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CHAPTER 7

Application of Molecular Methods to the Assessment of Genetic Mating Systems in Vertebrates

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CONTENTS

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SYNOPSIS

Verification of vertebrate mating systems is important for evaluating mating system and kin selection theory, and for estimating demographic and population genetic variables. Until recently, such assessments of "genetic" mating systems relative to "behavioral" or "social" ones in natural populations have been very difficult. Modern molecular genetics has provided a panoply of potentially useful methods to document parentage, and these vary in power, appropriateness, degree of difficulty, and expense. In this chapter I describe and evaluate the relative merits of a number of these potential genetic markers. I assess and compare the use of morphology, allozymes, single-copy nuclear DNA [scnDNA; assayed by restriction fragment length polymorphism [RFLP] or DNA sequence analyses], randomly amplified polymorphic DNA (RAPD), and variable number of tandem repeat DNA (VNTRs; i.e., minisatellites and microsatellites assayed by single-locus or multilocus probes, or by polymerase chain reaction amplification). In general, morphological, allozyme, and scnDNA variants provide low-resolution exclusion but lack the variability needed for easy assignment of parentage. RAPD protocols are simple, but dominance and sometimes low replicability of variable bands can hinder their use. Hypervariability of the many loci assayed in multilocus DNA fingerprinting provides much greater resolution, but inabilities to assign alleles to loci and to compare individuals on different gels limit this method mostly to small family groups. Single-locus VNTR methods generally render adequate variability and allow designation of alleles and among-gel comparisons. Microsatellite amplifications are superior in that very small and even degraded DNA samples are sufficient and alleles can be sized exactly, but they usually require considerable time, effort, and technical skill to develop for a limited set of related taxa. I conclude with a brief review and comparison of studies that have used these approaches to assess genetic mating systems in behaviorally monogamous, polygynous/promiscuous, and polyandrous vertebrates.

INTRODUCTION

Biologists have long been interested in the behaviors associated with mating and sexual selection (e.g., Darwin, 1871), but it is not until recently that we have had much more than a suspicion that the mating systems we infer from behavioral observations (i.e., the "social" or "behavioral" mating systems) are not necessarily the same as the ones discernible from genetic analyses (i.e., the "genetic" mating systems; Westneat et al., 1990; Dunn and Lifjeld, 1994). From the behavioral side this may be because it is often very difficult to follow the "private lives" of organisms in nature: copulations can be rapid and extra-pair mating may often be conducted covertly, thus requiring considerable effort to observe. In addition, it would be nearly impossible to observe the complete mating history of even a single individual free-roaming animal let alone a reasonable sample size of individuals.

Lastly, even if copulations are observed, there is almost no way to apportion or identify reproductive success if more than one male is involved.

From the genetic side of the equation, our ignorance has largely been because we have not had methodologies until recently that allow us to accurately evaluate genetic mating systems. The past decade has seen an incredible surge in the availability of molecular and other methods for the determination of parentage and kinship and, correspondingly, in the number of studies that document and compare genetic mating systems with social ones. It is my purpose here to review the various methods that have been developed to assess parentage, and to describe how and how well they have been applied to studies of mating systems in natural populations of vertebrates. I begin by briefly addressing the importance of documenting genetic mating systems. I then survey and compare the methods that have been used over the past two decades and discuss their strengths and weaknesses. I conclude with a general literature review and several specific case studies to demonstrate applications of the methods to the assessment of a variety of mating and unique social systems (e.g., cooperative breeders, brood parasites). The protocol by Sabine Loew and Robert Fleischer in Loew and Fleischer.1 contains a detailed methodology for multilocus DNA fingerprinting; another protocol by Robert Fleischer and Sabine Loew (Fleischer and Loew.2) provides a comprehensive protocol for constructing microsatellite-enriched plasmid libraries using hybridization selection (modified from Armour et al., 1994).

Rationale

Why is it important to know the genetic mating system? First, theories concerning the evolution of mating systems depend on accurate representations of the mating system and these may be particularly difficult to determine by observation in studies of nocturnal, fossorial, or otherwise secretive species. Models for mating system evolution require knowledge of the ratio of male reproductive success to female reproductive success and of the relative success of alternative reproductive tactics (Emlen and Oring, 1977; Payne, 1979; Trivers, 1985; Westneat et al., 1990), and strictly behaviorally based studies may provide biased and inaccurate estimates of these variables (e.g., Gibbs et al., 1990; Morton et al., 1990; Pemberton et al., 1992; Boness et al., 1993; Dunn and Lifjeld, 1994). In addition, in species where sperm storage or sperm competition may be important aspects of the mating system, molecular or other determinations of parentage are often essential (Smith, 1984; Birkhead and Møller, 1992; Oring et al., 1992). Many models of kin selection also require accurate assessments of relatedness (Trivers, 1985).

A correct determination of parentage is also needed to accurately estimate a number of variables of importance to studies of population dynamics and population and quantitative genetics. These include demographic variables such as lifetime reproductive success, effective population size, and the level of inbreeding in populations. Reliance on estimates of variance in reproductive success from behavioral methods alone could result in biased estimates of these variables. Lastly, estimates

of heritability of morphological or other characters may be significantly modified by unrevealed extra-pair parentage (e.g., Alatalo et al., 1984).

METHODS FOR DETERMINING PARENTAGE

A wide variety of methods exist for determining parentage in natural populations. I first provide a brief overview of some nongenetic methods, then present and discuss in greater detail several genetic approaches (Table 1). The goal of the genetic methods is to resolve variable, replicable, and easily assayed sets of markers that can be used to identify, with high probability, the parents of individual offspring. In some cases such markers only have the resolving power to exclude offspring from putative parents without also identifying extra-pair parents. Each of the methods also differs in their expense and degree of difficulty. I attempt to summarize these strengths and costs as equitably as possible and to recommend which methods I feel are best used for particular questions and/or systems. However, I would advise researchers not to choose a method because it is the most advanced, difficult, novel, or expensive if an easier, cheaper, or older method can just as effectively answer the questions of their study.

Nongenetic Methods

In the past, researchers have used nongenetic methods such as vasectomies (Bray et al., 1975), removal experiments, radioactive tracers (e.g., Wolff and Holleman, 1978; Tamarin et al., 1983), colored glass microspheres (Quay, 1988), fluorescent pigments (Ribble, 1991), and even antibody response to rare antigens (Glass et al., 1990) to exclude or identify one or both parents of an individual. Some of these methods allow only exclusion of paternity from a putative father (e.g., vasectomies, removals), while others involve difficult procedures or only determine matrilines

TABLE 1. Types of Genetic Markers/Methods that Have Been, or Could Potentially Be, Used to Document Parentage in Studies of Genetic Mating Systems

Morphology
Allozyme or protein variants
Protein electrophoresis / isoelectric focusing
Mitochondrial DNA / single-copy nuclear DNA / introns
Restriction fragment length polymorphism (RFLP)—random probes
Sequencing—control region / introns
Variable number tandem repeats (VNTRs)
Multilocus DNA fingerprinting
Single-locus VNTR probes
Single-locus amplified VNTRs/microsatellite
Randomly amplified polymorphic DNA (RAPD)

(e.g., antibody response). Few approach DNA methods in their level of resolution and none have been used extensively.

Phenotypic Marker Methods

Morphological Markers. Most studies that have taken advantage of heritable morphological variants for identification of extra-pair young have been conducted in captivity (where potential partners can be controlled; e.g., Burns et al., 1980). However, a small number of studies of wild birds (e.g., Mineau and Cooke, 1979; Alatalo et al., 1984; Norris and Blakey, 1989; Payne and Payne, 1989; Møller and Birkhead, 1992) have used analyses of inherited color polymorphism or heritability of quantitative morphological traits (e.g., tarsus length) to determine whether given nestlings were not likely the true offspring of one or both of their putative parents. While such studies often allow exclusion of offspring from putative parents, they rarely allow identification of extra-pair parents.

Protein Variant Markers. With the emergence of inexpensive protein gel electrophoresis in the 1970s, a number of researchers began to take advantage of allozyme variants to document genetic mating systems (e.g., Tilley and Hansman, 1976; Hanken and Sherman, 1981; Westneat, 1987; Pope, 1990; Bollinger and Gavin, 1991; Gowaty and Bridges, 1991; Xia and Millar, 1991) and/or brood parasitism in vertebrates (e.g., Fleischer, 1985; Brown and Brown, 1988; Smyth et al., 1993). Proteins can be analyzed from blood or other tissue samples, or from growing feathers (Marsden and May, 1984) or eggs (Fleischer, 1985; Smyth et al., 1993). Blood and feather samples are often the chosen tissue because they can be taken nondestructively. This, however, can limit the number and types of loci that can be screened, as other tissues (e.g., liver, muscle) generally have more expressed loci than plasma and erythrocytes. A number of researchers (e.g., Westneat, 1987) have used breast muscle biopsies to obtain sufficient numbers of allozyme loci for parentage analyses.

In allozyme methods, tissue extracts are wicked into starch gels or loaded into wells of polyacrylamide or cellulose acetate gels. Protein variants that differ in charge, conformation, or size are separated in the gel by electrophoresis using one of a variety of buffer systems varying in pH and ionic strength. Proteins are visualized by enzyme-specific or general protein stains applied to the whole gel or to a number of horizontal slices in the case of starch gels. The resultant bands are scored and interpreted as genotypes at mendelian-inherited loci (e.g., Fig. 1). There are several comprehensive protocols available for analysis of allozyme variability (e.g., Selander et al., 1971; Harris and Hopkinson, 1976; Evans, 1987; Murphy et al., 1990).

Researchers have found that the proportion of polymorphic allozyme loci is relatively low in many groups of vertebrates (e.g., Selander, 1976; Barrowclough et al., 1985; Nei, 1987). In addition, the few loci that are polymorphic often have low allelic diversity, with rarely more than 2–3 alleles per locus, and low heterozygosity. The low heterozygosity is in part caused by one allele having very high frequency (>80–90%), while others have very low frequencies. Such low levels of variation make it difficult for biochemical methods, along with any other low-

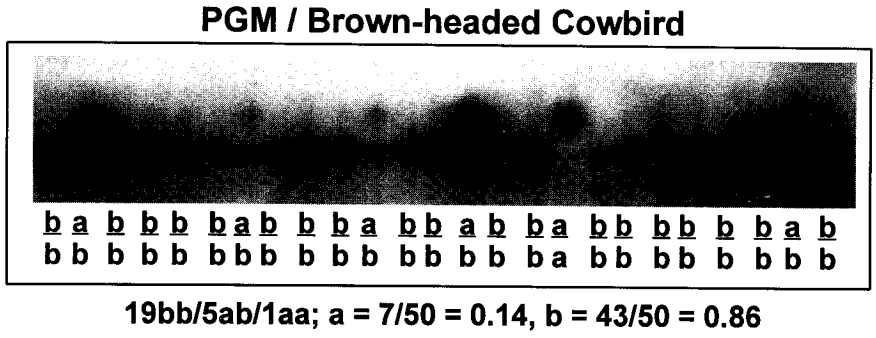

Figure 1. Typical allozyme result: diallelic phosphoglucomutase locus of the brown-headed cowbird. Genotypes are noted below each lane.

resolution method, to reliably exclude offspring from parents (Westneat et al., 1987; Burke et al., 1991). In addition, low variability makes identification of the individuals responsible for extra-pair offspring even less likely (Lewis and Snow, 1992).

Single-Copy DNA Methods

RFLP and Sequence Analyses. As methods of analyzing variation in DNA became less complicated and less expensive, it became apparent that molecular methods could also be applied to studies of genetic mating systems. Two common methods for documenting variation in both single-copy nuclear (scnDNA) and mitochondrial DNA (mtDNA) are restriction fragment length polymorphism (RFLP) analysis and DNA sequencing (see Hillis and Moritz, 1990; Hoelzel, 1992; and Avise, 1994, for applications and protocols). RFLP analyses use restriction endonucleases that cleave DNA at particular short sequences (usually 4–6 bp). If the sequences vary, the enzymes will not cut and variation can be revealed by electrophoretic separation of fragments in gel media. DNA sequencing is usually accomplished by electrophoretic separation of dideoxynucleotide-terminated, isotope-labeled, complementary strand DNA that is polymerase-synthesized from a primer annealed to single-strand template DNA (Sanger et al., 1977). Recently, additional methods involving PCR and a variety of screening methods have been developed (Karl and Avise, 1993; Lessa and Applebaum, 1993; Slade et al., 1994). None of these methods have yet been used extensively in parentage analyses, mostly because levels of intrapopulation variation in both exons and introns of nuclear genes appear to be too low for easy resolution of parentage. Both mtDNA and scnDNA have been used in parentage analyses: Quinn et al. (1987) used RFLP analysis with random single-copy nuclear probes to assess parentage in snow goose (*Anser caerulescens*) families; while Morin and Ryder (1991) used mtDNA RFLP, along with DNA fingerprinting, to reconstruct a pedigree of captive lion-tailed macaques (*Macaca silenus*). Avise et al. (1989) found a hypervariable region in mouse mtDNA and suggested it could be used to identify matrilines.

Variable Number of Tandem Repeat (VNTR) Markers

These methods take advantage of relatively small tandemly repeated sequences that are dispersed throughout the genome. Some VNTRs (referred to as minisatellites) have repeat lengths of 7–65 bp (Jeffreys et al., 1985; Shin et al., 1985; Nakamura et al., 1987; Vassart et al., 1987); microsatellites (sometimes called STRs for short tandem repeats or SSRs for simple sequence repeats; Edwards et al., 1992; Goff et al., 1992) are considered to have repeat lengths of 1–6 bp (Ali et al., 1986; Epplen, 1988). For both size classes of VNTRs, the primary source of variation is the difference in the number of repeats within an array. Also, for both types, replication slippage is thought to play a major role in generating additional repeats and variation in repeat number (Levinson and Gutman, 1987; Jeffreys et al., 1991; Schlötterer and Tautz, 1992). Minisatellite variation may also be produced by unequal sister chromatid exchange during mitosis or, less likely, unequal crossing-over between homologous chromosomes during meiosis (Smith, 1976; Jarman and Wells, 1989; Stephan, 1989; Wolff et al., 1991). Both classes of VNTR are perhaps arbitrary sections of a continuum, and it has been suggested that minisatellites originate from microsatellite duplications (Wright, 1994).

Multilocus DNA Fingerprinting. Multilocus DNA fingerprinting has been the primary method of choice for studies of genetic mating systems since its discovery in the mid-1980s until very recently (Jeffreys et al., 1985; Burke, 1989; Burke et al., 1991; Amos and Pemberton, 1992; Bruford et al., 1992). This has been because the extreme mutability and concomitant high allelic diversity and heterozygosity at minisatellite (and, to some extent, microsatellite) loci invariably permits exclusion of offspring from putative parents and usually also allows, with high probability, the assignment of excluded offspring to extra-pair parents (e.g., Burke et al., 1989; Gibbs et al., 1990; Westneat, 1990, 1993; Rabenold et al., 1990; Ribble, 1991; Smith et al., 1991; Oring et al., 1992; Stutchbury et al., 1994). In addition to high variability of markers, DNA fingerprinting is greatly simplified by the use of a large number of nearly "universal" minisatellite and microsatellite probes. Thus a major advantage of this method over others is that little or no preliminary work is required when one switches to a new species.

Multilocus DNA fingerprinting involves restriction digestion of samples of clean, high-molecular-weight genomic DNA with a tetranucleotide-recognizing restriction endonuclease followed by size fractionation in an agarose gel (Fig. 2; see Loew and Fleischer.1 for explicit protocol; also Jeffreys et al., 1985; Bruford et al., 1992). The digested DNA is denatured and transferred (i.e., capillary, vacuum, or pressure blotted) to a nylon or nitrocellulose membrane and then bound to the membrane by UV crosslinking and/or baking. Cloned or synthesized segments of DNA that contain the minisatellite or microsatellite sequences cited above are labeled (isotopically or nonisotopically) by random priming, nick translation, polymerase chain reaction, or 5′ or 3′ end-labeling. The probe is cleaned of excess labeled nucleotides, heat denatured, and hybridized to the DNA on the membrane. Some oligonucleotide probe protocols involve hybridization within a partially dried

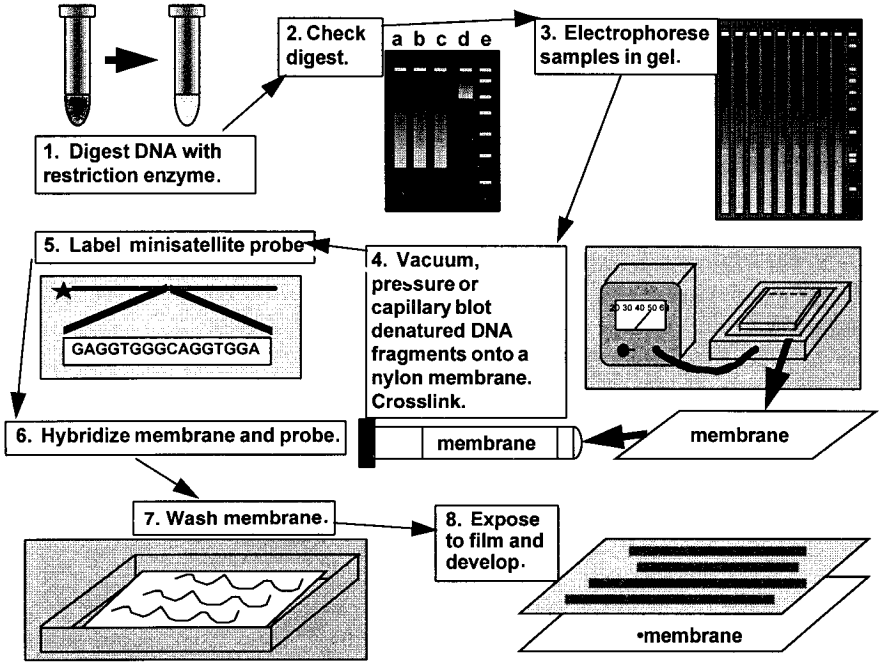

Figure 2. Abbreviated flowchart showing steps of multilocus DNA fingerprinting. In-depth protocols for multilocus DNA fingerprinting are in Loew and Fleischer.1.

gel (i.e., without transfer). X-ray film is exposed to the filter or gel for isotope and chemiluminescent methods and its development produces an autoradiograph in which 10–30 highly variable fragments (representing up to 25 or more separate loci) are usually visualized per lane (e.g., Fig. 3).

When controlled crosses or families with known pedigrees are subjected to multilocus DNA fingerprinting, the band distributions of parents and offspring generally conform to mendelian expectation (Fig. 3; e.g., Jeffreys et al., 1985, 1986, 1987; Nakamura et al., 1987; Longmire et al., 1988; Kuhnlein et al., 1989; Lang et al., 1993) with the occasional exception of a small percentage (usually $\ll 1\%$) of "excluding" fragments that are presumably shifted by mutation. Mutation rates for vertebrates have generally been observed in the range of 10^{-4} to 10^{-2}/gamete/generation for minisatellites and 10^{-4} to 10^{-3}/gamete/generation for microsatellites (Jeffreys et al., 1987, 1988a, 1991; Burke et al., 1989; Nürnberg et al., 1989; Kuhnlein et al., 1990; Westneat, 1990; Dallas, 1992; Dietrich et al., 1992; Kwiatkowski et al., 1992; Fleischer et al., 1994; Verheyen et al., 1994). One hypermutable human minisatellite locus (MS1; Jeffreys et al., 1988a), however, shows an astounding mutation rate of 0.05/gamete/generation. In a number of studies of vertebrates, one or more fragments have been found to be sex-linked (e.g., Rabenold et al., 1991; Ellegren et al., 1994) and have proved useful for identifying the sex of individuals in sexually monomorphic taxa.

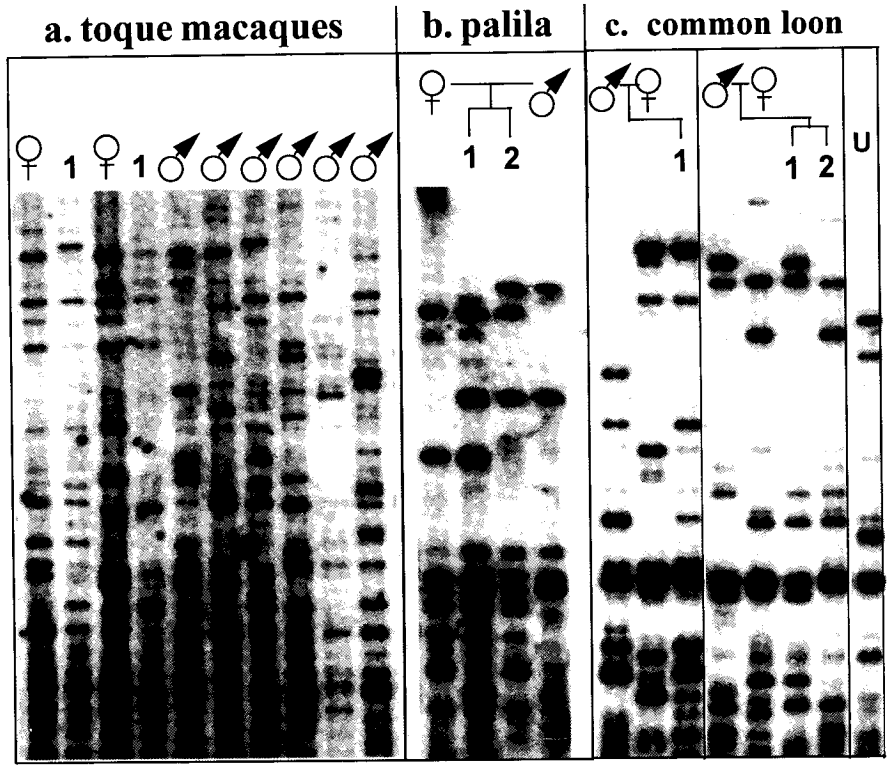

Figure 3. Examples of multilocus DNA fingerprints. (a) Multi-male group of toque macaques (S. Loew et al., *unpublished data*). Shown are two females and offspring followed by six potential fathers. (b) Nestlings (1,2) and putative parents of palila (Fleischer et al., 1994). (c) Two common loon families and an extra individual (W. Piper, *unpublished data*). Note that offspring profiles contain only fragments that are also found in parental ones.

In addition, with a few exceptions (e.g., Brock and White, 1991), most studies have revealed independent assortment of fingerprint fragments (e.g., Jeffreys et al., 1986, 1987; Jeffreys and Morton, 1987; Burke et al., 1991; Lang et al., 1993). The assumption of linkage can be assessed by a number of analyses (Burke et al., 1991; Amos et al., 1992; Bruford et al., 1992), including direct inspection of family pedigrees for co-segregation of fragments, statistical testing for nonrandom association of fragments, comparison of the widths or variances of the distributions of band-sharing among first-order relatives versus among unrelated individuals (Amos et al., 1992; Fleischer et al., 1994), and comparison of the predicted and actual mean band-sharing coefficient of first-order relatives. However, even if there is a fair level of linkage (e.g., 25% of fragments on average), there appears to be only a minor problem of incorrect parentage assignment (Amos et al., 1992).

Multilocus DNA fingerprints of putative family groups are usually scored for parentage analyses in three stages. First, extra-pair offspring are "excluded" from a

pair of putative parents if there are fragments in offspring profiles that are not present in one or both parental profiles. A single extra fragment in a profile is likely to have resulted from mutation or artifact rather than nonparentage based on the fit of novel fragment frequencies to a Poisson distribution (Westneat, 1990), but this likelihood should be worked out for each population under study. In families from normal populations it is typical to have five or more novel fragments per probe in extra-pair young. The power of the data to exclude can be further tested by comparing unexcluded offspring profiles to those of "sham" parents (i.e., adults in adjacent lanes) and producing a histogram of novel fragments. The number of unattributable fragments for the actual attending males is then compared to the histogram to indicate the likelihood of exclusion (Fleischer et al., 1994).

Second, coefficients of band-sharing (S of Lynch, 1990, or "unweighted" x of Jeffreys et al., 1985) are calculated between each putative parent and each offspring. These values are compared to a distribution of "background band-sharing" from comparisons of randomly sampled, putatively unrelated individuals. In normal outbred taxa, S usually ranges between 0.0 and 0.5 and averages 0.2–0.3 for randomly sampled, presumably unrelated individuals (Burke et al., 1991; Amos et al., 1992), although it has been found to be higher for some groups of organisms regardless of current inbreeding levels. If S for the male of an excluded pair falls within this range, and S for the female is greater than the range, the excluded offspring represents an extra-pair fertilization (EPF). If S is low for the female as well, the offspring is likely the result of intraspecific brood parasitism (ISBP).

Third, males from neighboring territories, from the same group in social breeders, or floaters are run on the same gel adjacent to the excluded offspring and female. These extra males should be included on the original gel if extra-pair fertilizations are expected or if no male was dominant for group breeders; or they can be run later on a second gel if the behaviorally assigned males are excluded. Each male is then subjected to stages 1 and 2, as above, by comparing his profile to those of the offspring and female. If a male cannot be excluded and has high band-sharing with the offspring, it is concluded that the individual is the actual father. It is very important to include equal amounts of digested DNA in each lane of a fingerprint. If any deviation must be made, it should be for offspring to have less DNA than parents so that extra fragments are not mistaken for nonparentage when they are actually artifacts of light fingerprints.

Additional advantages to multilocus DNA fingerprinting over other methods are that multilocus VNTR probes assay for as many as 10–25 highly variable loci with a single probing, and that filters can easily be reprobed with several independent (usually, but see Armour et al., 1990) probes. Thus a good deal of data can be accumulated rapidly. Several disadvantages include (1) the amount of DNA that is required (minimum of about 1 μg per individual, but usually 3–10 μg; however, these amounts are not as big a problem for birds or lower vertebrates because of their nucleated erythrocytes as they may be for small mammals and invertebrates), (2) the methodology involves more steps and can be more problematic than allozyme (or even than VNTR amplification) methods, (3) specific loci and alleles usually cannot be determined, and, perhaps most important, (4) all individuals

being compared should be run on a single gel (even if internal or between lane size standards are included). This is because intergel differences in fragment mobility make it nearly impossible to obtain accurate sizes and to standardize between gels. Thus, in contrast to prior optimism (Burke et al., 1991; Galbraith et al., 1991), I do not advocate scoring multilocus fingerprint similarity among individuals on different gels. [*Note:* This may be acceptable if similarity is high and a few individuals have all the variable fragments and can be used as markers on each gel. (e.g., Rave et al., 1994).] For situations where a large number of offspring are being tested (often for fish, amphibian, or reptile studies), or if there are a large number of potential fathers (or mothers), multilocus fingerprinting is generally less useful or reliable than microsatellite amplification (see below). In fact, the type of study for which multilocus fingerprinting may be best suited is for assessing parentage in monogamous vertebrates with small family sizes and low predicted EPF rates.

Single-Locus VNTR Probes. These probes assay only a single minisatellite "locus" but in protocol and otherwise are very similar to multilocus probes. The procedure begins by "lifting" or replicating onto nylon filters clones from genomic libraries grown on media-containing agar plates (genomic libraries are bacterial cells with engineered plasmid or viral "vectors" that contain random "inserts" of DNA isolated from a species of interest). Multilocus VNTR probes are then labeled and hybridized to the nylon filters in order to locate clones containing vectors with minisatellite-containing inserts (Wong et al., 1986; Nakamura et al., 1987; Armour et al., 1990; Hanotte et al., 1991; Bruford et al., 1992; Verheyen et al., 1994). The single-copy regions that flank the VNTR are the part of the probe that identifies a single locus. The isolated insert from the phage or charomid is used as a probe on nylon membranes bearing restriction enzyme-digested genomic DNA, as in the multilocus method above. The hybridization can include competitor DNA from a distant relative (to "soak up" the minisatellite part of the probe), or the insert can be restriction-digested and only the flanking regions used as a probe (and subcloned).

Such probes *usually* reveal size variants at single Mendelian loci (Fig. 4), but they can require a lot of effort to obtain and process: genomic library construction is not trivial, and even after screening and obtaining positive clones a considerable amount of fine-tuning remains. For example, in one study (Armour et al., 1990), only 12.4% of 185 positive clones assayed ended up providing useful, novel polymorphic VNTR probes. Single-locus VNTR probes, however, have an obvious advantage in allowing determination of allele frequencies, which makes them more amenable to classical population genetic analysis. On the other hand, their visualization requires precisely as much effort as in multilocus DNA fingerprinting, and exact sizing and scoring between gels can still be problematic.

Single-Locus Amplified VNTRs/Microsatellites. With the advent of the polymerase chain reaction (PCR), a method of producing highly variable single-locus genetic markers was developed for humans (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989). This method takes advantage of microsatellites and, rarely, of minisatellites (because their lengths are usually beyond the abilities of standard

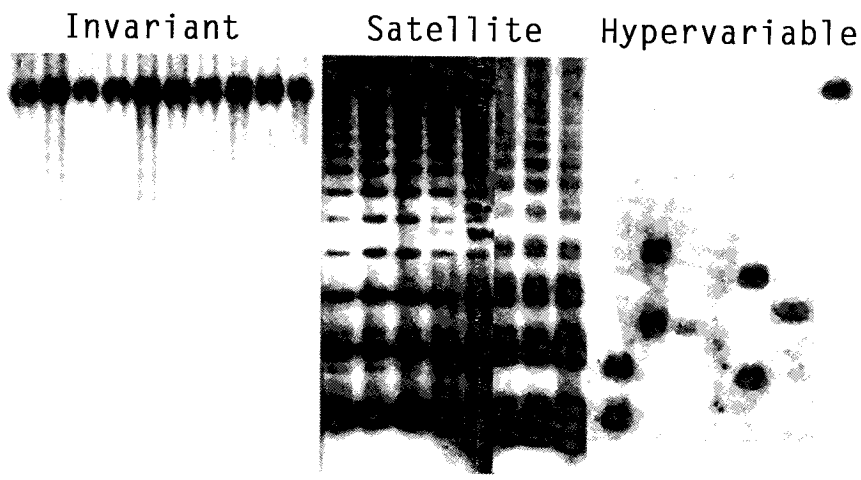

Figure 4. Potential results from hybridization with putative single-locus minisatellite probes. In some cases probes uncover no variability, satellite DNAs, or, preferably, hypervariability.

PCR and sizing is inexact; but see Jeffreys et al., 1988b; Horn et al., 1989; Decorte and Cassiman, 1991; Armour et al., 1992).

To develop primers for microsatellite amplification, microsatellite probes (the same ones used for multilocus fingerprinting; e.g., CAC_n, CA_n) are used to screen genomic libraries (usually in plasmid vectors) of the species of interest in order to locate clones that contain microsatellite and flanking sequences (Fig. 5; Rassmann et al., 1991; Ellegren et al., 1992; Hughes and Queller, 1993). A few methods for constructing libraries that are greatly enriched for microsatellite-bearing inserts have been developed (e.g., Ostrander et al., 1992; Armour et al., 1994); one such method (Armour et al., 1994) used successfully in my laboratory (Fig. 5d) is detailed in Fleischer and Loew.2. The regions that flank the repeat region are sequenced and the sequences are used to design synthetic oligonucleotide primers (Fig. 5a,b). These specific primers are then used in the polymerase chain reaction to amplify across the microsatellite to produce small products (usually <300 bp) that can be isotope-labeled and resolved on a polyacrylamide gel (Fig. 5c). Some laboratories visualize products with ethidium bromide or silver staining. The products can be sized exactly in multiples of the repeat length with a DNA sequence as a size marker and are highly variable, with up to 50 or more alleles and heterozygosities ranging up to 99% (Tautz, 1989; Amos et al., 1993).

Another way in which microsatellites have been identified is by "probing" GenBank or other DNA sequence computer databases with microsatellite sequences

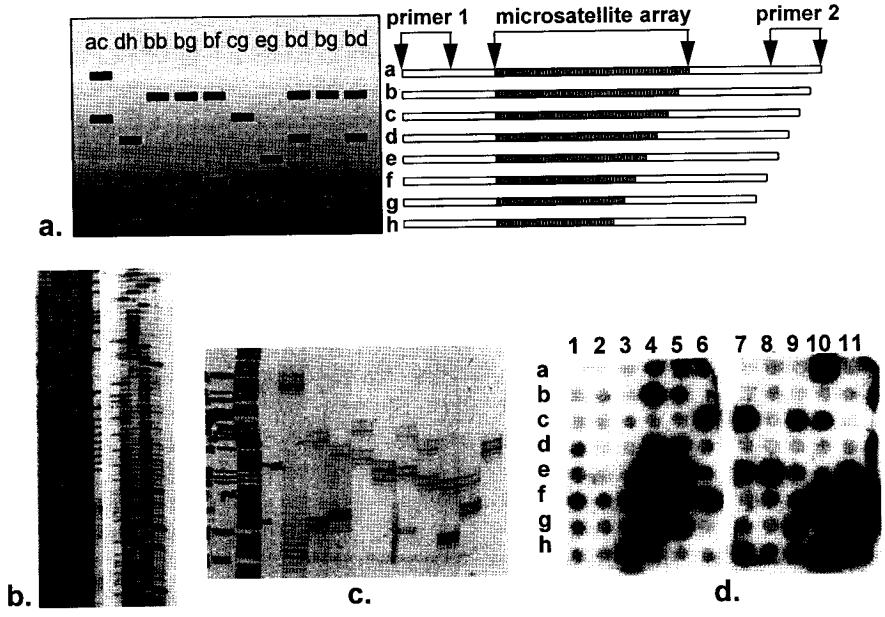

Figure 5. (a) PCR-amplified microsatellite bands (alleles) differ in length based on the number of microsatellite repeat units. Variation is assayed by amplification with two flanking region primers followed by electrophoretic separation of the products (see text). The bands in the gel correspond to the amplified products displayed on the right and differ in size by multiples of the repeat unit. (b) Examples of CA/TG_n repeat and GGA_n repeat, both isolated from a lizard genomic library (N. Zucker et al., *unpublished data*); note how the sequencing enzyme does not always read clearly through the dinucleotide repeat. Primers are designed from the flanking region sequences. (c) Example of microsatellite amplifications of blue-headed wrasses (L. Woonink et al., *unpublished data*). (d) Microtiter plate replica filters from a giant kangaroo rat (*Dipodomys ingens*) genomic library screened with labeled microsatellites (protocol of Armour et al., 1994; Fleischer and Loew.2). Positive clones increased from $\leq 1\%$ in a normal library to over 30%; most of these yielded long microsatellite arrays (>10 repeats; S. Loew et al., *unpublished data*).

(e.g., Moore et al., 1991; Stallings et al., 1991). Microsatellites can often be found in introns of protein coding genes sequenced for other reasons. Also, if one is fortunate (e.g., Morin and Woodruff, 1992; Gotelli et al., 1994), microsatellites developed for a domestic or laboratory species may work with closely related species in natural populations.

Initially microsatellites were developed for humans (above) and domesticated or laboratory organisms (e.g., Love et al., 1989; Fries et al., 1990), but now amplifiable microsatellites have been developed for a growing number of wild vertebrate taxa, including fish (e.g., Goff et al., 1992), amphibians (e.g., Scribner et al., 1994), reptiles (e.g., N. Zucker, S. Loew, and R. C. Fleischer, *unpublished data*), mammals (e.g., Schlötterer et al., 1991; Paetkau and Strobeck, 1994; Taylor et al.,

1994), and birds (Ellegren, 1992; Hanotte et al., 1994; McDonald and Potts, 1994). Based on these studies, avian microsatellites appear to be as variable as those of mammals, having a range of 1–15 alleles and 40–92% heterozygosity per locus, but birds seem to have fewer microsatellites in their genome than other vertebrates (*personal observation;* T. Glenn and C. Hughes, *personal communications*), making it more difficult to isolate them from genomic libraries without enrichment (e.g., Fleischer and Loew.2).

The primary advantage to microsatellites is that each locus and allele can be typed exactly and put into a database: thus individuals run on different gels at different times can be directly compared. In addition, microsatellites can be amplified from very small amounts of DNA, including partly degraded DNA from shed hair (Morin and Woodruff, 1992), museum specimens (Ellegren, 1991; Taylor et al., 1994), and feces (Constable et al., 1995). The only real disadvantages to microsatellite amplification are (1) they are not as mutable (Nürnberg et al., 1989; Dallas, 1992) hence as variable as minisatellites and (2) primers can be difficult and time-consuming to develop; they are not universal, and they usually will cross-anneal only with species that are fairly closely related (same family or sometimes order; Schlötterer et al., 1991; Moore et al., 1991; Stallings et al., 1991; Ellegren, 1992; Hanotte et al., 1994; Garza et al., 1995; but see FitzSimmons et al., 1995). Therefore, until microsatellites are developed for a broad spectrum of taxa (5+ years from now?), they are likely to be most useful for long-term, intensive studies of single species or related groups of species, for which the development time and effort required will be repaid. A couple of other minor problems recently noted include nonamplifying or "null" alleles (Pemberton et al., 1995), which may predominate when primers are used beyond the taxon for which they were developed, and hidden variability (Gertsch et al., 1995). Although amplified microsatellites would be useful for any study of genetic mating systems, they are essential for studies of mating systems in taxa such as brood parasites or aquatic spawners, for which assignment of offspring to parents would be extremely difficult with multilocus fingerprinting.

Thus far, only a few studies have been published that use amplified VNTRs (microsatellites) to assess genetic mating systems in either nonhuman vertebrates (primates: Takasaki and Takenaka, 1991; Sugiyama et al., 1993; Morin et al., 1994; pinnipeds: Amos et al., 1995; birds: Primmer et al., 1995; bears: Craighead et al., 1995; and cetaceans: Amos et al., 1993) or invertebrates (see Strassmann et al., Chapter 8 in this volume). Most studies to date that utilize amplified microsatellites in vertebrates deal with forensics and linkage mapping in humans and domesticated species (e.g., Love et al., 1989; Fries et al., 1990; Edwards et al., 1992; Dietrich et al., 1992; Goff et al., 1992; Ostrander et al., 1993) or analyses of genetic structure and relatedness in natural populations (e.g., Bowcock et al., 1994; Gottelli et al., 1994; McDonald and Potts, 1994; Taylor et al., 1994). As noted above, however, amplified microsatellites are superior in many ways to other methods of assessing parentage in natural populations, and we should see a rapid increase in their use and in the rate of publication of results.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD analysis (sometimes called AP-PCR, or arbitrarily primed PCR; Welsh and McClelland, 1990) uses random sequence priming to identify polymorphism in putatively random, anonymous DNA sequences. In its most common form (Williams et al., 1990), a single, randomly constructed 10-base oligonucleotide primer is used in a PCR reaction. The amplification requires that complementary, inverted primer sites occur in two locations along a continuous DNA sequence, and that they flank a region which is short enough (<2–3 kb) to be amplified. The resultant products (from 0 to 10 or more different-sized fragments) are electrophoresed in an agarose or polyacrylamide gel, stained with ethidium bromide or silver, and photographed. Some modified protocols have used isotope labels, denaturing polyacrylamide gels, and autoradiography to increase resolution and repeatability (e.g., Welsh and McClelland, 1990; McClelland et al., 1994).

The phenotype of the polymorphism is usually dominant and seen as the presence or absence of a fragment: presumably one variant amplifies and the other cannot because of deletions or substitutions in one or both priming sites, or large insertions or deletions between the priming sites (Williams et al., 1990). Most reports to date indicate that fragment inheritance is mendelian (e.g., Williams et al., 1990; Levitan and Grosberg, 1993), but some researchers have found artifactual variation seen as noninherited fragments (Riedy et al., 1992; Ellsworth et al., 1993; Ayliffe et al., 1994). In one study (Ayliffe et al., 1994), the extra, nonparental fragment was identified as a heteroduplex product of two variants that differ by 38 bp. Hadrys et al. (1993) dealt with this potential problem by amplifying a "synthetic offspring" that included equal amounts of DNA from both parents, and presumably produced any heteroduplex or chimerical products for comparison to actual offspring profiles. Other researchers (e.g., Jones et al., 1994) control artifacts by rigorous standardization of conditions such as DNA quantity and quality, buffer components, and amplification parameters. DNA degradation, in particular, can be a problematic cause for missing bands.

The dominance of RAPD markers is perhaps the greatest drawback to their use for parentage analysis because considerably more loci are required to identify parents than with codominant markers (Lewis and Snow, 1992; Milligan and McMurry, 1993). Interestingly, the probability of assignment increases with increasing recessive allele frequency of RAPDs (Lewis and Snow, 1992). Extra-pair parentage can be identified only if both parents lack a fragment (i.e., are recessive homozygotes) and an offspring has the fragment. Alternative methods of analysis include calculation of band-sharing coefficients (Hadrys et al., 1993) and cluster analysis (Levitan and Grosberg, 1993). The latter allowed clear identification of parents while minimizing the effects of artifactual fragments. To date, nearly all published studies of parentage using RAPD markers have dealt with plants or invertebrates. Unless methods develop to increase replicability and codominance of alleles, RAPD analysis will not likely be the usual method of choice for studies of genetic mating systems.

APPLICATIONS OF METHODS TO MATING SYSTEMS

Monogamy

Monogamy is the most common behavioral mating system in birds (>90%; Lack, 1968) and perhaps the rarest in mammals (<5%; Kleiman, 1977; Boness et al., 1993) and lower vertebrates (Birkhead and Møller, 1992). Thus most applications of genetic markers to assess whether behavioral monogamy equates with genetic monogamy have dealt with birds (but see Ribble, 1991; Dixon et al., 1994), and mostly with birds of temperate rather than tropical regions.

All parentage studies to date of a wide variety of behaviorally monogamous, *non*passerine birds have revealed relatively low EPF rates: the mean percentage of offspring resulting from EPF for 14 species is 4.0 ± 6.3% (range of 0–18%; snow goose, *Anser caerulescens;* blue duck, *Hymenolaimus malachorhynchos;* mallard, *Anas platyrhynchos;* swift, *Apus apus;* fulmar, *Fulmarus glacialis;* black vulture, *Coragyps atratus;* shag, *Phalacrocorax aristotelis;* sparrowhawk, *Accipiter nisus;* and oystercatcher, *Haematopus ostralegus,* from Appendix of Møller and Birkhead, 1994; short-tailed shearwater, *Puffinus tenuirostris,* from Austin et al., 1993; Cory's shearwater, *Calonectris diomedea,* from Swatschek et al., 1994; merlin, *Falco columbarius* from Warkentin et al., 1994; unhelped bee-eaters, *Merops apiaster,* and red-cockaded woodpeckers, *Picoides borealis,* from Haig et al., 1994). Thus genetic mating systems for nonpasserine birds mostly match behavioral mating systems.

Behaviorally monogamous passerine birds, on the other hand, exhibit a much higher variance in EPF rate: ranging from 0% to a high of 58% of offspring. The proportion of EPF may be related to sedentary versus migratory status, and perhaps also to living in the tropics. The few tropical species assessed thus far show very low rates of EPF per nestling (e.g., no evidence for EPF in the common myna, *Acridotheres tristis,* Telecky, 1989; the palila, *Loxioides balleui,* Fleischer et al., 1994; and the dusky antbird, *Cercomacra tyrannina,* C. Tarr et al., *unpublished data*). Sedentary monogamous species (including the tropical ones above) have relatively low levels of EPF (6.0 ± 6.2% of offspring, range of 0–17%, n = 17 studies of 14 species; values for jackdaws, *Corvus monedula;* Siberian jay, *Perisoreus infaustas;* zebra finch, *Taeniopygia guttata;* house finch, *Carpodacus mexicanus;* chaffinch, *Fringilla coelebs;* and dunnock, *Prunella modularis,* obtained from Appendix of Møller and Birkhead, 1994; great tit, *Parus major,* from Blakey, 1994; blue tit, *Parus caeruleus,* from Kempenaers et al., 1992; and both tit species from Gullberg et al., 1992; monogamous corn bunting, *Milaria calandra,* from Hartley et al., 1993; house sparrow, *Passer domesticus,* from Wetton et al., 1992, and Burke et al., 1991; and bull-headed shrike, *Lanius bucephalus,* from Yamagishi et al., 1992). Migratory monogamous species show, on average, a higher rate of EPF (23.2 ± 15.1% of offspring, range of 0–58%, n = 17 species); wheatear, *Oenanthe oenanthe;* eastern bluebird, *Sialia sialis;* reed warbler, *Acrocephalus schoenobaenus;* willow warbler, *Phylloscopus sibilatrix;* tree swallow, *Tachycineta bicolor;* purple martin, *Progne subis;* barn swallow, *Hirundo rustica;* cliff swallow,

Hirundo pyrrhonota; white-crowned sparrow, *Zonotrichia leucophrys;* dark-eyed junco, *Junco hyemalis;* indigo bunting, *Passerina cyanea;* and hooded warbler, *Wilsonia citrina,* from Appendix of Møller and Birkhead, 1994; house wren, *Troglodytes aedon,* and field sparrow, *Spizella pusilla, recalculated* from Price et al., 1989, and Petter et al., 1990, respectively; Wilson's warbler, *Wilsonia pusilla,* from Bereson et al., in press; Kentucky warbler, *Oporornus formosus,* from M. V. McDonald et al., *unpublished data;* and monogamous reed bunting, *Emberiza schoeniclus,* from Dixon et al., 1994). In summary, a remarkable number of these behaviorally monogamous taxa have turned out to have genetically promiscuous mating systems.

There have been a number of hypotheses proposed to account for the variation in rates of EPF in behaviorally monogamous species, and they generally include characteristics such as nesting density or mate guarding ability (reviewed in Westneat et al., 1990), sexual dimorphism (Møller and Birkhead, 1994), nesting synchrony (Westneat et al., 1990; Birkhead and Møller, 1992; Stutchbury and Morton, 1995), variation in male fertility (Sheldon, 1994), and habitat occludedness (Bereson et al., 1995). Almost certainly a multivariate approach will be necessary when assessing the contributions of various factors to EPF rate, and phylogenetic nonindependence should not be ignored. Only a small number of socially monogamous mammal species have been tested with molecular methods. Data from allozymes (Foltz, 1981) and DNA fingerprinting (Ribble, 1991) revealed no EPFs in *Peromyscus polionotus* and *P. californicus,* respectively, while an allozyme study on a third species, *P. leucopus,* showed direct evidence for multiple paternity (Xia and Millar, 1991).

Polygyny/Promiscuity

Most fish, amphibians, reptiles, mammals, and some birds fall into the categories wherein males typically mate with more than one female, while females do not (polygyny) or do (promiscuity or polygynandry) mate with more than one male. These mating systems are somewhat broadly defined and can include variants such as simultaneous and serial polygyny, harem polygyny, multi-male groups/communal breeders, scramble mating, aquatic spawning, and lek mating. In addition, promiscuous mating systems, in particular, can be more difficult to assess with molecular methods than monogamous or polygynous systems, primarily because there can be many more individuals to survey to exclude as parents. Thus "inclusion" of parentage is usually the priority for studies involving multiple males, unlike monogamy, in which parentage exclusion is the first priority.

Examples of classical polygyny that have been assessed by molecular methods include studies of red deer (*Cervus elaphus;* Pemberton et al., 1992), threespine sticklebacks (*Gasterosteus aculeatus;* Rico et al., 1992), red-winged blackbird (*Agelaius phoenecius;* Gibbs et al., 1990; Westneat, 1993), bobolink (*Dolichonyx oryzivorous;* Bollinger and Gavin, 1991), corn bunting (*Miliaria calandra;* Hartley et al., 1993), and harem-polygynous fur seals (*Arctocephalus* spp.; S. Goldsworthy

et al., *unpublished data*). Both monogamous and polygynous corn buntings showed no evidence of EPF, whereas the other taxa all had significant rates of EPF. Remarkably, both red-winged blackbird studies revealed nearly identical rates of extra-pair fertilization (28% and 24%), but Gibbs et al. (1990) found that apparent reproductive success did not correlate with realized (or actual) reproductive success, while Westneat (1993) did. In the red deer and both red-winged blackbird studies total reproductive success of males was determined, and overall variance in reproductive success was greater than that predicted from behavioral data alone.

Another common assessment involving polygyny or promiscuity is of paternity in multi-male groups. Mammalian examples include primates (e.g., Pope, 1990; De Ruiter and van Hooff, 1993; Morin et al., 1994; S. Loew et al., *unpublished data*), lions (Packer et al., 1991), mongooses (Keane et al., 1994), and whales (Amos et al., 1991, 1993). Questions subject to DNA analysis included: Do all males in a group mate, or only dominant ones? Does relatedness of males affect their likelihood of paternity? In the primate, lion, and mongoose studies, all or nearly all paternity could be ascribed to males within the group, and multilocus DNA fingerprinting or allozyme (Pope, 1990) methods were generally adequate to resolve parentage. In all but one of the above studies the dominant (Pope, 1990) or high-ranking males obtained nearly all reproductive success. In lions and mongooses dominant males shared paternity primarily with *unrelated* high-ranking subordinates, suggesting match to models of power-sharing. However, in pilot whales (*Globicephala melas*), Amos et al. (1991, 1993) surprisingly found, from both multilocus fingerprinting and microsatellite amplification analyses, that virtually no offspring fetuses analyzed could have been fathered by males present in the pod at the time of capture. Amos et al. inferred that males remain in their natal pods with their families, mate only with unrelated females in other pods, but do not mate with the related females in their own pod.

Avian examples generally involve cooperatively breeding species. Researchers have used molecular methods to assess mating systems within natural populations of a variety of species, including woodpeckers, bee-eaters, fairy wrens, stripe-backed wrens (see Table 1 of Haig et al., 1994; Mulder et al., 1994), rallids (e.g., Jamieson et al., 1994; Lambert et al., 1994), and cuckoos (e.g., Quinn et al., 1994). Brood parasites such as cowbirds and cuckoos often represent a special case among birds in that both parents need to be identified with molecular methods. Hahn and Fleischer (1995) found evidence with multilocus DNA fingerprinting that some female brown-headed cowbirds associate with their own juvenile offspring at feeding sites.

Polyandry

Strict polyandry is a relatively rare social mating system and most applications of molecular methods have been conducted on polyandrous birds such as the dunnock (Burke et al., 1989), the Galapagos hawk (Faaborg et al., 1995), and the spotted sandpiper (*Actitis macularia;* Oring et al., 1992). In Galapagos hawks, Faaborg et al. (1995) used multilocus fingerprinting to confirm that offspring of a female were

fathered in an egalitarian manner by most or all of the males in a group. Oring et al. (1992) provided some of the most compelling data for long-term sperm storage in a natural population. Female spotted sandpipers in Minnesota lay clutches serially for males to incubate. Males compete to be the incubator for a female's first clutch of the season, in spite of no observed fitness benefits accruing from being first. Eggs from first clutches only rarely have evidence of EPF, but eggs from later clutches show as high as 14% EPF, nearly all of which cannot be excluded from a female's prior mates. Many of these eggs could only have been fertilized by stored sperm because some previous mates disappeared from the study site long before the eggs were laid. This discovery, like many other examples presented above, could not have been made without the use of highly variable and reliable genetic markers.

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