this would be labor intensive at the outset, particularly if DNA libraries must be screened for the simple-sequence repeats. Primers developed for these loci were once thought to be species-specific, although several research groups have found that primers developed for one species will also amplify a corresponding locus in closely related species (e.g., mammals: Moore et al. 1991, Schlötterer et al. 1991, Rubensztein et al. 1995; birds: Ellegren 1991, 1992, Primmer et al. 1996). As more researchers develop and screen such primers across species, those that work well within a family or order are accumulating (e.g., Primmer et al. 1996). However, the application of microsatellite markers to questions requiring individual identity usually requires multiple primer development, unless one's study species is in a well-characterized taxon. For long-term population studies or systems in which very large sample sizes are anticipated, the ease with which analyses can be performed subsequent to development of primers makes this approach attractive.

Statistical approaches to analysis of microsatellite data in the context of individual identity should be based on number of loci screened, allele frequencies, and population size, as in calculations of probability of detection using allozymes or nuclear RFLPs (discussed in the following section [*Parentage*]). An important difference is that microsatellite allele frequencies are likely to be much smaller because there can be many alleles per locus. The likelihood of a second individual having the same genotype as a focal individual is easily estimated. For example, if the focal individual is found to be heterozygous at four microsatellite loci, at each of which are found four equally common alleles, the likelihood of another individual having an identical genotype is equal to the probability of finding the same genotype at the first locus ( $2pq = 2(0.25)(0.25) = 0.125$ ), multiplied by the likelihood of finding an identical genotype at the second locus (0.125), multiplied by the likelihoods for the third and fourth loci, or  $0.125^4 = 2.44 \times 10^{-4}$ . The likelihood of another individual having a *different* multilocus genotype from the focal individual is  $(1.0 - 2.44 \times 10^{-4})^n$ , where  $n$  is the number of individuals in the population. If the population contained 100 individuals, more than 97% of the individuals would be expected to have genotypes different from that of the focal individual. Adding a fifth locus with four equally common alleles increases this value to 99.7% ( $[1.0 - 3.05 \times 10^{-5}]^{100}$ ). Once the necessary number of polymorphic microsatellite loci is calculated and primers have been developed, multiple loci can be screened simultaneous-

ly in multiplex PCR reactions to facilitate processing (Luty et al. 1990, Edwards et al. 1991, Hazan et al. 1992).

### Parentage

Special requirements of markers for examining parentage depend upon whether the goal is exclusion or assignment. Those involving assignment will require much higher levels of resolution, attainable either through multiple applications of single-locus markers or high-resolution multilocus markers. For accurate assignment studies, very thorough population sampling must be carried out to ensure that a reasonable proportion of the potential parents are included.

*Exclusion.*—The most robust analyses for exclusion of nonparents involves sampling complete nuclear families. In this case, the probability of detection (likelihood of detecting a nonparental putative parent) can be calculated based on allele frequencies and number of loci screened (Chakravarti and Li 1983, Westneat et al. 1987).

1. *Allozymes.*—Classic exclusion studies of parentage used allozymes (Birdsall and Nash 1973), in which the probability of detection depends on allele frequencies. For example, assuming Hardy-Weinberg equilibrium, a single polymorphic locus with two equally common alleles will allow parental exclusions from among the reproductive adults in the population only for the 50% of the young that are homozygous, as adults with any genotype could have contributed to heterozygous young. For each homozygous young, only 0.25 of the possible parental adults can be excluded (the complementary homozygotes). This does not narrow the field appreciably, but samples can be quickly and inexpensively run once sufficient numbers of polymorphic loci have been identified. For exclusion analyses in which putative parents are identified a priori, even heterozygous young can be used to detect nonparentage when both putative parents are identical homozygotes. The application of allozymes to exclusion parentage analyses has been frustrating in some groups (e.g., birds) with typically low levels of protein polymorphism (Barrowclough et al. 1985, Mumme et al. 1985, Evans 1987), although more recent studies have benefitted from earlier workers' identification of polymorphic allozyme loci (Brooker et al. 1990, Bollinger and Gavin 1991). As noted earlier, the percentage of allozyme loci that are polymorphic varies among species, but it is generally higher among plants than among animals (Table 1).

2. *RFLPs.*—Nuclear RFLPs can be used in exclusion analyses (Quinn and White 1987) by adopting the same analytical approach as allozymes, with the probability of detection approaching 100% as the number of RFLPs increases (Soller and Beckmann 1983). Mitochondrial RFLPs are not appropriately applied in parentage analyses since they are almost always mater-

nally inherited. Of course, mitochondrial RFLPs could be used in maternal exclusion analyses, but with extremely low power. Since different mitochondrial types do not recombine, numerous mothers within a population would be expected to share mitochondrial lineages.

3. *VNTRs.*—Multilocus minisatellite fingerprinting is an efficient means of paternity exclusion because it screens multiple polymorphic loci simultaneously (Wetton et al. 1987, Burke and Bruford 1987). These loci are present in large numbers per genome and are highly polymorphic.

In the hypothetical average population discussed earlier, the mean allele frequency ( $q$ ) across the dozens of loci screened by a minisatellite probe was 0.15. An offspring correctly placed with actual parents should share slightly more than half of its fragments with each parent  $((1 + q - q^2)/(2 - q))$  or 0.61 (Jeffreys et al. 1985b, Georges et al. 1988), and every fragment in the offspring banding pattern should be attributable to a fragment found in either or both of the putative parental patterns. On the other hand, any nonparent should be easily detected. An offspring tested against one parent and one nonparent should have, on average, 0.61 of its fragments accounted for by the actual parent, leaving 0.39 to be tested against the nonparent. Of that 39% (7.8 bands of 20 on average), it will share 28% or 2.2 on average with the nonparent, leaving 7.8 - 2.2 or 5.6 bands unattributable to either putative parent. These statistical approaches to exclusion analyses are straightforward (Jeffreys et al. 1985a, Georges et al. 1988), but require sampling of complete families. For families incompletely sampled, exclusions rest on statistical assignment of categorical relatedness (that is, asking whether an individual can be excluded as a first-order relative of the young in question), after calibrating the scale of similarity by dyads of "known" relatedness (Kuhnlein et al. 1990).

Other disadvantages of multilocus fingerprinting include the difficulty of scoring banding patterns even for technically excellent autoradiographs with large numbers of resolvable bands. This difficulty stems from the complexity of the patterns, which is paradoxically their greatest advantage. For systems with high levels of polymorphism (mean allele frequencies measured across all loci screened are often as low as 0.05–0.15), there are frequently no fragments common to all individuals on a gel, meaning that there are no "landmark" fragments against which to judge the relative positions of other fragments. As a result, accuracy of comparisons falls dramatically with distance between lanes on a gel (Piper and Parker Rabenold 1992). Lanes containing samples from putative parents must be placed near those of offspring and must therefore be run repeatedly in particularly large families. Alternatively, internal standards can be run in each lane. Even correctly assigned parents will occasionally produce an

offspring with one (and rarely more) band not attributable to either parental type owing to the high mutation rate that generates new-length alleles at these loci (Jeffreys et al. 1988).

The most efficient approach for exclusion analyses might involve single-locus highly polymorphic PCR-based markers such as microsatellites. Simple patterns allow scoring across multiple lanes within gels and across gels, assuming accurate estimates of sizes allowed by high-resolution polyacrylamide sequencing gels. The accumulation of a small number of highly polymorphic loci should allow high probability of detection of exclusions (e.g., Morin et al. 1994). Paternity exclusion probabilities can be calculated for each locus based on the allele frequencies observed in the study population (Chakravarti and Li 1983, Morin and Woodruff 1992).

4. *RAPDs*.—Use of random amplified polymorphic DNA in studies of parentage is still so new that it is difficult to evaluate. Some have supported this application (Hadrys et al. 1993, Milligan and McMurry 1993), but the technique has rarely been used with natural populations (but see Wagner et al. 1996 in which RAPDs were used effectively as an auxiliary technique alongside multilocus minisatellite VNTRs). Disadvantages of RAPDs in this application include Mendelian dominance of the markers and the reduction this causes in exclusionary power (Lewis and Snow 1992). Applications of RAPDs to known pedigrees show that it is sometimes hampered by unexplained, perhaps artifactual, fragments (Riedy et al. 1992). However, for species producing large clutches or broods of offspring, the effect of artifactual bands that could lead to false exclusions in an offspring-by-offspring analysis can be minimized by a pooled "synthetic offspring" approach that allows testing of proportionate representation of different potential parents (Hadrys et al. 1993).

*Assignment*.—Assignment of parentage requires that all but the true parents of an individual can be excluded with statistical confidence. In theory, any technique that assays sufficient variation relative to the size of the population should allow assignment of parentage. In practice, however, paternity assignment is often impractical in natural populations of promiscuous organisms (this includes all outcrossing plants). If one's goal is to compare the relative fertilization rates of different males, rather than identify who their offspring are, then likelihood estimates should be used to obtain the most accurate data per unit effort (Devlin et al. 1988, Roeder et al. 1989, Milligan and McMurry 1993, Snow and Lewis 1993). Lower resolution markers, such as allozymes, RFLPs, and RAPDs may be adequate for this type of analysis, but highly polymorphic, single-locus VNTRs would provide more statistical power. The most feasible study systems for parentage analysis seeking assignments are animal species in which the pool of possible fathers is very small (on the order of <20

individuals), and in which behavioral observations can be utilized to further narrow the pool of candidates.

1. *Allozymes and RFLPs*.—With these markers allele frequencies for each locus can be used to calculate the number of loci needed to exclude all nonparental adults. This is equivalent to a probability of detection of 100%, which various models suggest will be approximated with use of 10 loci, each with three or more alleles of equal frequency (e.g., Westneat et al. 1987, Brown 1990). Probability of detection remains below 90% for use of 15 loci with two equally frequent alleles, and continues to fall as frequency of alleles becomes more skewed (Westneat et al. 1987, Wrege and Emlen 1987). Unless multiple polymorphic loci are available, statistical assignment will be difficult or impossible. Examination of data from allozyme studies (Table 1) suggests that these requirements can rarely be satisfied.

2. *VNTRs*.—For reasons similar to those given in the previous section, minisatellites and microsatellites are likely to be the preferred approach for attempts to assign parentage (Fig. 2). Use of minisatellites, especially multilocus probes, is subject to the same cautions presented above regarding positioning of individual lanes on gels to be near enough to other lanes of interest. This limitation may be prohibitive in systems in which promiscuity is suspected, or in which there is a large number of equally likely potential parents. For these situations (probably most sexually reproducing organisms would fall in this class), a possible route is a two-stage approach similar to that used by Gibbs et al. (1990). An initial screening of the population using a highly polymorphic single-locus marker is used to group adults into pools of potential parents with respect to each offspring. Once the pool has been thus narrowed, the potential parents can feasibly be run on the same multilocus minisatellite gel to identify actual parents. The typical statistical power of this technique to distinguish parentage even among very closely related potential parents is impressive. In Stripe-backed Wrens, for example, the calculated error rate at which uncles were misassigned as fathers using two minisatellite multilocus probes hybridized sequentially with a single genomic blot was  $3.6 \times 10^{-5}$  (Rabenold et al. 1991).

3. *RAPDs*.—The problems associated with the still-new application of RAPDs to exclusion analyses, above, will make it an even less suitable technique when the goal is assignment. The "synthetic offspring" approach requires large numbers of putative offspring and provides an estimate, not a count, of the representation of different putative parents in a clutch (Hadrys et al. 1993). The application of maximum likelihood methods to RAPD data for estimating paternal reproductive success is an intriguing possibility (Milligan and McMurry 1993).



*Estimating relatedness among individuals or differentiation among groups*

Population ecologists are often concerned with describing the nature of their study population and its interactions with other groups of the same species. Is it effectively connected with neighboring populations through dispersal, or are these units isolated? Are smaller "neighborhoods" effectively isolated within the population? These questions ask about the levels at which populations are structured and are best approached by making estimates of genetic similarity within and between the different hierarchical levels, beginning with pairs of individuals. Questions involving inbreeding or outbreeding rates seek coefficients of relatedness (the proportion of genes identical by descent) between individuals, while studies of genetic substructuring of populations quantify the distribution of genetic variance within and among clusters or demes. Traditionally, studies of the latter type use techniques that provide allele frequencies for calculating Wright's *F* statistics (Wright 1978). Estimates of relatedness at the individual level (e.g., the coefficient of relatedness of two individuals drawn at random from a population) will require markers assaying maximum variation in order to distinguish pairs that have many elements in common (close kin) from those that do not. In contrast, the study of relatedness among social groups or larger subpopulations should generally require more conservative markers that would have elements common to members of groups, with sufficient variation to reveal any divergence between groups.

*Allozymes and RFLPs.*—Techniques assaying simple variation among alleles at single loci are appropriately applied to studies of genetic substructuring of populations. Methods for estimating relatedness at individual and higher levels from diploid genotypes at single allozyme loci are well developed (e.g., Pamilo and Crozier 1982, Hamrick 1989, Pamilo 1989, Queller and Goodnight 1989). Accumulation of data across several loci makes possible the detection of substructuring of populations at different hierarchical levels (e.g., within and between families, colonies, neighborhoods, or populations) (Queller et al. 1992, Leberg 1996). Use of nuclear RFLP data in allozyme paradigms is appropriate; it is perhaps more likely that RFLPs would reveal polymorphisms since they screen anonymous sites in the genome that are less likely to be subject to stabilizing selection than allozyme variants. Should allozymes or RFLPs not reveal sufficient polymorphism within the study population, isolation of single minisatellite or microsatellite loci of high heterozygosity should provide necessary resolution (Wong et al. 1986, Bruford et al. 1992, Warner et al. 1992).

Animal mtDNA RFLPs are typically extremely well suited to studies of genetic divergence of populations over large geographic areas (e.g., Avise et al. 1987, Seutin et al. 1993). The small size of the mitochondrial

genome, its predominantly maternal inheritance, and relatively high rate of base-pair substitutions (especially in the D-loop) make it a valuable tool for studying relationships among subspecies and higher taxonomic units. Within species, sufficient variation is often revealed via mitochondrial RFLPs to examine genetic structure among populations (e.g., Avise et al. 1987, Quinn and White 1987).

*VNTRs.*—Multilocus minisatellites were heralded as a tool that would allow fairly precise estimates of genetic relatedness of two individuals drawn at random from a population. However, the variance in the distributions of proportions of bands shared by dyads of varying levels of relatedness is great enough to limit its use in this regard (Lynch 1988). Presumably, this variance derives from variation in number of scorable bands per lane as well as "background" or nonhomologous band sharing among nonrelatives. However, such similarity indices based on band-sharing values can be used to compare relatedness within and among groups where such precision is not necessary (Lynch 1990), and variance in distributions of similarity indices can be reduced by combinations of multiple probes, enzymes, and scorers to the point where nonoverlapping distributions have been obtained for at least first-order relatives, second-order relatives, and a category combining lesser relatives (Piper and Parker Rabenold 1992). Thus, it is possible in some systems to use multilocus VNTRs to describe relatedness of pairs of individuals drawn at random, but this requires careful calibration of the technique in the population under study. More progress has been made in application of VNTRs, particularly multilocus minisatellites, to estimating average relatedness within social groups (Reeve et al. 1992).

Novel analyses based on allelic information from microsatellite loci have provided evaluation of relatedness within and between social groups of organisms with intractable demography like pilot whales (Amos et al. 1993). Microsatellites have several advantages over other techniques: (1) they provide information from individual loci and therefore are amenable to calculations of allele frequencies necessary for population studies; (2) they typically exhibit high levels of gene diversity and so are expected to routinely assay high levels of polymorphism; and (3) once primers have been developed, the protocol consists of PCR and electrophoresis, eliminating time-consuming gel blot hybridizations. Microsatellite markers are currently being used to define relationships among populations (e.g., Bowcock et al. 1994), and much progress is being made in development of analytical approaches to this application that take into account the stepwise nature of mutations at microsatellite loci (Goldstein et al. 1995, Slatkin 1995).

Single-locus information is obtainable from minisatellites as well (Bruford et al. 1992), but typically



these markers must be identified by gel blot hybridization with single-locus probes. Furthermore, minisatellites tend to be more variable than microsatellites (Bruford et al. 1992), making them perhaps too variable for population-level work. If markers from individuals of one cluster are so variable that they have few common elements, distinguishing that cluster from another becomes difficult. Available evidence suggests that microsatellite VNTRs are potentially the most widely applicable single technique that we have discussed.

**RAPDs.**—New analytical approaches to RAPD markers may render them useful in studies of genetic differentiation of populations. Russell et al. (1993) used RAPD markers in study of cocoa populations by partitioning variability into within- and between-population components using Shannon's index of phenotypic diversity (King and Schaal 1989). Similarity values (based on proportion of bands shared in RAPD profiles) were used to generate a similarity matrix (Nei and Li 1979) for use in cluster analysis. An alternative approach focuses on single variable bands for each locus, avoiding the errors associated with calculation of similarity indices (Haig et al. 1994). Data accumulated in this way across loci were treated as phenotypic  $+/-$  data and subjected to AMOVA (Analysis of Molecular Variance; Excoffier et al. 1992) to describe the organization of phenotypic patterns at group and population levels. The tremendous advantage of RAPDs is the technical simplicity of the production of these markers. Also, their application to population structure and phylogeny is not as constrained by the occasional artifactual band as is their application to studies of parentage. However, these advantages are offset by the need to sample both more individuals (2–10 times more) and more loci to accommodate for the lack of complete genotypic information caused by dominance (Lynch and Milligan 1994).

**DNA sequencing.**—Determining the nucleotide sequence of a DNA fragment is reliably applied to questions at population and taxonomic levels (Nei 1987). Sequencing avoids the problems inherent in protein electrophoresis of allelic variants that go undetected owing to redundancy in the genetic code or similar migration distances on a gel. For use as genetic markers, DNA sequencing may often involve very laborious and expensive procedures, and has been applied to population-level questions infrequently. Although highly variable fragments of the genome can be identified, problems with linkage disequilibrium are a major concern. Population ecologists are most often interested in questions about the particular organism or group of organisms they study. Polymorphism within a single gene assayed by DNA sequencing reveals the evolutionary history of *that single gene*, which may be quite different from the evolutionary history of the population (Nei 1987, Avise 1994). Thus, many genes must be sequenced if reliable inferences about a population

are to be obtained, and the technical feasibility of DNA sequencing quickly becomes a serious concern.

Despite these limitations, great advances are being made in understanding the evolutionary process of DNA sequence variation by sequencing particular DNA fragments such as mitochondrial DNA control regions (Taberlet 1996). For population ecologists, the extent to which this provides insight into the evolution of other DNA markers such as allozymes, VNTRs, and RFLPs is important. Furthermore, if adaptation of particular characters are of interest to a population ecologist, identification and sequencing of particular genes that are involved in the expression of that character may be profitable. However, this leads into the realm of molecular biology and evolution. At the current time, DNA sequencing for population ecologists is one of the most technically challenging and expensive techniques available.

#### CONCLUDING REMARKS

The main purpose of this review is to provide population ecologists with an informed appreciation of both the advantages and the disadvantages of working with a variety of molecular markers. In certain areas of ecology and evolutionary biology, rapid progress is being made because newly available markers can identify individuals, populations, genetic strains, or closely related species. Thus, it is becoming increasingly important for population biologists to be able to understand and evaluate molecular data, and to know whether their own research questions could be addressed with molecular techniques.

Here we have described the types of molecular markers that seem best suited for different levels of questions. If the anticipated results from a particular technique seem worth the effort and expense involved in obtaining them, we recommend contacting a colleague who has the technique up and running. Arranging a visit to a working laboratory to observe the protocol firsthand can save months of time in simply getting a given technique to work. All too often, the information one needs to begin using a new technique is not fully reported in journal articles, but rather exists in the written or oral traditions of different laboratories. This is particularly true when the goal is to discover the best protocol for a new species by tinkering with extraction techniques, PCR conditions, electrophoretic methods, etc.

For many field biologists with limited funding, molecular markers are undoubtedly too costly and labor intensive to consider (e.g., Weatherhead and Montgomerie 1991; but see Pemberton et al. 1991). To some extent, these obstacles can be lessened by sharing equipment, moving temporarily to another laboratory (after working out a collaborative arrangement), or arranging to have the work done on a contractual basis in a competent laboratory. We expect that molecular

tools will become increasingly accessible as students trained in these applications become more numerous, and as technological innovations in molecular biology and the biotechnology industry make molecular data more affordable for the average researcher.

## ACKNOWLEDGMENTS

We thank Daniel Crawford, Nidia Arguedas, Susan Kephart, Louise Lewis, and Paul Lewis for commenting on the manuscript. Our work was supported by NSF grants BSR-8818038, BSR-9111848, and DEB-9322544 to P. G. Parker, and BSR-8906667 and DEB-9306393 A. A. Snow.

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