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Molecular Primatology

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THE UTILITY AND PROMISE OF MOLECULAR PRIMATOLOGY

The fundamental aim of molecular primatology is to describe and interpret the patterns of molecular variation found within and between primate taxa. There are a number of areas in contemporary primatology where molecular studies are particularly useful.

First, molecular studies provide a means for quickly and efficiently generating data that can be used for inferring phylogenetic relationships among groups of organisms. One advantage of molecular data is that the alternative states of a molecular character are typically far less ambiguous than for many morphological traits (e.g., a nucleotide base that is "adenine" versus a molar crest that is "high"). Another advantage of molecular over morphological data is that, at least for some loci, reasonably good estimates of the rate of change (mutation rates) for deoxyribonucleic acid (DNA) and amino acid sequences are available. If these rates of change are reasonably consistent over evolutionary time (i.e.,

approximate a so-called molecular clock), researchers can infer the timing of lineage splitting events within a phylogeny. Over the last decade, molecular phylogenies for extant primates have largely replaced morphology-based ones.

Second, molecular studies provide a direct way to investigate numerous features of primate social systems that are not easily observed in the field. For example, using various kinds of molecular markers, researchers can conduct parentage assessments, document kin relationships among individuals within or between social groups, examine population substructuring, assess levels of inbreeding within and gene flow between populations, and evaluate sex biases in dispersal behavior, allowing inferences to be made about mating systems, social organization, and dispersal patterns that are typically impossible to make based on observational studies alone (Di Fiore 2003).

Third, molecular techniques can contribute significantly to applied primate conservation efforts both in the wild and in captivity. For example, researchers might use genotyping of noninvasively collected samples (e.g., feces, hair from nests) to perform molecular mark–recapture studies for determining population density and sex composition or, if species-specific markers are available, to assay the presence or absence of a particular species in an area. Similarly, with genotype data, captive colony managers and zoo personnel can make more informed decisions about which animals to breed in order to minimize inbreeding and preserve the genetic variation present in captive populations. Routine health monitoring and veterinary care for captive primates benefit from molecular studies as well. A variety of polymerase chain reaction (PCR)–based tests are already available that allow animals to be quickly screened for various hereditary disorders as well as for infection by numerous pathogens.

Fourth, molecular studies of primates are increasingly relevant to questions of human health and disease as well as to the tracking and management of disease outbreaks in wild animal populations. Primates are natural reservoirs and zoonotic sources for a variety of current and emerging human diseases (e.g., ebola, Marburg, simian or human immunodeficiency virus, herpes simiae virus) (Daszak et al. 2000, Hahn et al. 2000). Molecular studies of both pathogen and host populations can lead to better understanding of the source and mode of transmission of these diseases, as well as the factors responsible for differential susceptibility to various pathogens.

Finally, the current era of *genomics* (the study of the complete DNA sequence of the genome of an organism) holds promise for evolutionary biologists to be able to undertake far more complete and sophisticated comparisons of the organization, function, and evolution of genomes across taxa. Nearly complete genome sequences are now known for a variety of organisms, ranging from microbes to invertebrates to mammals, including mice (Mouse Genome Sequencing Consortium 2002), modern humans (International Human Genome Sequencing Consortium 2001, 2004), and our close relative the chimpanzee (Chimpanzee Sequencing and Analysis Consortium 2005), with the completed cow, dog, rat, and rhesus macaque genomes expected soon. Comparative genomic studies are providing insights into the history of genes associated with important diseases in humans and are shedding light on a variety of candidate genes that have been implicated in the development of many aspects of biology and behavior that appear to distinguish humans from the great apes. For example, despite being extremely closely related genetically and sharing close to 99% of their genomes in coding regions, humans and chimpanzees differ notably in many aspects of life history, physiology, behavior, and, fundamentally, their capacity for symbolic and acoustic communication. Molecular studies are now beginning to reveal some of the important molecular differences between modern humans and chimpanzees (Gagneux and Varki 2001).

BREADTH OF THE FIELD

Current use of the term *molecular primatology* typically equates molecular studies with studies of DNA, the fundamental molecule of genetic heredity. Indeed, studies of primate DNA have burgeoned in the last decade as researchers have increasingly employed genetic techniques (particularly microsatellite genotyping and direct DNA sequencing) to investigate mating systems and social structure (Altmann et al. 1996; Keane et al. 1997; Gagneux et al. 1997b, 1999; Gerloff et al. 1999; Nievergelt et al. 2000; Laundhart et al. 2001; Constable et al. 2001; Vigilant et al. 2001; Utami et al. 2002; Radespiel et al. 2002; Kappeler et al. 2002; Wimmer et al. 2002), dispersal patterns (Morin et al. 1994, Melnick and Hoelzer 1996, Di Fiore 2002, Di Fiore and Fleischer 2005), and within-group social behavior, kin recognition, and relatedness (Goldberg and Wrangham 1997, Alberts 1999, Hohmann et al. 1999, Mitani et al. 2000, Vigilant et al. 2001, Widdig et al. 2001, Di Fiore and Fleischer 2005). Genetic data are also contributing increasingly to attempts to infer phylogenetic relationships among all major radiations of primates (strepsirhines, Yoder et al. 1996, Yoder 1997, Yoder and Yang 2004; New World monkeys, von Dornum and Ruvolo 1999, Schneider et al. 2001; Old World monkeys, Stewart and Disotell 1998, Disotell 2000, hominoids, Arnason et al. 1996, Ruvolo 1997, Satta et al. 2000), as well as to evaluate the demographic (Gagneux et al. 1999; Storz et al. 2002a,b) and biogeographic (Rosenblum et al. 1997a,b; Goldberg and Ruvolo 1997; Evans et al. 1999; Collins and Dubach 2000a,b; Hapke et al. 2001; Gagneux et al. 2001; Tosi et al. 2000, 2003; Cortés-Ortiz et al. 2003; Wildman et al. 2004) histories of various primate groups.

However, the true scope of molecular primatology is much broader than simply the study of DNA sequence-level genetic variation, and the discipline has deep historical roots (Table 22.1). First, there are many other types of biomolecules besides DNA that investigators might use to explore patterns of natural variation within and between primate species,

Table 22.1 Brief Overview of Significant Developments in Molecular Primatology

DEVELOPMENT	REFERENCE(S)
Discovery of human blood groups	Landsteiner (1900)
Early immunological experiments on blood serum cross-reactivity	Nuttal (1904)
First correct assessment of the human chromosome number ($2n = 46$)	Tjio and Levan (1956)
Immunological cross-reactivity experiments to elucidate the phylogeny of hominoid primates: results suggested a much more recent common ancestry for humans and great apes than previously thought	Sarich and Wilson (1967)
Dramatic improvements in cytogenetic techniques for staining metaphase chromosomes	Caspersson et al. (1968)
Phylogenetic studies of many of the major primate radiations using immunological methods	Goodman and Moore (1971), Goodman et al. (1974), Dene et al. (1976a,b, 1980), Baba et al. (1979), Sarich and Cronin (1976)
Early applications of amino acid sequence data to primate phylogenetic studies	Goodman et al. (1975), Goodman (1976), Hewett-Emmett et al. (1976), Romero-Herrera et al. (1976), Tashian et al. (1976)
Allozyme methods applied to studying the population structure, dispersal pattern, and mating system of a variety of wild primates (e.g., baboons, lion tamarins, howler monkeys, rhesus macaques, long-tailed macaques)	Jolly and Brett (1973), Brett et al. (1976), Melnick et al. (1984a,b), Forman et al. (1987), Pope (1990, 1992), de Ruiter et al. (1992, 1994)
Cytogenetic studies of hominoid primates suggest a sister-taxon relationship for humans and chimpanzees to the exclusion of gorillas	Yunis and Prakash (1982)
DNA–DNA hybridization studies of the phylogenetic relationships among African great apes provide additional support for a sister-taxon relationship between humans and chimpanzees	Sibley and Ahlquist (1984, 1987)
Development of PCR, which allows in vitro cloning of specific fragments of the genome from a complex DNA sample	Saiki et al. (1985, 1988), Mullis and Faloona (1987)
Evolutionary relationships among hominoids investigated using 2D allozyme electrophoresis: results provide additional support for a chimpanzee–human clade within the hominoids	Goldman et al. (1987)
RFLP studies of mitochondrial DNA variation suggest a recent African origin for all modern humans	Cann et al. (1987), Vigilant et al. (1991)
Demonstration of the utility of noninvasive sampling (e.g., hair, buccal cells, feces) as a source of DNA for PCR-based genotyping and sequencing studies of primates	Higuchi et al. (1988), Takasaki and Takenaka (1991), Morin and Woodruff (1992), Sugiyama et al. (1993), Constable et al. (1995)
Minisatellite DNA "fingerprinting" applied to paternity testing in captive and semi-free-ranging primates (e.g., ring-tailed lemurs, mandrills, Barbary macaques, rhesus macaques, stump-tailed macaques)	Periera and Weiss (1991), Dixson et al. (1993), Paul et al. (1993), Berard et al. (1993, 1994), Bauers and Hearn (1994)
Widespread use of PCR-based genotyping and sequencing in molecular ecological and phylogenetic studies of primates	1995 to present

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; 2D, two-dimensional.

from various types of ribonucleic acid (RNA) to regulatory enzymes to cell membrane proteins to "classic" serum protein markers. Moreover, there are numerous ways in which many of these molecules can be modified following transcription and translation through posttranslational modifications (e.g., addition of sugars, phosphates, sulfates, fatty acids, etc.). The study of variation among and within taxa in all of these molecules also falls under the rubric of "molecular primatology" and can contribute to our understanding of the evolutionary relationships among primate taxa, the selective pressures which have acted on populations within taxa, and the differences in physiology and behavior observed between taxa. Additionally, advances in the study of chromosomes (e.g., cytogenetic techniques such as chromosome painting through fluorescence in situ hybridization, or FISH) and new computational methods for mining and comparing large sets of sequence data are providing greater insights into variation among primate taxa in genome structure. Among such findings are the discovery of differences among taxa in the type, frequency, and distribution within the genome of various kinds of repetitive DNA, transposons (i.e., mobile pieces of DNA, or "jumping genes"), and endogenous retroviruses (Gagneux and Varki 2001, Trask 2002). Finally, prompted by insights from comparative genomic and gene expression studies, we have come to realize the significance of variation in both coding and noncoding (e.g., promoter and enhancer binding sites, pre-messenger RNA [mRNA] splicing signals present in introns and at intron/exon boundaries) regions of the genome for producing phenotypic variation, either through their regulation of transcription or through their effects on pre-mRNA splicing (Hastings and Krainer 2001, Modrek and Lee 2002, Cartegni et al. 2002).

As these loci play a central role in the link between genotype and phenotype and likely explain much of the evolutionarily significant phenotypic variation seen among closely related primate taxa, they should be important targets for future research.

In the remainder of this chapter, our aim is to provide a brief but broad summary of modern molecular primatology. We begin by summarizing some of the major laboratory methods that are currently used to analyze molecular variation, dividing our discussion into four sections: variation at the level of the genome, variation at the level of the gene or DNA sequence, variation in gene structure and expression, and variation in other types of non-DNA molecules. We then briefly detail the kinds of samples primatologists should collect in the field in order to be able to carry out these types of molecular studies and provide some methodological suggestions for sample collection and storage. Finally, we point to a number of resources where students and researchers new to this field can find more information on laboratory and analytical methods.

MODERN TECHNIQUES FOR ASSAYING MOLECULAR VARIATION

Depending on the question being addressed, researchers will be interested in different levels of molecular variation. For example, comparative karyotypic or cytogenetic data are useful for broad phylogenetic studies as well as for comparing genome organization across taxa. Data on variation between taxa in a variety of non-DNA molecules (e.g., cell surface proteins) and comparative data on gene expression can provide insights into the fundamental biological differences among nonhuman primate taxa. DNA sequence data, like cytogenetic data, are useful for evaluating the phylogenetic relationships among a group of taxa and can also be used to examine intraspecific variation (e.g., for phylogeographic studies) if the loci under investigation evolve rapidly enough. Finally, for detailed analyses of identity, parentage, and relatedness within populations of a particular species, researchers would be most interested in assaying variation at very rapidly evolving and hypervariable loci such as microsatellites or minisatellites.

Variation at the Level of the Genome

Studying the organization and comparative composition of whole genomes is the purview of the fields of cytogenetics and comparative genomics. Classical cytogenetics uses precipitated metaphase chromosomes, which are harvested from actively dividing cells and then dropped on a glass slide, causing the chromosomes to form a spread or splatter. The chromosomes can then be treated with enzymes (e.g., trypsin) to partially digest the histone proteins around which DNA is coiled and then stained with various dyes to create banding patterns that reflect the different base pair composition of particular areas (e.g., GC-rich bands, which contain the majority of active genes). The species-specific number and appearance (morphology) of such chromosome spreads is called a *karyotype*.

Studies of the primate genome classically done by cytogenetic analysis are rapidly evolving to merge with molecular genetic techniques such as fluorescent nucleotide probe hybridization on whole chromosomes (FISH) (Ried et al. 1993; Wienberg and Stanyon 1995, 1997, 1998). FISH studies use PCR to produce chromosome-specific probes from flow cytometry–sorted chromosomes of a particular species. Fluorescent dyes incorporated in the amplified fragments can then be used to "paint" the target chromosomes of other species, where the fluorescent probes will hybridize to complementary sequences found on any of the chromosomes of the species under investigation. This technique allows the detection of major rearrangements in chromosomes between taxa by highlighting where genetic material with similar sequence has been maintained through evolution (Fig. 22.1). The use of reciprocal chromosome painting via FISH has produced insights into chromosome evolution within a number of major primate groups, including strepsirhines (Müller et al. 1997, Stanyon et al. 2002), platyrrhines (Stanyon et al. 2000, 2001; Neusser et al. 2001), colobines (Bigoni et al. 2003, 2004), and hylobatids (Arnold et al. 1996; Koehler et al. 1995a,b; Müller et al. 2003).

At a more detailed level, comparative genomic studies have revealed a surprising degree of variation among taxa in genome organization due to dynamic processes of inter- and intrachromosomal segmental duplications. These duplications, which range in size from a few thousand to a few million base pairs, are widespread within the genome; and their prevalence suggests that the genome is much more plastic than previously thought (Samonte and Eichler 2002, van Geel et al. 2002). While segmental duplications are more common in gene-poor regions of the genome, some duplications also involve regions containing functional genes, and some of these appear to have surprisingly high rates of evolutionary change (e.g., the *morpheus* gene family, Johnson et al. 2001). Recent work is focusing on the relationship between chromosomal rearrangements and the quick pace of sequence evolution in genes located near rearrangement sites (Navarro and Barton 2003, Marquès-Bonet et al. 2004). The jury is still out, however, on the importance of such effects for gene expression evolution and its potential role in the speciation process (Zhang et al. 2004).

Variation at the Level of the Gene or DNA Sequence

Modern techniques for studying DNA sequence-level variation take advantage of a relatively small set of fundamental concepts of molecular biology: the cutting of DNA with restriction enzymes, the joining of DNA fragments with DNA ligase, the separation (denaturing) and joining (annealing) of complementary strands of DNA by heating and cooling, the replication of DNA using DNA polymerases,

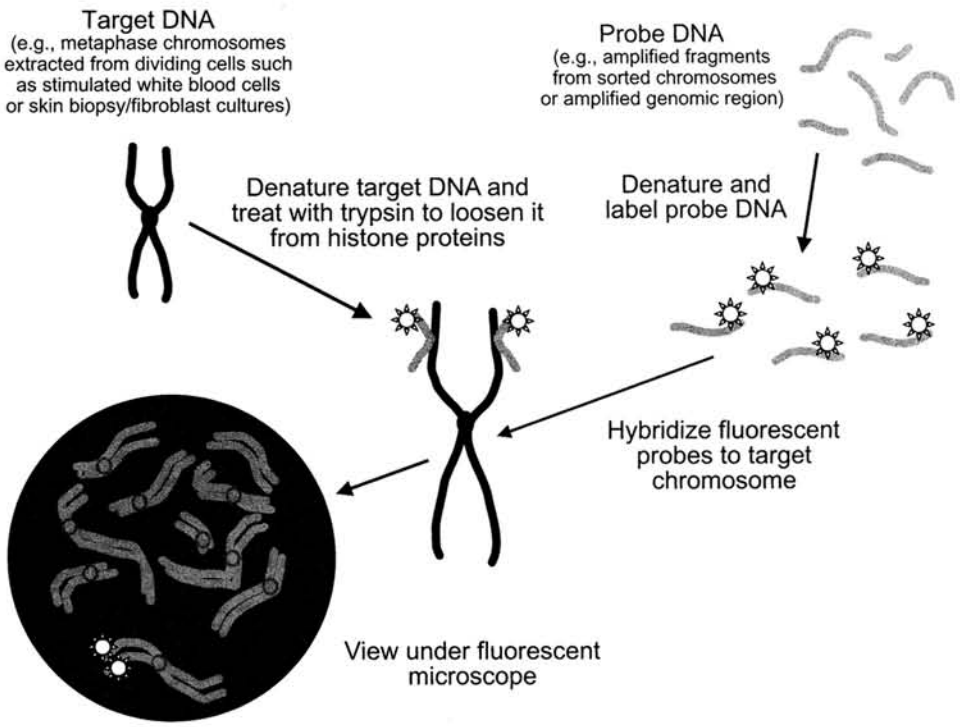


Figure 22.1 Fluorescence in situ hybridization (FISH). This technique allows the localization of DNA sequences on chromosomes with the help of fluorescent dyes attached to short DNA probes. It can be used to study chromsome rearrangements (as in reciprocal chromosome painting) or to detect the precise chromosomal location of a known stretch of DNA, such as a fragment of genomic DNA containing a gene of interest.

and the separation of fragments of DNA by size through gel electrophoresis. Since there are many excellent reviews of the basic molecular biological concepts applied in modern genetic analysis, we will not undertake such a task and instead direct the reader to those reviews (Hoelzel 1998, Hillis et al. 1996, Ferraris and Palumbi 1996, Baker 2000). We focus here on only a small set of techniques that are currently used to generate marker data (e.g., genotypes) and DNA sequences.

The workhorse tool of virtually all modern genetic studies is PCR (Saiki et al. 1985, 1988; Mullis and Faloona 1987). PCR is essentially a tool for in vitro cloning, allowing the many-million-fold amplification of a specific target fragment of the genome from the midst of a complex DNA sample. PCR takes advantage of the discovery of various thermostable polymerases whose activity is not degraded by the high temperatures required to denature double-stranded DNA prior to replication. In PCR, template DNA and DNA polymerase are mixed with free nucleotides (deoxynucleotide triphosphates, dNTPs) and *primers*, short oligonucleotides of DNA that are complementary to the sequence flanking the region to be amplified, thus providing the double-stranded template necessary for the polymerase to function properly (Fig. 22.2).

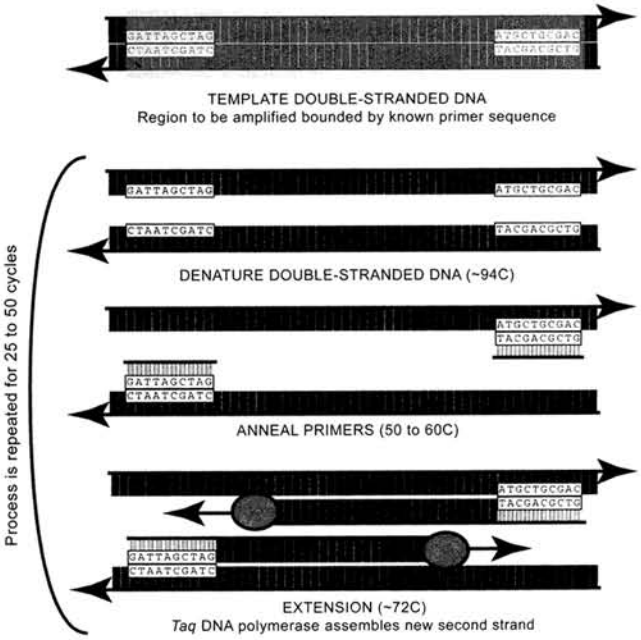


Figure 22.2 The polymerase chain reaction (PCR). The most common element in almost all modern genetic studies of primates, PCR takes advantage of a heat-stable DNA-replicating enzyme isolated from a hot-spring bacterium (*Thermophilus aquaticus*) from Yellowstone National Park to generate large numbers of identical copies of a given target DNA sequence.

Microsatellite Genotyping

Microsatellites, also known as simple sequence repeats or short tandem repeats, are one class of molecular markers known more generally as variable number of tandem repeat (VNTR) loci. These loci consist of a short 1–6 bp motif repeated in series a variable number of times (Fig. 22.3).

Microsatellite regions tend to evolve far more rapidly than other portions of the genome, presumably due to slippage of DNA polymerase as DNA is replicated during the process of gametogenesis (Levinson and Gutman 1987, Kruglyak et al. 1998, Toth et al. 2000). The various alleles present in a population at a given microsatellite locus can be assayed through PCR amplification of the locus followed by electrophoretic

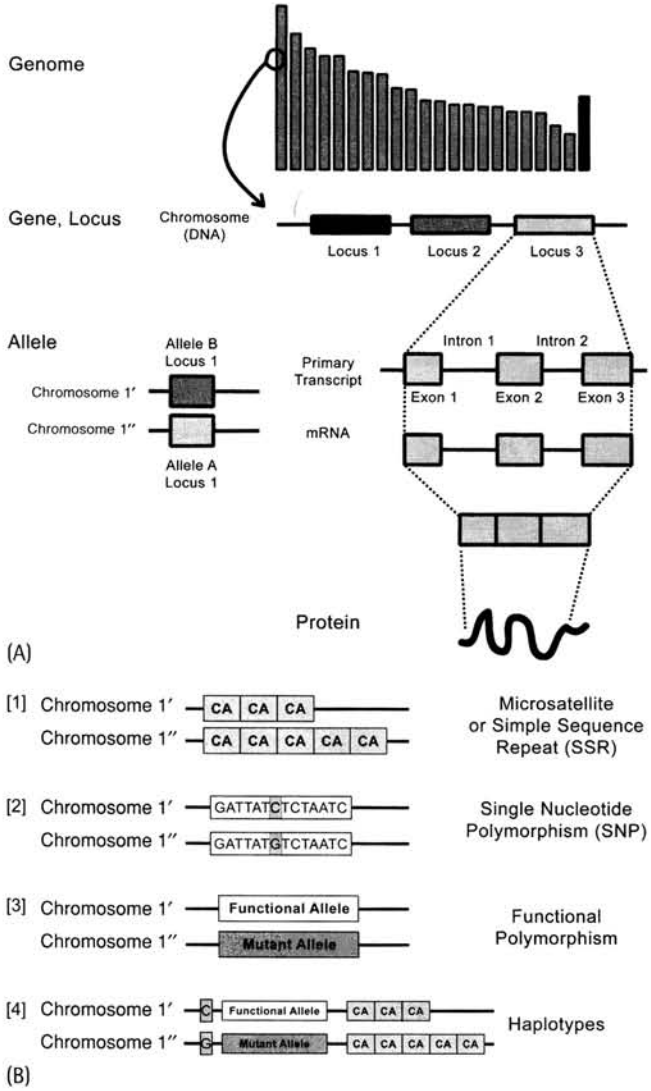


Figure 22.3 Genome and genetic marker vocabulary. A: Schematic overview of genome and gene structure. B: Various classes of molecular genetic markers. Each line represents a region of homologous DNA on one member of each autosomal chromosome pair. From top to bottom: *(1)* Microsatellite marker locus involving the repeat of a dinucleotide (CA) motif. This individual is a $(CA)_3/(CA)_5$ heterozygote. *(2)* Biallelic single-nucleotide polymorphism, or SNP, site. This individual is an A/G heterozygote. *(3)* Functional polymorphism. This individual carries one "normal" and one mutant copy of a protein-coding region of DNA. An example might be an individual heterozygous for the normal (Hb^A) and sickle (Hb^S) alleles coding for the β chain of a hemoglobin molecule. *(4)* Alternative haplotypes at a locus. The term *haplotype* refers to a set of markers that are "linked" or inherited together most of the time because they are located close to one another on the same chromosome and therefore less likely to undergo recombination during the process of gamete formation.

separation of the *amplicons* (the millions of copies of the same DNA fragment produced by PCR). Because alleles will differ in length due to the presence of different numbers of repeats of the microsatellite motif, these fragments will migrate to different positions in a gel during electrophoresis and individual genotypes can be scored directly, without the need for sequencing (Fig. 22.4A).

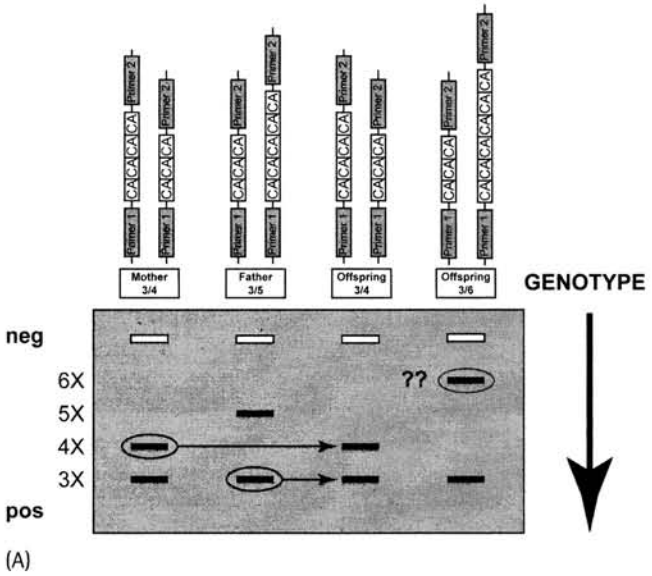
In the last decade, PCR-based microsatellite genotyping has been increasingly used to provide insight into various aspects of the behavioral biology of nonhuman primates, including mating systems, dispersal patterns, and population genetic structure (Di Fiore 2003, de Ruiter 2004). One of the limitations of PCR-based microsatellite genotyping, however, is that primers need to be available for amplifying variable microsatellite loci. Many such primer pairs are known for a variety of hominoid and cercopithecoid taxa since primers used in human genotyping and linkage studies often amplify homologous regions in other catarrhines. Researchers working on more distantly related primates, however, often need to invest considerable time and money isolating novel microsatellites either from taxon-specific *genome libraries* (sets of short fragments of DNA covering the entire genome that are inserted into biological vectors and cloned; e.g., Strassman et al. 1996, Hamilton et al. 1999, Paetkau 1999) or from subsets of genome fragments generated through a variety of PCR procedures (e.g., Fisher et al. 1996, Lunt et al. 1999, Zane et al. 2002). An up-to-date database of microsatellite loci used in primate studies has been compiled by Di Fiore (2003, www.nyu.edu/projects/difiore/yearbook/appendix.html), and a similar database is currently under development by INPRIMAT (www.inprimat.org), an international consortium to promote primate molecular biology research.

Single Nucleotide Polymorphism Genotyping

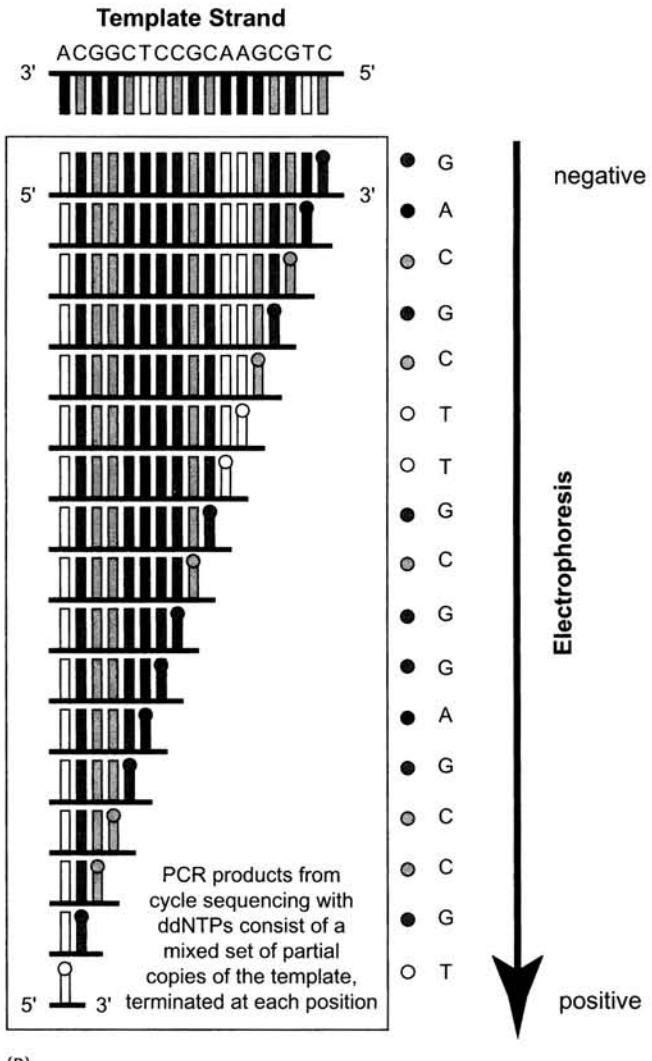
Even faster methods of detecting genetic variation have been developed that do not require separation of PCR products via electrophoresis. Much emphasis has been placed recently on the search for single nucleotide polymorphisms (SNPs, or "snips") in the human genome for use as molecular markers in large-scale linkage studies. SNPs, as their name implies, are single nucleotide positions in the genome that differ between different chromosomes in a population (Fig. 22.3). Most SNPs are biallelic, existing in only two alternative forms. In humans, SNPs occur approximately once every 1,000–2,000 bp in the genome, and over 1.4 million SNPs have already been putatively identified in the roughly 2.85 billion bp comprising the completed sequence of the euchromatic portion of the human haploid genome (International Human Genome Sequencing Consortium 2001, 2004; International SNP Map Working Group 2001).

SNPs have been used extensively in human studies to examine population genetic structure and migration patterns. For example, a number of studies have used Y chromosome SNP data to trace the spread of modern humans around the globe (Underhill et al. 2000, 2001; Hammer and Zegura 2002). As yet, however, SNPs have not been used widely in studies on nonhuman primates, primarily due to the lack of known markers. In the coming years, this will likely change as improved protocols for isolating SNPs are developed (e.g., Aitken et al. 2004).

Because of their utility in linkage studies in humans, a number of high-throughput, automated methods have been derived to directly assay variation at multiple SNP loci



(A)



(B)

simultaneously, including the use of allele-specific extension on DNA microarrays (Pastinen et al. 2000), hybridization of different alleles to complementary oligonucleotides bound on microarrays (Hacia et al. 1999), and the detection of alternative alleles created via PCR using mass spectrometry (Haff and Smirnov 1997, Bray et al. 2001). Reviews of these and other automated SNP typing methods are provided by Gut (2001) and Syvänen (2001).

Direct DNA Sequencing

The most detailed form of genetic variation that molecular primatologists can assay is variation in an actual DNA sequence between individuals or among taxa. Modern phylogenetic studies commonly use direct DNA sequencing to generate DNA sequence data for a particular site (locus) of interest and then compare the sequence composition, the distribution of variable sites, and the relative genetic distance between individuals or groups of sequences. Modern DNA sequencing relies on the controlled replication of a DNA template of interest using PCR. Template DNA is mixed with polymerase and other reaction components, along with free nucleotides (A, T, G, and C) in the form of dNTPs. Some small fraction of these free nucleotides are di-deoxynucleotides (ddNTPs), which, when randomly incorporated into a growing DNA strand, prevent further extension as they lack the 3′-hydroxyl group needed to form a phosphodiester bond with a subsequent nucleotide (Sanger et al. 1977). The result of the sequencing reaction is thus a mixed population of copied DNA fragments differing in length by a single base pair, from 1 base to the full complementary length of the template. These fragments are then separated by electrophoresis, allowing the sequence of DNA bases to be determined based on which ddNTP is incorporated at the end of each fragment (Fig. 22.4b). Studies of sequence variation are useful at multiple levels (e.g., for inferring phylogenetic relationships among higher-order taxa, such as species or genera, as well as for inferring intraspecific phylogenies that can be used in phylogeographic studies).

Figure 22.4 (*left*) Molecular genetics techniques routinely used in primatological studies. A: Microsatellite genotyping by electrophoretic separation of amplied DNA fragments. Fragments are separated by size in an electrophoretic medium such as a polyacrylamide gel. DNA is negatively charged and will migrate toward the positive pole in a gel. Microsatellite alleles are codominant, allowing genotypes to be scored directly. Offspring must inherit one allele from each parent, thus *Offspring 2* in this example appears to have been fathered by an unsampled individual. B: Direct DNA sequencing by the dideoxy method, which causes DNA replication of a template to be terminated when a dideoxynucleotide base analog is incorporated into a growing DNA strand (Sanger et al. 1977). Electrophoresis separates a mixture of partially copied DNA sequence fragments differing in size by one base pair in a medium sensitive enough to resolve these differences. In modern automated sequencing, the four possible dideoxynucleotide triphosphates (ddNTPs; A, T, G, and C) are each tagged with a different fluorescent molecule, allowing the type of nucleotide at the terminal position of a DNA fragment to be resolved by laser detection. From shortest to longest fragments, the order of the passing fragments gives the complementary sequence of the template DNA.

Alternative Methods for Detecting Sequence Variation

Several alternative methods have been developed as shortcuts for rapidly evaluating whether sequence variation exists within a population at a given locus without having to directly sequence every individual sample. These include a variety of techniques—single-strand confirmation polymorphism analysis (SSCP), denaturing gel gradient electrophoresis analysis (DGGE), and conformation-sensitive gel electrophoresis analysis (CSGE)—that take advantage of the fact that fragments of DNA differing by as little as a single base pair will migrate differently through a variety of electrophoretic media. Amplified DNA fragments from a sample can also be screened for variation using the more recently developed technique of denaturing high-performance liquid chromatography (DHPLC) (Xiao and Oefner 2001). All of these methods can thus be used to rapidly screen a population of samples for variation and to identify a set of representative variants for subsequent direct sequencing. They can also be used as less costly alternatives to microarrays and mass spectrometry for rapidly genotyping individuals at well-characterized variable loci, such as SNP sites.

Alternative methods for fast typing of well-characterized sequence variants, such as SNPs, include "real-time" or "quantitative" PCR and flow cytometry. Real-time PCR is a modified form of PCR that, in addition to the two oligonucleotide primers bracketing the target sequence, incorporates a third oligonucleotide that is complementary to a short segment of the target. This third oligonucleotide, or "probe," carries both a fluorescent dye molecule and a "quenching" molecule, which prevents the dye from emitting light as long as the probe is intact. During PCR amplification, the DNA polymerase, which also has exonuclease activity, digests the central probe as it works its way down the template synthesizing a new complementary strand (Fig. 22.5). Digestion of the probe separates the fluorescent dye from the quencher, causing it to emit light when stimulated by a laser. The intensity of the fluorescent emission is proportional to the amount of template DNA present at each cycle; thus, the initial quantity of template in an experimental sample can be evaluated by comparing its amplification profile to that of a standard template of known DNA quantity. A simpler and cheaper form of real-time PCR uses the nucleic acid dye Cyber green and simply measures the change in the total amount of DNA (PCR product) over time, without the need for a highly specific probe. The coupling of allele-specific amplification (e.g., for an SNP locus) with real-time PCR detection allows for fast and efficient screening of a population for sequence variation.

Flow cytometry is a general procedure that can be used for the analysis of a variety of particulate samples, from fragments of DNA to organelles to bacteria to whole eukaryotic cells (Fig. 22.6). The technique relies on samples being kept as a suspension in a "sheath" fluid, the flow of which is controlled such that the particles to be studied flow through a chamber one-by-one at a rate of several hundred

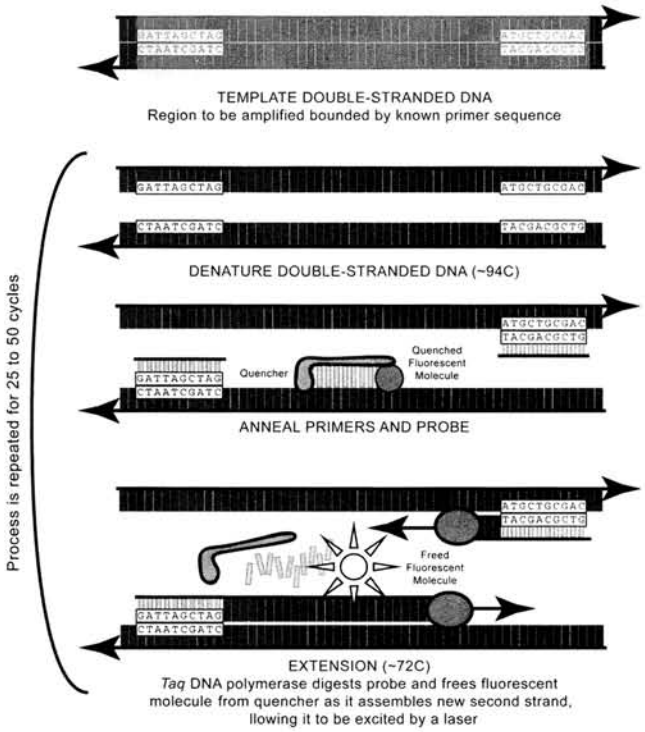


Figure 22.5 Real-time or quantitative polymerase chain reaction (Q-PCR). In addition to flanking PCR primers, a third oligonucleotide is used that is complementary to a portion in the middle of the target region to be amplified and contains both a fluorescent and a "quencher" molecule. As replication proceeds, the DNA polymerase digests this third primer and releases the quencher molecule, allowing the fluorescent molecule to emit detectable light when stimulated by a laser. The intensity of this light can be measured to provide an accurate estimate of the number of amplified copies of a DNA sequence present after each cycle of replication.

per second. Laser light illuminates each passing particle, and a collection of light detectors (photomultipliers) measures the emission on the other side of the chamber. This procedure allows the rapid determination of particle size and granularity and, based on the emission profile, can be used to analyze samples by detecting molecules present on the particles and using fluorescence intensity to quantify their abundance. Methods for SNP genotyping by flow cytometry make use of microspheres that are tagged with a distinct fluorescent color and that have short, allele-specific oligonucleotide capture probes attached to them. Populations of microspheres bearing probes for the alternative alleles at an SNP site are then hybridized to sample DNA subject to allele-specific PCR, which also incorporates a different fluorescent marker. The flow cytometer is then used to determine the presence of each alternative allele, based on the combined microsphere and allelic fluorescent signals recorded. Taking advantage of the fact that microspheres with 100 distinct fluorescent tags are commercially available (Luminex Corp., Austin, TX), Ye et al. (2001) have demonstrated the potential of the flow-cytometric method for multiplex genotyping of DNA samples at up to 15 SNP loci simultaneously.

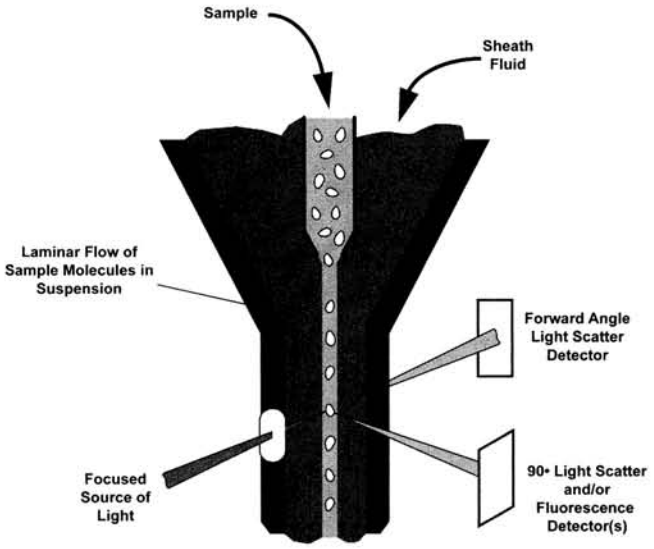


Figure 22.6 Flow cytometry (literally "cell measuring") is a technique used to measure various aspects of individual particles or cells at a rate of several hundred per second. Sample particles in suspension are injected into a sheath fluid (usually an isotonic buffer or medium). By adjusting the pressure on the sheath and sample channels, the sample fluid can be directed to flow through the center of the sheath fluid column without mixing and contain a focused stream of separated molecules. These molecules flow one-by-one past a source of illumination (e.g., a laser). The amount of light scattered in the forward and side directions can be detected and used to characterize the size and granularity of the sample particles. Additionally, particles of interest can be assayed and quantified by measuring the color and amount of light emanating from laser-stimulated fluorescent probes used to stain those particles. Flow cytometry can be applied to many different tasks, from identifying and sorting different types of cell (on the basis of their forward and side scatter illumination profiles) to evaluating what stage of the cell cycle particular cells are in (based on fluorescent quantification of the amount of DNA present in the cell) to sorting chromosomes (based on fluorescent quantification of two different dyes, one that binds preferentially to GC-rich areas of chromosomes and one that binds to AT-rich areas) to multiplex genotyping of a mixture of polymerase chain reaction–amplified DNA fragments (based on the incorporation of allele-specific fluorescent molecules and their binding to similarly tagged microspheres).

Variation in Gene Structure and Expression

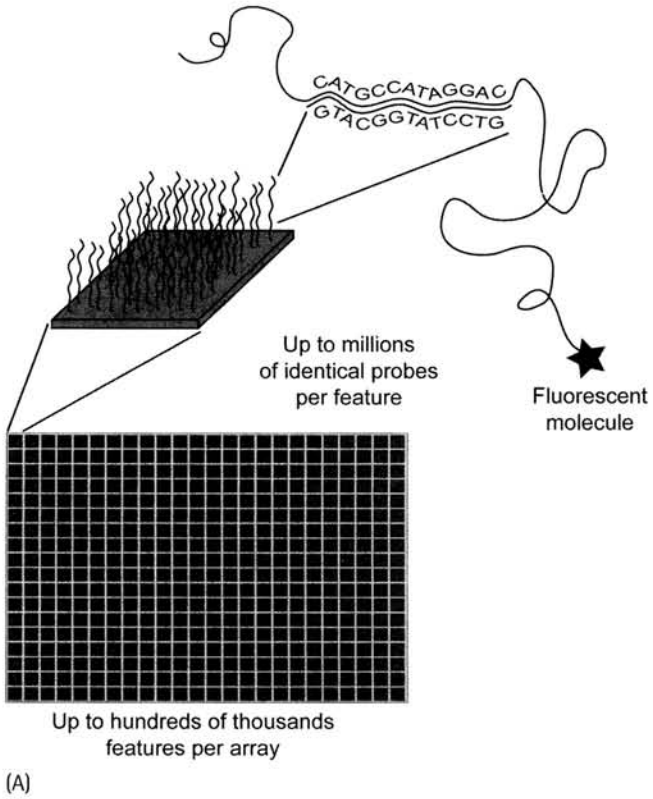
Studying the structure and expression of genes requires the copying of large stretches of chromosomal DNA (up to several tens of thousands of base pairs) that contain the full coding sequence as well as the regulatory regions and noncoding exons and introns of a gene. This is because regulation of gene expression is governed by the binding of various transcription factors (mostly proteins) to well-defined binding regions upstream from the gene itself. Variants of basic PCR procedures, such as "long-distance" or "long-range" PCR, make such amplification possible. Amplified stretches of that genomic DNA can then be probed for the presence of known sequence elements (e.g., regulatory sequences). This is done by separating the fragments on agarose gels, transferring the fragments to a membrane by Southern blotting, and screening the membrane with labeled probes for the DNA regions of interest. Such information is crucial for understanding how genes are structured.

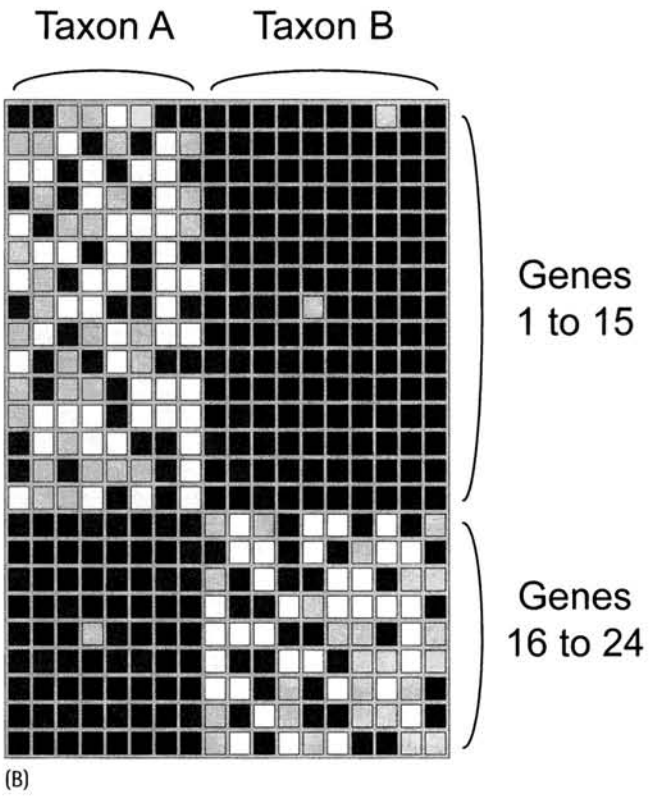
Furthermore, many genes can be "read" in more than one way by the process of *alternative splicing* (the joining together of different exonic portions of a primary mRNA transcript in different ways). The DNA sequence at splice sites can provide information on the total number of potential transcripts (mRNAs) generated by alternative ways of transcribing the same gene. Different patterns of mutation in the exons and introns, for example, can be used to infer selection affecting a particular gene or even a particular functional region of the protein (Sonnenburg et al. 2004).

In order to more directly study the nature of expressed genes and the process of alternative splicing of genetic messages to generate more than one product from the same gene, mRNA can be extracted from a tissue of interest, separated by electrophoresis on agarose gels, and then transferred to a membrane. There, it can be probed with single-stranded DNA oligonucleotides labeled with either radioactive or fluorescent biomolecules to determine what genes (and combinations of genes) and in what quantity contribute to the mRNA transcripts expressed in particular tissues. Single-stranded mRNA, which is far less stable than DNA, can also be copied back into DNA via reverse-transcription PCR (RT-PCR), which allows it to be studied more easily. Real time RT-PCR can also be used to quantify the genetic message by providing an estimate of the amount of starting mRNA template isolated from a particular tissue (Cáceres et al. 2003). The term *transcriptomics* is used to describe such studies of the complete collection of genetic messages, or "transcripts," present in a given tissue at a given time.

Increasingly, transcriptomic studies are being carried out using microarray technology to measure the presence and relative quantity of different mRNAs that are expressed in specific tissues or cell types. This involves the hybridization of reverse-transcribed mRNA to arrays of unique short oligonucleotide or complementary DNA (cDNA) probes specific to genes of interest. These arrays may be commercially available "DNA chips" containing thousands of probes or homemade dot plots of a much smaller number of probes bound to a membrane or slide (Fig. 22.7). Karaman et al. (2003) used this procedure to evaluate the relative expression of a large number of genes in gorillas, bonobos, and humans by interrogating Affymetrix (Santa Clara, CA) U95Av2 DNA chips (which contain short probes for over 10,000 human gene transcripts) with mRNA isolated from multiple fibroblast cell lines (cultures of skin cells initiated from biopsy samples) of each of these three taxa. Their analysis identified a number of candidate genes (many involved in metabolic pathways, neural signal transduction, and cell and tissue growth) that were differentially expressed among these species, providing some directions for future research on the underlying causes of phenotypic differentiation within hominoids.

In independent studies using similar DNA chips, Enard et al. (2002) and Cáceres et al. (2003) compared the expression of the same set of human genes in the brains of humans, chimpanzees, orangutans, and macaques and compared





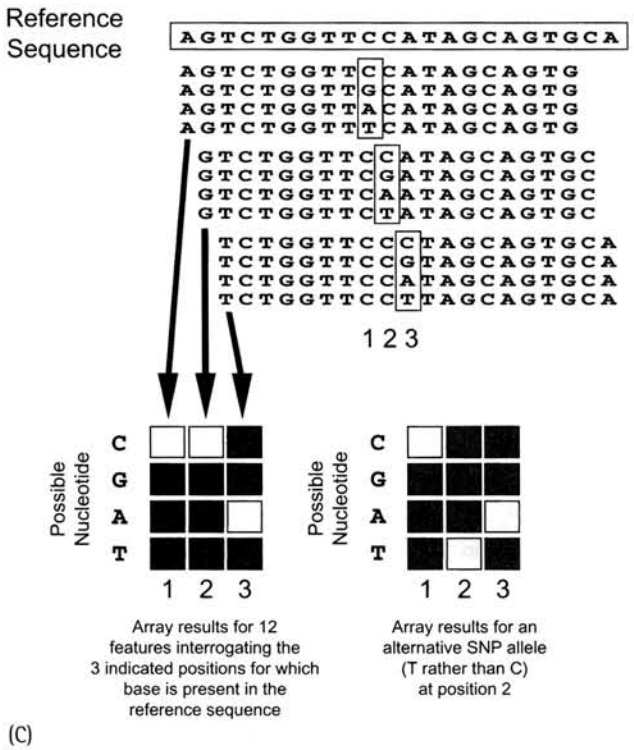


Figure 22.7 DNA microarrays and gene chips. Microarrays are contraptions allowing miniature hybridization experiments of sample DNA against an array of up to thousands of known DNA probes fixed in a grid. A: Single-stranded DNA oligonucleotides are either blotted onto a small, flat substrate (e.g., a glass slide) in well-defined positions (a "DNA microarray") or synthesized directly on a silica-based chip using photolithography (a "gene chip"). Multiple copies of the same oligonucleotide probe bound to the same region of the microarray or chip constitute a "feature." A single photolithographically produced chip may contain up to several hundred thousand features. The microarray or chip is washed with a denatured nucleic acid sample of interest that has been tagged with a radioactive or fluorescent label. Sample DNA or RNA will hybridize only where a complementary sequence is found and can be detected based on the corresponding luminescent signal. B: As discussed in the text, microarrays and chips can be used in comparative studies of gene expression. As a hypothetical example, a cDNA-based microarray containing probes for 24 genes (*rows*) is used to interrogate fluorescently labeled mRNA extracted from tissue samples of 18 individuals from two different taxa (columns 1–8, taxon A; columns 9–18, taxon B). Lighter coloration for a feature indicates that more of the labeled mRNA sample bound to the probe. In this case, genes 1–15 show greater expression in taxon A, while genes 16–24 show greater expression in taxon B. C: Microarrays and chips can also be used for single nucleotide polymorphism (SNP) genotyping of polymerase chain reaction–amplified samples. In this case, features consist of staggered sets of oligonucleotides that span a known reference sequence containing the SNP site. For each of these sets, there are four features on the array, one corresponding to each potential nucleotide present at a "query" position located in the middle of the oligonucleotide. Amplified samples will hybridize only to the feature containing the appropriate complementary base at each query position, which allows for rapid SNP genotyping. A similar procedure can be used for "resequencing" an entire DNA amplicon.

these results to those for other body tissues. In both studies, nearly 170 genes were expressed differentially in the brains of chimpanzees and humans, with humans showing higher expression levels (upregulation) for the vast majority of these genes, many of which appear to be related to cell growth and maintenance and to neural function. These differences in expression levels, however, did not extend to heart or liver tissue, where only just over half of the genes with different expression levels were upregulated in humans (Cáceres et al. 2003). If real, these differences in expression

levels suggest a possible neurological basis for some of the behavioral and cognitive adaptations thought to be associated with the divergence of humans from other African apes. Interestingly, the locations of these upregulated genes appear to be associated with areas of chromosomal rearrangement and segmental duplication between humans and chimpanzees (Khaitovich et al. 2004, Marquès-Bonet et al. 2004). A similar study by another group demonstrated that genes expressed in cortical tissue are upregulated in all of the African great apes relative to macaques, though in humans to a greater extent than in either chimpanzees or gorillas (Uddin et al. 2004). Preuss et al. (2004) provide a comprehensive review of these and other microarray studies of gene expression differences among primates.

One of the important limitations of the microarray approach is that, to date, it is still impossible to detect subtle changes (e.g., less than twofold differences) in gene expression levels (Forster et al. 2003). A further limitation is that these studies are heavily biased toward "prominent" messages (i.e., those genes that are expressed at very high levels, producing high copy numbers of the same mRNA transcript). Other genes with relatively low and much more narrowly localized expression may nonetheless have important phenotypic consequences (e.g., glycosyl transferases and other protein-modifying enzymes).

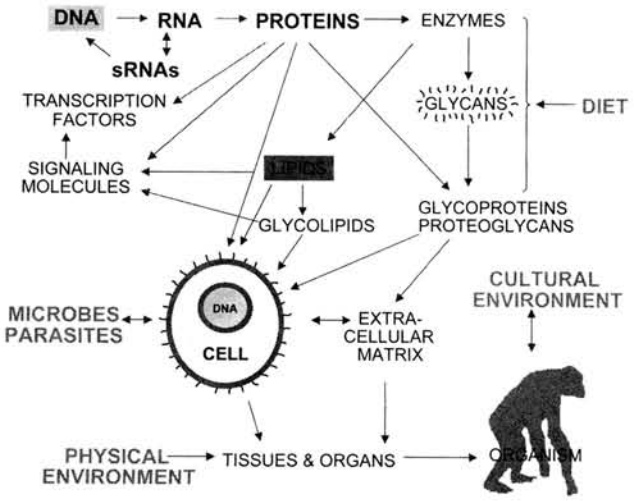


Figure 22.8 Molecules in context. Living organisms are made up of four classes of molecules—nucleic acids (DNA and RNA), proteins, lipids, and carbohydrates/sugars—which exist in various combinations. The assembly, distribution, and abundance of the final molecules are affected by many factors, ranging from genetic control to cellular dialog within the organism to interactions with the biotic and abiotic environment faced during development. DNA (*light gray shading*) is found in the nucleus (and mitchondria, not shown), lipids (*dark gray shading*) are prevalent in the nuclear and cellular membranes, and carbohydrate-containing glycans (*black threads*) are attached to proteins expressed on the surfaces of cells. (Figure kindly provided by A. Varki.)

Assaying Variation in non-DNA Molecules

In addition to nucleic acids (DNA and RNA), the cells of living organisms contain several other classes of important biomolecules, including proteins, lipids, and sugars, which exist in various combinations (Fig. 22.8). Techniques from other fields of molecular biology used to study these other kinds of molecules are starting to find wider application in primatology, even if to a much lesser degree than genetic methods. For example, protein comparisons have become much more sophisticated since the days of early allozyme studies, contributing to the rapidly expanding field of *proteomics* (the study of the complete set of proteins found in a particular type of cell or tissue). Soluble and extracted proteins can be separated in two dimensions via isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a membrane and probed with various labeled antibodies to assay the presence of particular proteins as well as their electro-phoretic signatures. An example of this approach is the comparison of soluble proteins in the blood plasma of great apes and humans (Gagneux et al. 2001). Sugar molecules on glycoproteins can be assayed similarly, by probing with carbohydrate-specific lectins and certain antibodies that bind to these molecules (Boenisch 2001). A more recent approach, multidimensional protein identification technology (MudPIT), is based on the partial digestion of proteins followed by mass spectrometry, which allows the identification of membrane proteins and their post-translational modification (Wu et al. 2003).

Variation in soluble or extracted proteins can also be screened immunologically using the enzyme-linked immunosorbent assay (ELISA). In this technique, the presence of a protein or protein variant in a sample is detected via its reactivity with antibodies that are labeled with a *chromogenic* (color-producing) enzyme. In a variant of ELISA, the line immunoassay (LIA), probes consisting of known proteins are blotted on a membrane, over which a sample is then washed. This technique has been used to test for the presence of antibodies against simian immunodeficiency virus in a large number of wild primates in Africa (Peeters et al. 2002). Animals that tested positive for antiviral antibodies in their serum or urine were then further investigated with PCR or RT-PCR to confirm the presence of the virus and to characterize its nucleotide sequence (Santiago et al. 2003).

Flow cytometry is also increasingly being used for studying the comparative cell biology of primates. The process can be characterized as a form of "liquid ELISA" as it allows the study of molecules on and inside living or fixed cells by taking advantage of fluorescently labeled probes that can be bound to the molecule of interest. Flow cytometry has recently been used to document differences among several primate species in the distribution and abundance of immune molecules present on white blood cells and in the ability of these cells to bind certain well-defined ligands (Brinkman-van der Linden et al. 2000). Analyses of primate sperm are also now conducted with the help of flow cytometry as this powerful method allows for the quantitative analysis of large numbers of cells in a very short time (~10,000 per minute) (Shankar et al. 2004).

The detection and visualization of specific molecules in and on cells can also be accomplished using immunohistochemical methods. These methods rely on cells left intact in the architecture of a particular tissue or isolated from such tissue by enzymatic digestion, and they are sensitive enough to detect extremely low amounts of target molecules. This sensitivity is critical because certain components of a tissue may be present at very low concentration (and thus relatively underrepresented in a tissue extract) and yet be of the utmost biological importance (e.g., receptors found only on the apical side of a single-cell epithelial cell layer such as hormone receptors, olfactory receptors, endogenous lectins, or glycoconjugates) (Gagneux et al. 2003). Additionally, proteins with known genetic sequence can be cloned in vitro (i.e., by copying and pasting their genes in mRNA or cDNA to form plasmids, which are then introduced into a bacterial vector and replicated) and then expressed in an expression system (e.g., Chinese hamster ovary cells) for comparative functional and/or structural studies.

Fibroblast lines established from skin biopsy samples form precious repositories of live cells, which can be stored indefinitely and regrown as needed as a source of DNA for genetic studies or for use in comparative cytogenetic, transcriptomic, and proteomic studies as well as studies of gene expression in various tissues. With support from the National Science Foundation, numerous institutions, such as the Frozen Zoo of the Zoological Society of San Diego, the Coriell Institute, and Princeton University, are currently collaborating to establish the Integrated Primate Biomaterials and Information Resource (www.ipbir.org) containing a large collection of such cell lines from animals with known provenience and, often, known life histories. Additionally, for hominoid primates, immortal cell lines created from white blood cells form a crucial resource for comparative studies of gene function, particularly for studies of the many important genes of the immune system, such as *MHC* and *KIR* (Adams and Parham 2001, Rajalingam et al. 2001). These lines are created from immune system B cells via transformation using Epstein-Barr virus (EBV). Unfortunately, however, there are no methods of immortalizing B cells from the majority of nonhuman primate species, as EBV has a fairly restricted host range, and other appropriate transforming viruses remain to be identified.

Finally, although little explored to date, interest is growing in the comparative study of the *microbiome* (the complete set of microbial symbionts and parasites living in and on an animal) of humans and other primates. For example, molecular genetic techniques, in combination with classical methods from microbiology such as serology, can be used to identify the types and strains of bacteria inhabiting the bodies of healthy and sick primates, as well as potential adaptations of the host primate genome to their presence (e.g., Stewart et al. 1987, Zhang et al. 2002). This field promises to uncover a vast and brand new realm of primate biodiversity, one with clear significance for understanding aspects of primate evolution relevant to health and diseases.

FIELD METHODS: SAMPLE TYPES AND TECHNIQUES

In order to take advantage of any of these molecular techniques, it is crucial to collect appropriate samples. The collection, exportation, and importation of nonhuman primate samples are regulated by a variety of national and international laws and conventions. For both practical and ethical reasons, it is imperative that researchers comply fully with these regulations. For the United States, permission to import various nonhuman primate samples needs to be obtained from one or more of the following agencies: the Centers for Disease Control and Prevention of the U.S. Public Health Service, the U.S. Fish and Wildlife Service, and the U.S. Department of Agriculture. Also, as many nonhuman primates appear in Appendices I and II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES), the U.S. Fish and Wildlife Service requires that CITES export permits be obtained from the country of origin for most primate samples. Furthermore, nonhuman primates, by virtue of their close taxonomic proximity to humans, can carry a large number of pathogens of great concern (e.g., herpes simiae and polio viruses in Old World monkeys; simian immunodeficiency virus, ebola, and anthrax in great apes). Before embarking on a molecular study utilizing primate samples, researchers must be trained in safe laboratory practices and follow special primate protocols that are now required at many research institutions, and they must be vaccinated against a number of infectious agents likely to be encountered in primate tissues or secretions.

Sampling for Genetic Analyses

For genetic analyses of wild primates, a variety of sample types have been used as sources for DNA, including blood, tissue, hair, feces, urine, semen, buccal swabs, and masticated food wadges. There are, of course, advantages and disadvantages associated with the use of different sample types. Blood and tissue are the best sources for large quantities of high-quality genomic DNA, but they are the most difficult samples to acquire. Blood collection requires that animals be immobilized, often under anesthesia, which may be difficult to do in field conditions. Additionally, collectors must have received professional veterinary training in how to draw blood without injuring the animal. Tissue sampling (e.g., collection of ear punches) can also be done on anesthetized animals and requires less training and practice to perform safely. Nonetheless, any such invasive procedures carry risks to the health of both animal and researcher. Such procedures will also be subject to Institutional Animal Care and Use Committee approval, which may require the presence of professional animal-care technicians during sampling. The logistical complexities associated with anesthetizing and collecting blood or tissue from wild animals may preclude the use of these kinds of sample if large numbers of samples are required.

Biopsy darts propelled from a compressed air- or CO_2-powered pistol or rifle are a minimally invasive alternative for retrieving small tissue samples that are suitable sources of large amounts of high-quality genomic DNA. This technique has been successfully used in studies of several primate species, including leaf monkeys (Rosenblum et al. 1997a), woolly monkeys (Di Fiore 2002, Di Fiore and Fleischer 2005), and spider monkeys (Di Fiore unpublished data). Darts can be made by hand from standard 3 cc syringes and 16-gauge needles (e.g., Henry Schein) or purchased from commercial suppliers (e.g., Pneudart, Williamsport, PA).

Some of the earliest field studies of primates to employ molecular techniques used noninvasively collected hair samples as a source of DNA (e.g., Morin et al. 1994; Gagneux et al. 1997b, 1999; Goldberg and Wrangham 1997; Mitani et al. 2000). Unfortunately, it is now clear that shed hairs are generally a very poor source of nuclear DNA, though follicle-containing plucked hairs are more suitable. A variety of creative techniques have been employed to collect fresh hair samples from wild nonhuman primates, including shooting animals with flat-tipped darts that have sticky tape mounted on the front, presenting animals with food bait wrapped in tape, and coating the edges of the entrance to a food corral with tape to capture an animal's hair as it enters (Valderrama et al. 1999).

Fecal samples have also been used as a source of DNA for numerous genetic studies of wild primates. Feces typically contains small numbers of cells sloughed from the epithelial lining of an animal's lower digestive tract as a bolus of fecal material passes through. An extraction of DNA from feces will thus contain a complex mix of DNA from a variety of sources, including the animal itself, as well as gut microbes and plants or insects that the animal consumed. PCR allows the preferential amplification of a specific target sequence present at low copy number (i.e., a piece of the genome of the animal) from amidst this complex sample. The low template copy number in hair and fecal samples poses a potential problem for determining reliable genotypes on the basis of fecal DNA due to allelic dropout (Taberlet et al. 1996, 1999; Gagneux et al. 1997a). However, real-time PCR can be used to prescreen fecal DNA extracts, as well as extracts from any other noninvasive samples, to identify those containing sufficient template for subsequent analysis (Morin et al. 2001). Over the last several years, improved methods for storing (Roeder et al. 2004, Nsubaga et al. 2004) and extracting DNA from fecal samples (e.g., commercially available DNA extraction kits, such as the QIAgen™ DNA Stool Mini Kit; Qiagen, Chatsworth, CA) have been developed. As a result, fecal samples are now routinely used as a source of DNA for molecular analysis in a number of field studies (e.g., Vigilant et al. 2001, Bradley et al. 2004). Coupled with pre-amplification methods (Piggott et al. 2004) and new methods for whole-genome amplification from very limited copy number templates (Dean et al. 2002), fecal samples will probably become even more widely used in future studies.

Although blood, tissue, hair, and feces are the most commonly used samples as sources of DNA for genetic studies, several additional sample types might also be used. For example, in a study of captive Japanese macaques, Hayakawa and Takenaka (1999) demonstrated that urine could be used as a source of DNA for microsatellite genotyping and that it yielded concordant genotypes to fecal samples from the same individuals (see also Valière and Taberlet 2000). More recently, Domingo-Roura et al. (2004) demonstrated the efficacy of amplifying mitochondrial DNA and microsatellite loci from DNA extracted from the semen of wild Japanese macaques. DNA extracted from buccal cells collected either via cheek swabs or from chewed food wadges (Takasaki and Takenaka 1991, Sugiyama et al. 1993, Vigilant et al. 2001) has also been used on occasion for microsatellite genotype and mitochondrial DNA sequencing. Woodruff (2004) and Goossens et al. (2003) provide excellent overviews of the use of non-invasive techniques for sampling primates for genetic studies.

Sampling for Other Kinds of Molecular Study

Depending on the questions being asked, primatologists might be interested in collecting a variety of other samples from trapped or darted animals for studies of other biomolecules. These include saliva, scent gland secretions, milk, nose swabs, vaginal swabs, copulatory plugs, semen, skin biopsies, and microbial samples from the gastrointestinal tract.

Samples for immunohistochemical studies are most valuable when they are rapidly frozen in an appropriate medium (e.g., embedded in optimal cutting temperature [OCT] compound and flash-frozen in a dry ice and 2-methylbutane/isopentane slush). Such treatment will maintain the cellular architecture of the tissue, and subsequent frozen sectioning on a cryostat allows for the detection of proteins and nucleic acids in situ. Immediate fixation in buffered formalin will result in the loss of a large number of protein epitopes as these are denatured by the preservative but will maintain much of the cellular architecture, which can be studied by embedding the fixed samples in paraffin for sectioning at a later point in time. Samples taken for other kinds of biochemical analysis (e.g., proteomics, lipid composition) should ideally be kept and transported frozen. Samples of glandular secretions for chromatographic analysis can be collected with a sterile dry swab and then stored in an air-tight glass vial for long periods of time in the field.

Many kinds of sample typically collected for nucleic acid extraction also provide abundant opportunities for the study of other molecules. For example, because certain hormones (e.g., prolactin, testosterone, cortisol, progesterone, estradiol) and metabolic by-products (e.g., ketones from metabolism of fat) are present in feces and urine, these samples can be used for endocrinological studies of female reproductive cycles (reviewed in Hodges and Heistermann 2003), for examining the hormonal correlates of social status (van Schaik et al. 1991, Strier et al. 1999), or for investigating

seasonal changes in energy budgets (Knott 1997, 1998). It is important to keep aliquots of the various fractions created during DNA extraction as a potential source for some of these other biomolecules. Unlike larger and more fragile molecules, mono- and oligosaccharides are generally robust and subject to slower decay, unless there is bacterial or fungal contamination of the samples. As such contamination almost always renders the samples useless, an aseptic technique should be followed in the field for preserving these molecules. Skin biopsies, if taken with proper caution (aseptic techniques) and kept in cell culture medium, can survive at room temperature for a week to 10 days and still yield viable fibroblast cultures. Alternatively, if liquid nitrogen is available, skin biopsy samples can be minced and frozen in dimethyl sulfoxide (DMSO) in the field to prevent crystal formation and resulting cell death and then shipped in dry ice (Houck et al. 1995).

Sample Storage

Storing samples while in the field can present challenges as electricity for running refrigerators or freezers for preserving samples is typically not available. However, many sample types can be kept for long periods of time at room temperature without substantial degradation of DNA and other molecules of interest if stored under the proper conditions. For example, all samples potentially containing DNA and RNA can be kept at room temperature for several weeks if stored in a sufficient volume of RNA*later*® (Ambion, Austin, TX), which is a nonflammable, nontoxic buffer that will maintain RNA and DNA for later isolation and amplification. Table 22.2 summarizes a set of appropriate field storage conditions (most at room temperature) for a range of sample types and includes recipes for a number of several useful field buffers. Whenever possible, these samples should be transferred to appropriate long-term storage temperatures (e.g., 4°C–20°C) as soon as possible.

A BRIEF NOTE ON LABORATORY AND ANALYTICAL METHODS

Because a number of excellent publications present detailed protocols for many of the laboratory methods discussed above—and because very few of these protocols are specific to primates—we saw little need to repeat that information here. Instead, we have compiled a short list of useful references where readers can go for more detail on how to implement some of the general methods discussed above (Table 22.3). In addition to these, Humana Press' *Methods in Molecular Biology* series and a variety of titles from Cold Spring Harbor Laboratory Press provide even more detail on using particular methods (e.g., RT-PCR) for specific applications. Table 22.3 also highlights a set of excellent sources providing overviews of important methods for analyzing molecular data generated using the techniques described above.

Table 22.2 Suggested Field Storage Conditions for a Variety of Sample Types

SAMPLE TYPE	USED FOR	STORAGE MEDIUM	RECIPE	HOW TO USE AND REFERENCE
Blood	DNA	Blood field buffer	0.2M NaCl, 0.1M EDTA, 2% SDS, store at RT	Mix 1 volume whole blood with 5 volumes of buffer and store at RT
	DNA	STE buffer	0.1M NaCl, 10 mM Tris, 1 mM EDTA, adjust pH to 8.0 with HCl and store at RT	Mix whole blood with equal volume of buffer and store at RT (Goossens et al. 2003)
	DNA	Citrate blood buffer	0.48 g citric acid, 1.32 g sodium citrate, 1.47 g glucose, add ddH_2O to 100 ml and store at RT	Mix 1 volume whole blood with 6 volumes of buffer and store at RT
	DNA	Filter paper cards (e.g., Whatman FTA® cards)		Place drops of sample on card, allow to air dry, and store in airtight container at RT with desiccant; FTA® cards are impregnated with chemicals that lyse cells, denature proteins, and protect DNA from damage; suitable for long-term storage
	DNA	Queen's lysis buffer	10 mM NaCl, 10 mM Tris, 10 mM EDTA, 1% *n*-lauroylacrosine, adjust pH to 8.0 with HCl and store at RT	Mix whole blood with 20 volumes of buffer (e.g., 50 µl in 1 ml) (Seutin et al. 1991)
	White blood cells, DNA	ACK lysis buffer, followed by storage in field buffer or PBS	150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, adjust pH to 7.2–7.4 with HCl, sterilize with 0.22 mm filter, and store at RT	Mix 1 volume of pellet with 5 volumes of ACK lysis buffer, vortex, spin, and remove supernatant; repeat until no more red blood cells are visible in the remaining pellet of concentrated white blood cells; resuspend the pellet in 5 volumes of field buffer at RT or frozen in PBS
Blood serum	Proteins (albumin, globulins, hormones)			Collect whole blood into special serum separator or blood collection tubes; allow blood to coagulate at RT for 2–4 hr, then carefully pipet off liquid phase (serum) and store in liquid nitrogen or at −80°C

Table 22.2 *(cont'd)*

SAMPLE TYPE	USED FOR	STORAGE MEDIUM	RECIPE	HOW TO USE AND REFERENCE
Tissue	DNA	NaCl-saturated DMSO	20% (wt/vol) DMSO, 0.25M EDTA, NaCl to saturation, adjust pH to 7.5 with HCl and store at RT	Score tissue with razor to expose more of surface to solution, immerse sample and store at RT (Seutin et al. 1991)
	DNA	90%–100% ethanol		Score tissue with razor to expose more of surface to solution; immerse sample and store at RT (Note: DNA yield will be lower and DNA more degraded [fewer large fragments] using this storage medium)
	DNA	Queen's lysis buffer	See above	Store small tissue biopsies in 1 ml of buffer (Bruford et al. 1998)
	DNA, RNA, proteins	RNA*later*®		Follow manufacturer's protocols for sample preparation and storage
	Histology	OCT (optimal cutting temperature) compound	Available from several commercial suppliers (e.g., Pelco)	Embed sample in OCT compound, flash-freeze in dry ice and 2-methylbutane slush by floating it in a small plastic sample holder/boat, and store in liquid nitrogen or at –80°C
Hair	DNA	Paper or glassine envelopes		Collect sample using flame-sterilized forceps, being careful to include root; store envelopes in airtight container desiccated with silica gel beads
Feces	DNA	RNA*later*® buffer	Available from several commercial suppliers (e.g., Ambion)	Add 1 ml of sample to 5 ml of buffer and shake vigorously to homogenize; buffer should cover sample
	DNA	70%–100% ethanol		Add 1 ml of sample to 5 ml of buffer and shake vigorously to homogenize; buffer should cover sample
	DNA	Desiccating silica gel beads	Available from several commercial suppliers (e.g., Sigma)	Place sample in airtight plastic bag or collection tube and add sufficient silica gel beads to thoroughly desiccate sample (6–8× weight of sample)
	DNA	90% ethanol and desiccating silica gel beads		Place 200–250 mg of feces in 4 ml ethanol for 1 day, then decant ethanol and place sample on filter paper in a collection tube containing 25–30 ml of silica gel beads and store at RT (Roeder et al. 2004)
	DNA	DETs solution	20% (wt/vol) DMSO, 0.25M EDTA, 100 mM Tris, NaCl to saturation, adjust pH to 7.5 with HCl and store at RT	Add ~2 g wet weight sample to 1 ml of DETs solution (Frantzen et al. 1998)
Buccal swabs	DNA	Plastic sealable bag containing desiccating silica beads		Using a sterile foam-tipped swab or nylon cytology brush, rub up and down along the inner cheek of the subject for ~15 sec; allow to air dry for 10–15 min, then remove tip and store in airtight environment with desiccating silica beads
		Filter paper cards (e.g., Whatman FTA® cards)		Collect sample as above and then express directly onto card; allow card to air dry and store in airtight container at RT with desiccant
		Cell lysis buffer (or Queen's lysis buffer or STE buffer)	0.1M NaCl, 0.1M Tris, 10 mM EDTA, 1% SDS, store at RT	Collect sample as above, then swish tip around in buffer thoroughly, express excess fluid against side of collection tube, and store at RT
Skin biopsy	Fibroblast cell culture	Sterile growth medium	Alpha MEM (GIBCO BRL) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 200 mM L-glutamine	Aseptic technique for skin biopsy collection from a properly anesthesized animal: (*1*) Clean area on the skin with alcohol swabs (*2*) Shave area clear of any hair and repeat cleaning with second sterile alcohol pad (*3*) Using sterile forceps, pinch and lift the skin (*4*) Using a new scalpel blade, cut a 5 × 5 mm piece of skin (*5*) Place the cut sample directly into a flask containing sterile medium (*6*) Disinfect cut and suture if necessary
		DMSO for freezing on site of minced tissue	Alpha MEM (GIBCO BRL) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 200 mM L-glutamine 10% (wt/vol) DMSO	

RT, room temperature; EDTA, ethylene diamide tetra acetate acid (a chelating agent); SDS, sodium dodecyl sulfate (a detergent for lysing cells); PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; STE,—Sodium-Tris-EDTA solution; ACK,—ACK buffer; DETs,—DMSO-EDTA-Tris-Sodium solution; ddH_2O, double-distilled water; MEM, minimal essential medium.

Table 22.3 Texts Providing Laboratory Protocols and Overviews of Theoretical and Analytical Methods Useful to Molecular Primatologists

TITLE	REFERENCE	DESCRIPTION
DNA Fingerprinting	Burke et al. (1991)	Provides a thorough introduction to minisatellite DNA fingerprinting and an early overview of DNA typing in general
Genetic Data Analysis II	Weir (1996)	Offers a comprehensive overview of the statistical analysis of population genetic data (evaluating linkage between loci, selection, inbreeding, population structure, etc), also provides an overview of methods used in phylogeny reconstruction
Fundamentals of Molecular Evolution	Graur and Li (2000)	Provides a clearly written overview of many aspects of molecular evolution, including the structure of genes and genomes, genome evolution, population genetics, and methods of phylogeny reconstruction
Inferring Phylogenies	Felsenstein (2004b)	Provides a comprehensive, readable overview of phylogenetic methods
Molecular Cloning	Sambrook and Russell (2001)	Serves as a central source of useful, basic protocols for manipulating and studying DNA in vitro, including DNA extraction, polymerase chain reaction, electrophoresis, visualization, and vector cloning; also a comprehensive source of basic buffer and reagent "recipes"
Molecular Genetic Analysis of Populations, 2nd ed.	Hoelzel (1998)	Provides a short but comprehensive set of laboratory protocols for finding and assaying variation in allozymes and DNA
Molecular Markers, Natural History, and Evolution, 2nd ed.	Avise (2004)	Offers a comprehensive theoretical and practical overview of the various kinds of molecular markers evolutionary biologists might be interested in and of the utility of these markers for investigating many aspects of a taxon's biology and history
Molecular Methods in Ecology	Baker (2000)	Provides a general overview of both basic and newly developed molecular biology techniques that can be useful in behavioral and ecological research that is accessible to nonspecialists
Molecular Systematics	Hillis et al. (1996)	Provides a large set of protocols useful for assaying variation in a variety of molecule types, including allozymes, entire chromosomes, and DNA fragments; also includes thorough overviews of many of the analytical techniques used for inferring phylogenies using molecular data
Molecular Zoology	Ferraris and Palumbi (1996)	Overviews a number of zoological research programs that use molecular techniques as a central research tool and provides a compendium of laboratory protocols relevant to these techniques

One set of laboratory techniques that is more specific to primates involves those used to genetically determine the sex of a DNA sample. A number of methods for sex typing primate DNA have been developed. Most commonly, these involve the coamplification of homologous fragments of the nonrecombining region of the sex chromosomes that show fixed sequence length differences between the X and the Y. For several hominoids, an assay developed by Sullivan et al. (1993), which relies on the presence of a 6 bp difference between the X- and Y-borne copies of the gene for the tooth enamel protein amelogenin, has proved effective for molecular sex typing (Bradley et al. 2001). However, this assay does not work effectively outside of the Hominoidea (Ensminger and Hoffman 2002), nor is it effective for the genus *Pongo* (Steiper and Ruvolo 2003). Wilson and Erlandsson (1998) developed a PCR-based assay effective for a broad range of anthropoid primates; however, it relies on the co-amplification of two fragments of ~700 and ~1,100 bp in length from the X- and Y-borne copies of the zinc finger protein gene, and these are too large to amplify reliably from many types of noninvasively collected sample. Fredsted and Villesen (2004) have designed a similar assay based on the amelogenin locus and involving amplicons of >1,300 bp that appears to be effective in a number of strepsirhines as well as humans. Finally, Di Fiore (in press) has recently developed a novel sex-typing assay that is effective in a broad range of anthropoid primates. This assay uses multiplex PCR to coamplify fragments of the amelogenin gene from the X chromosome and the sex-determining region of the Y gene (*SRY*) from the Y chromosome. The advantage of this assay is that the target fragments are short enough (<200 bp) to reliably amplify from degraded DNA samples.

Finally, there now exists a wealth of software packages useful for analyzing various kinds of molecular data, many of which are publicly available for downloading from the internet. These include packages for multiple sequence alignment, phylogeny inference (using a variety of algorithms, including distance, parsimony, and likelihood-based methods), basic population genetic analyses, and paternity assignment (Table 22.4). Additionally, some journals (e.g., *Molecular Ecology Notes*, *Bioinformatics*) routinely publish notes on new software suitable for particular types of analysis.

CONCLUSIONS

Molecular primatology has already contributed much to our understanding of primate evolution. For the future, the

Table 22.4 Software Packages Useful for the Analysis of Molecular Data

PROGRAM	DESCRIPTION	REFERENCE	PLATFORM AND SOURCE
Sequence alignment			
CLUSTAL	Program for multiple alignment of nucleotide and protein sequence data using a progressive sequence alignment algorithm, can also be used for inferring the phylogenetic relationships among sequences using the neighbor-joining method	Higgins et al. (1996)	Web: www.ebi.ac.uk/clustalw/ Windows, Mac, Linux (via X Windows): ftp-igbmc.u-strasbg.fr/pub/ClustalX/
MALIGN	Program for multiple alignment of nucleotide sequence data that builds phylogenetic trees as it constructs an alignment and uses parsimony criteria to assess the best alignment	Wheeler and Gladstein (1994)	Windows, Linux, Sun: research.amnh.org/scicomp/projects/malign.php
TreeAlign	Program for multiple alignment of nucleotide and protein sequence data that builds phylogenetic trees as it constructs an alignment and uses approximate parsimony criteria to assess the best alignment	Hein (1994)	Web: bioweb.pasteur.fr/seqanal/interfaces/treealign-simple.html Unix: ftp.ebi.ac.uk/pub/software/unix/
DNASIS	Commercially available program for multiple alignment of nucleotide and protein sequence data using a progressive sequence alignment algorithm, also used for archiving of sequence data and for various kinds of sequence analysis (e.g., protein structure)		Windows, Mac: www.oligo.net/dnasis.htm
Sequencher	Commercially available program for multiple sequence alignment and editing and for archiving of sequence data		Windows, Mac: www.genecodes.com/
Phylogenetic inference			
PAUP*	Very widely used and general-purpose program for phylogenetic analysis; calculates numerous measures of genetic distance between taxa according to a user-specified model of evolution; performs phylogeny estimation using distance, parsimony, and likelihood methods	Swofford (2002)	Windows, Mac, Unix, DOS: paup.csit.fsu.edu/
MEGA	General-purpose program for DNA and protein sequence analysis, calculates various measures of genetic distance between sequences from molecular data according to a user-specified model of evolution, performs phylogeny estimation using distance and parsimony methods	Kumar et al. (2001)	Windows: www.megasoftware.net/
PHYLIP	Large set of programs for phylogeny estimation that can use sequence data, allele frequency data, or character data; performs phylogeny estimation using distance, parsimony, and likelihood methods	Felsenstein (2004a)	Windows, Mac, Linux: evolution.genetics.washington.edu/phylip.html
MrBayes	Program to estimate phylogenetic trees based on Bayesian inference	Ronquist and Huelsenbeck (2003)	Windows, Mac, Unix: morphbank.ebc.uu.se/mrbayes/info.php
PAML	Set of programs for phylogeny estimation using maximum likelihood methods and implementing many different models of sequence evolution	Yang (1997)	Windows, Mac, Linux: abacus.gene.ucl.ac.uk/software/paml.html
TREE-PUZZLE	Program to estimate phylogenetic trees from DNA and protein sequence data using a maximum likelihood quartet-puzzling method	Schmidt et al. (2002)	Windows, Mac, Linux: www.tree-puzzle.de/
Population genetics and analysis of population structure			
GENEPOP	General-purpose software package for population genetic analysis; calculates allele frequencies as well as observed and expected genotype frequencies and evaluates whether genotype data deviate significantly from Hardy-Weinberg equilibrium expectation; also calculates various estimators of population differentiation and gene flow among populations and can be used to test for linkage among loci; web version includes useful routines for converting among file formats used by different population genetics programs	Raymond and Roussett (1995)	Web, DOS: wbiomed.curtin.edu.au/genepop/
FSTAT	Versatile general-purpose program for population genetic analysis; calculates allele frequencies as well as observed and expected genotype frequencies and goodness of fit to Hardy-Weinberg equilibrium expectations; also calculates various estimators of population subdivision and inbreeding as well as estimators of genetic distance and relatedness between populations using allelic data; can also be used to test for linkage among loci and to evaluate potential sex biases in dispersal	Goudet (1995, 2001)	Windows: www.unil.ch/izea/softwares/fstat.html
RSTCALC	Program for analysis of population structure and gene flow specifically designed for use with microsatellite marker data	Goodman (1997)	DOS: helios.bto.ed.ac.uk/evolgen/rst/rst.html

Table 22.4 *(cont'd)*

PROGRAM	DESCRIPTION	REFERENCE	PLATFORM AND SOURCE
ARLEQUIN	Versatile multiplatform program for population genetic analysis; calculates allele frequencies as well as observed and expected genotype frequencies, various estimators of population subdivision and gene flow, and various estimators of genetic distance between populations using allelic, haplotype, or sequence data; conducts tests for goodness of fit to Hardy-Weinberg expectations and can also be used to perform assignment tests and to test for isolation by distance	Schneider et al. (2000)	Windows, Mac, Linux (via Java): lgb.unige.ch/arlequin/
Structure	Program for analysis of population structure using multilocus genotype data from various kinds of genetic markers, can evaluate whether a population is subdivided and can be used to assign individuals to subpopulations or to investigate hybridization and admixture	Pritchard et al. (2000)	Windows: pritch.bsd.uchicago.edu/
GeneClass	Program to detect migrants into a population and to assign individuals to a population based on multilocus genotype data, also useful for converting among several file formats used by different population genetics programs	Piry et al. (2004)	Windows, Linux: www.montpellier.inra.fr/URLB/index.html
Identity and parentage analysis			
RELATEDNESS	Estimates pairwise relatedness between individuals or average pairwise relatedness between groups of individuals using a regression-based estimator of relatedness	Queller and Goodnight (1989)	Mac: gsoft.smu.edu/GSoft.html
KINSHIP	Evaluates user-specified hypotheses about pedigree relationships using likelihood methods and can be used for parentage assignment	Goodnight and Queller (1999)	Mac: gsoft.smu.edu/GSoft.html
CERVUS	Conducts parentage assignments using likelihood-based methods that allow for incomplete sampling of potential parents and for some error in genotyping, also can be used to test for the existence of null alleles at a locus and for evaluating whether genotype data deviate significantly from Hardy-Weinberg expectations	Marshall et al. (1998)	Windows:helios.bto.ed.ac.uk/evolgen /cervus/cervus.htm

promise of combining molecular genetic data with information gleaned from other biomolecules, such as proteins, lipids, and carbohydrates and their various combinations, is likely to reveal additional important insights into primate adaptations. The acute conservation crisis faced by most nonhuman primate species makes it imperative that we use all the tools at our disposal to further the understanding of primate biology and support conservation management decisions with key scientific knowledge. Both molecular and whole-organism primatologists have much to gain from the many possibilities for fertile collaboration.

We conclude this chapter by making one caveat. While the various techniques described here clearly constitute an impressive set of tools for examining and interpreting the molecular variation seen within and among different primate taxa, implementation of any of these techniques often requires substantial investment in time, energy, and resources to become proficient with both the theoretical and practical aspects of a particular kind of analysis. Thus, we caution field workers to beware of succumbing to the idea that it is easy to simply "do genetics on the side." To be sure, molecular data can provide important insights into many aspects of the biology of wild primates that may be difficult to realize through field studies alone, but the rationale for adding a molecular component to any field study must be clearly scientifically motivated and worth the investment. This is especially true when the collection of samples for molecular analyses requires anything but completely noninvasive procedures. Thus, as a point of caution, we urge students starting out in the field to not underestimate the workload involved in the molecular part of field projects, and we suggest that students undertake their laboratory training before taking off to the field to start a project. Likewise, we caution molecular biologists to not underestimate the crucial importance of reliable behavioral and ecological data from the field. Some of the most provocative molecular findings may be meaningless without their context in natural history.

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