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POLYMERASE-CHAIN-REACTION (PCR) ANALYSIS OF MICROSATELLITES—A NEW APPROACH TO STUDIES OF GENETIC RELATIONSHIPS IN BIRDS

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ABSTRACT.—Genomic DNA libraries of the Barn Swallow (*Hirundo rustica*) and the Pied Flycatcher (*Ficedula hypoleuca*) were screened for the presence of dinucleotide microsatellite repeats. Two thymine-guanine repeats, "$(TG)_n$," and two thymine-cytosine repeats, "$(TC)_n$," were isolated and sequenced from the two species, respectively. Polymerase-chain-reaction (PCR) analysis of 25 unrelated Barn Swallows and 10 unrelated Pied Flycatchers revealed 3 to 15 alleles per locus and heterozygosities in the range of 0.46 to 0.89. Mendelian inheritance was confirmed for all four loci in 10 Pied Flycatcher and 2 Barn Swallow families comprising a total of 240 meioses. The occurrence of nonparental alleles in offspring from two Barn Swallow families was consistent with extrapair fertilization as revealed by a parallel DNA-fingerprinting analysis. A DNA amount approximately corresponding to 0.01 μl blood was used for PCR analysis. DNA was also prepared from feathers and approximately 1% of a preparation from a single remex or rectrix was sufficient for PCR amplification. One of the primer pairs from the Barn Swallow also amplified a polymorphic locus in the House Martin (*Delichon urbica*) and in the Bank Swallow (*R. riparia*). *Received 3 October 1991, accepted 16 June 1992.*

DURING THE last 10 to 15 years, molecular genetic techniques have opened new perspectives in biological research. Several ornithological fields provide good illustrations. For example, DNA-DNA hybridization has clearly contributed to avian systematics (Sibley and Ahlquist 1990), polymorphic patterns of mitochondrial DNA (mtDNA) have aided in differentiation studies of species and populations (Kessler and Avise 1985), and polymorphisms of random genomic DNA segments have been used to resolve questions about parentage (Quinn et al. 1987). Perhaps most spectacularly, DNA fingerprinting, using minisatellite (Burke and Bruford 1987, Wetton et al. 1987) or synthetic simple-repeat probes (Ellegren 1991a), has proved to be the most sensitive method for determining the genetic relationships of individuals in a population (Burke 1989). Moreover, the isolation of locus-specific minisatellite probes has simplified such studies (Gyllensten et al. 1989, Hanotte et al. 1991).

Recently, a new type of genetic marker, microsatellites, has received considerable attention in genome analyses (Litt and Luty 1989, Tautz 1989, Weber and May 1989). Microsatellites are tandem stretches of mono-, di-, tri- or tetranucleotide sequence motifs that frequently occur as randomly dispersed repetitive elements in eukaryotic genomes. The most abundant dinucleotide variant in mammals, thymine-guanine repeats "$(TG)_n$," shows copy numbers in the order of 10^5 (Hamada et al. 1984a), on average occurring every 30 kb (kilobase pairs) in the human genome (Stallings et al. 1991). Considering all possible simple motifs there is probably one microsatellite every 6 kb in man (Beckmann and Weber 1992). Although their evolutionary conservation indicates a functional or structural significance, there is yet no conclusive evidence for suggested roles as hot-spot regions for recombination (Pardue et al. 1987), gene regulators (Hamada et al. 1984b), or sequences stimulating packing and condensing of chromosomes (Stallings et al. 1991).

The allelic variability at microsatellite loci is usually high, with heterozygosities approaching 90%. As for minisatellites, the polymorphism is due to varying number of repeat units (typically in range of 10–50), which can be revealed by *in vitro* amplification of the DNA segment of interest using the polymerase chain reaction (PCR; Saiki et al. 1988) and subsequent gel electrophoresis (reviewed by Beckmann and Soller 1990, Weber 1990, Litt 1991). In humans, microsatellites have become invaluable tools serving as genetic markers in the human genome (HUGO) project, for instance facilitating

the localization of genes with impacts on genetic diseases (Froguel et al. 1992, Goto et al. 1992). The hypervariable nature of these markers also makes them highly suitable for identity or parentage testing (Edwards et al. 1991, 1992, Ellegren et al. 1992).

In addition to work on humans, microsatellites have been analyzed in the mouse (*Mus musculus*; e.g. Love et al. 1990), some domestic animals (e.g. Fries et al. 1990, Crawford et al. 1990, Ellegren et al. 1992, Winterø et al. 1992), and whales (Tautz 1989, Schlötterer et al. 1991). In this study, I report the molecular cloning of avian microsatellites. Four dinucleotide repeats were isolated from the Barn Swallow (*Hirundo rustica*) and the Pied Flycatcher (*Ficedula hypoleuca*), and the polymorphism was investigated by PCR amplification of DNA from unrelated individuals and families.

Methods

Clone isolation.—The procedure followed Ellegren et al. (1992). Shortly, size-fractionated (200–500 base pairs) genomic libraries were constructed from Barn Swallow DNA digested with *Alu*I, *Hae*III and *Hin*fI in the plasmid vector pBluescript SKII+, and from Pied Flycatcher DNA digested with the same enzymes in the phage vector M13mp18. The libraries were transferred to nylon membranes and screened with radioactively labelled polynucleotide $(TG)_n$ and poly(thymine-cytosine), $(TC)_n$, probes for the presence of microsatellite-containing clones. Positive clones were isolated and sequenced.

Polymerase-chain-reaction analysis.—Microsatellite-flanking PCR primers were synthesized on the basis of the derived sequence information. Primer labelling (end-labelling with gamma-^{32}P-dATP) and PCR reagents were as previously described (Ellegren et al. 1992). PCR (10 μl) was carried out with approximately 50 ng template DNA and 2 pmol primer (one primer labelled) in small Eppendorf tubes or in microtiter plate wells using a thermal cycler (Coy Laboratory Products, Inc., or Techne) in the following way: 1 cycle of 95°C for 2 min, 55°C for 30 s and 72°C for 1 min; 25 to 29 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 1 min. After the final cycle, a prolonged extension step of 5 min was added.

I mixed 1 μl of the product from the amplification reaction with 95% formamide and dye, heated to 75°C and separated in 6% denaturing polyacrylamide gels (standard "sequencing" gels). After electrophoresis, gels were dried and amplification products visualized by autoradiography for 2 to 24 h. The sizes of alleles were determined by electrophoresis of an accompanying sequence reaction. Amplification of the cloned alleles also aided in size determination and served as a positive control. A negative control involving PCR master mix without added DNA was always included.

DNA preparation.—DNA was either prepared from venous blood or from single feathers. In the former case, 1 μl blood (frozen in EDTA capillars and newly thawed) was diluted to 1 ml with water and incubated at room temperature for 15 min. After centrifugation at 10,000 × *g* for 2 min, the supernatant was carefully removed (leaving about 25 μl) and discarded. A 5% Chelex (Biorad) solution was added to a final volume of 200 μl and incubated at 56°C for 15 min. The mixture was vortexed for 10 s, boiled for 8 min and then vortexed again. Following centrifugation as above, 1 to 5 μl of the supernatant were taken to PCR and the remainder frozen. Before being used again, the sample was vortexed and centrifugated. Chelex is a chelating styrene divinylbenzene resin that forms multicoordinated complexes with bivalent metal ions that otherwise may catalyze the breakdown of DNA during boiling (cf. Ellegren 1992).

Various types of feathers (primaries, secondaries and remiges) were used for DNA preparation. Feathers were collected from adult birds one to three weeks before analysis and were stored at room temperature between when they were collected and analyzed. Approximately 5 mm of the root part of a single shaft was cut into a few pieces with a surgical blade and transferred to 200 μl 5% Chelex and 2.5 μl proteinase K (8 mg/ml). The mixture was incubated at 56°C for at least 4 h, and an additional portion of proteinase K was added one-half way through the incubation step. After this treatment the sample was handled as above (vortexing, boiling, vortexing and centrifugation) and 1 to 5 μl was taken to PCR. When handling and preparing the feathers the possible risk of contamination was carefully avoided by using a separate surgical blade for each feather. Unless otherwise stated, data will refer to analysis of DNA prepared from blood.

Results

Isolation of microsatellites.—Screening of about 2,000 recombinant plasmid clones from the Barn Swallow library with $(TG)_n$ and $(TC)_n$ probes yielded two TG-positive clones (STG1 and STG4). Sequence analysis of these clones revealed one perfect stretch of 12 TG repeats and one compound structure of GA-GT repeats. Screening of approximately the same number of phage clones from the Pied Flycatcher library yielded two TC-positive clones (PTC2 and PTC3). These consisted of eight and 12 perfect TC-repeats, respectively, and were derived from different loci. Primers (21 or 24 bp) for PCR amplification were synthesized according to the DNA sequences flanking the repeat regions,

TABLE 1. Repeat structure and primer sequences at two Barn Swallow (STG1 and STG4) and two Pied Flycatcher (PTC2 and PTC3) microsatellite loci.

Locus	Primer A (5'-3')	Repeat structure	Primer B (5'-3')
STG1	TGAACAATGAGAGAACTGATGCAA	$(GT)_{12}$	CATCACTCAGATGAGAAACTGGAA
STG4	CATCAAGAGAGGGATGGAAAGAGG	$(GA)_6(GT)_7A(TG)_3$	GAAAAGATTATTTTTCTTTCTCCC
PTC2	TGATCGAAAGACCTGTAAGAT	$(TC)_8$	ATCAGCGTTAGACCAATACTCTTA
PTC3	GTGTTCTTAAAACATGCCTGGAGG	$(CT)_{12}$	GCACAGGTAAATATTTGCTGGGCC

following recommendations given by Luty et al. (1990). Repeat structures and primer compositions are indicated in Table 1.

PCR amplification and variability.—PCR analysis of Barn Swallow and Pied Flycatcher DNA using primers designed to flank the microsatellites gave amplification products of varying but approximately expected size. No individual exhibited more than two intense bands consistent with a locus-specific amplification. Each "main" band was associated with one or a few fainter bands appearing immediately below (i.e. being shorter than the main product; Fig. 1). These "extra" bands seem to be inevitable artifacts in PCR amplification of dinucleotide microsatellites, and are presumed to be the result of slippage events as the *Taq* polymerase synthesizes the complementary strand of the repeat region (e.g. Litt and Luty 1989, Weber and May 1989, Luty et al. 1990). Cloning experiments have demonstrated that extra bands lack one or a few repeat units (Luty et al. 1990, Riess et al. 1990), a situation supported by the fact that extra bands occur with multiples of the length of the repeat unit. The possibility that the extra bands would reflect a genetic heterogeneity at the genomic level can be ruled out by noting that amplifications of cloned microsatellite alleles display extra band patterns identical to genomic amplifications (e.g. Ellegren et al. 1992).

Analysis of unrelated individuals revealed extensive polymorphism at all four avian microsatellite loci (e.g. Fig. 1). Ten Pied Flycatchers showed four different alleles at the PTC2 locus (expected heterozygosity [H_{exp}] = 0.49) and eight alleles at the PTC3 locus (H_{exp} = 0.76). A more extensive screening of 25 unrelated Barn Swallows revealed heterozygosities of 0.89 and 0.46 at the STG1 and STG4 loci, respectively. As many as 15 alleles were found at STG1. The observed allele frequencies at the Barn Swallow loci are summarized in Table 2.

The extra bands of one allele sometimes were in juxtaposition to the other allele (e.g. heterozygous individuals 2 and 6 in Fig. 1 show allele size differences corresponding to one and two repeat units, respectively). This situation did not prevent scoring since the occurrence of an adjacent and slightly shorter allele was recognized as a strong signal caused by the combined radiation from an extra band and a main band.

Inheritance pattern.—To investigate the inheritance pattern of the avian microsatellites, they were applied to families previously analyzed by DNA fingerprinting (data and DNA kindly provided by Håkan Tegelström, Department of Genetics, Uppsala University). In 10 Pied Flycatcher (n = 52 offspring) and 2 Barn Swallow (n = 8 offspring) families, DNA fingerprinting using the Jeffreys 33.15 probe (Jeffreys et al. 1985) had failed to detect any illegitimate offspring (the probe reveals highly variable DNA fingerprints in the two species; Gelther et al. 1992, Smith et al. 1991). In microsatellite amplifications of these families, every offspring allele at the PTC2, PTC3, STG1 and STG4 loci was also present in either of the parents; for homozygous offspring, the allele was found in both parents (Fig. 2). Data were consistent with every parent transmitting one allele to each offspring and, thus, autosomal inheritance was indicated in a total of 240 meioses (208 for Pied Flycatcher and 32 for Barn Swallow). However, sex linkage could not be completely excluded for STG1 since offspring were unsexed and the female appeared to be homozygous in both families.

Detection of extrapair fertilization.—Given the assumption that microsatellites are stably inherited, the hypervariable nature of these markers suggests that they could be useful for detecting extrapair paternity or intraspecific brood parasitism in birds. In two Barn Swallow families, DNA fingerprinting had demonstrated two (of three) and four (all) illegitimate offspring, respectively (Tegelström unpubl. data; extrapair paternity is known from previous DNA studies of the Barn Swallow [Smith et al. 1991]). An analysis of the segregation pattern at the STG1 and STG4 microsatellite loci in these two

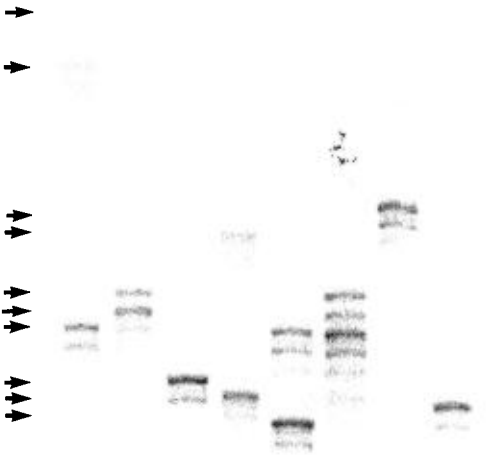

Fig. 1. PCR amplification of the STG1 microsatellite locus in eight unrelated Barn Swallows. Alleles indicated by arrows.

TABLE 2. Estimated allele frequencies at the Barn Swallow STG1 and STG4 microsatellite loci (n = 25 individuals).

Allele size (bp)	Frequency
STG1	
200	0.02
228	0.05
230	0.16
232	0.11
234	0.05
236	0.04
238	0.07
240	0.11
242	0.20
244	0.02
252	0.02
256	0.05
260	0.05
262	0.07
268	0.02
STG4	
135	0.14
137	0.70
139	0.16

families revealed nonparental alleles in all six illegitimate offspring. One of the female alleles was present in all offspring, whereas the two males apparently did not transmit alleles to any offspring (Fig. 3).

The presence of nonparental microsatellite alleles may either be due to false parentage and/or to mutation. Here, false parentage was known from previous experiments and, thus, it is reasonable that the observed microsatellite patterns resulted from it. The lack of male-specific alleles suggests that all offspring in these families were the outcome of cuckoldry. Since the mutation rate at microsatellite loci seem to be significantly lower than at minisatellite loci (Kwiatkowski et al. 1992), the presence of a nonparental microsatellite allele may in practice be used as a marker of false parentage (see Discussion).

Analysis of feathers.—As an alternative to blood sampling, feathers were evaluated for their suitability in serving as a DNA source for PCR analysis. By a simple protease/boiling procedure, DNA was prepared from about 5 mm of the root part of single remex and rectrix from the Barn Swallow. Using approximately 1% of a preparation as starting material in PCR reactions, autoradiography signals of suitable strength were obtained after overnight exposure (Fig. 4). The strength of the signals varied between individuals suggesting different DNA yields from the preparations.

Cross-species analysis.—Primers flanking the Barn Swallow microsatellites were also applied to DNA prepared from the House Martin (*Delichon urbica*) and from the Bank Swallow (*R. riparia*; in both cases, feather preparations used). The annealing temperature in the PCR reaction was decreased to 45°C to allow a certain mismatch between the primers and the heterogenous sequences. Whereas no specific amplification product was obtained at STG1, the STG4 locus yielded a distinct two-allele polymorphism in six unrelated individuals of both species (data not shown). The House Martin and Bank Swallow alleles were identical to two of the Barn Swallow alleles, but the 135 bp allele that showed a frequency of just 0.14 in the Barn Swallow was present in 10 of 12 chromosomes in the other species. Whether these shared alleles reflect a common ancestry of alleles or are due to a *de novo* generation of alleles of the same size cannot be resolved.

DISCUSSION

Previous hybridization studies have indicated the frequent occurrence of dinucleotide repeats in avian genomes (Hamada et al. 1982),

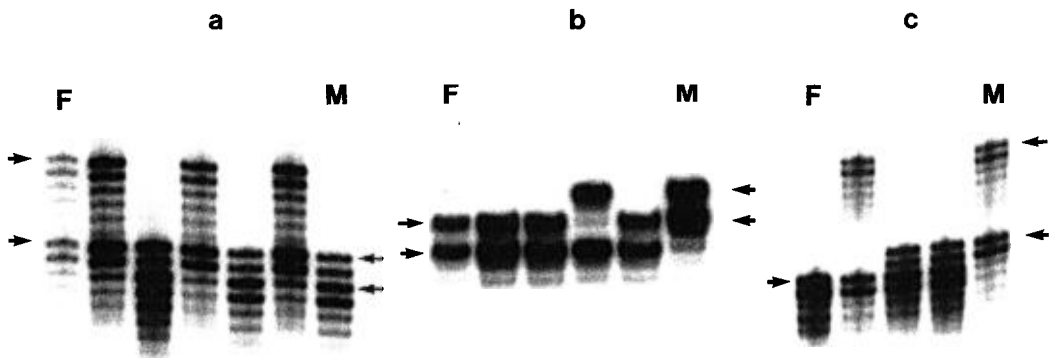

Fig. 2. PCR amplification of avian microsatellite loci in families with parentage confirmed by DNA fingerprinting. F is female, M is male, arrows on each side indicate alleles of female and male origin, respectively. Analysis of: (a) a Pied Flycatcher family at PTC3 locus, (b) a different Pied Flycatcher family at PTC2 locus, and (c) a Barn Swallow family at STG1 locus.

but this constitutes the first report of the molecular cloning of such sequences. Present data show that microsatellites in the Barn Swallow and the Pied Flycatcher display a considerable degree of polymorphism (i.e. hypervariability), a fact suggesting that this type of marker could be useful for monitoring the genetic variability in populations as well as for investigating close genetic relationships (e.g. parentage).

In the latter case an important prerequisite is that alleles are stably inherited without a significant element of mutation to new length al-

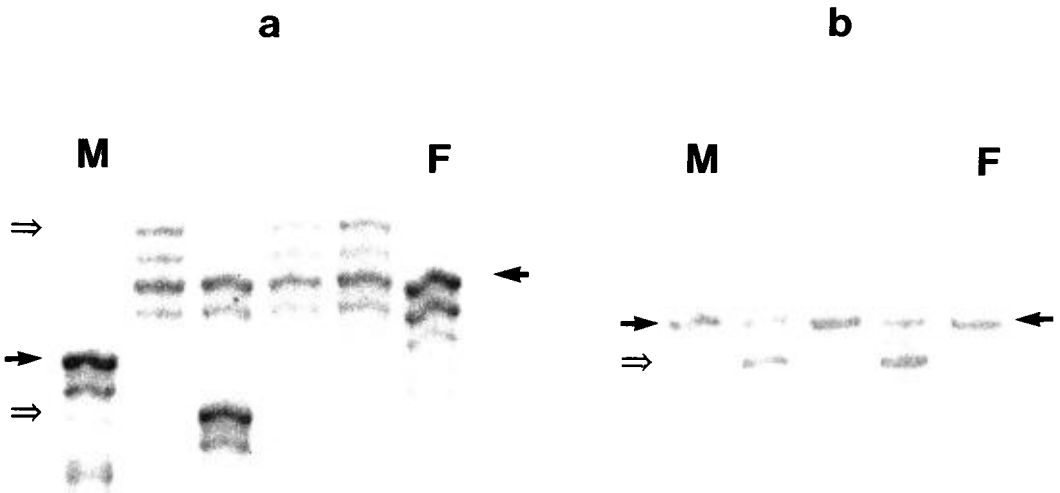

Fig. 3. Detection of extrapair fertilization in two Barn Swallow families with aid of PCR-analyzed microsatellite polymorphisms. M (male) is putative father, F (female) is putative mother, solid arrows on each side refer to male and female alleles, respectively, and open arrows indicate nonparental alleles. (a) Analysis of a Barn Swallow family at STG1 locus, and (b) analysis of a different family at STG4 locus. DNA fingerprinting had revealed that all offspring in family *a* and offspring 1 and 3 in family *b* were illegitimate.

leles. I found no mutation among 240 meioses (with parentage confirmed by DNA fingerprinting), indicating that mutation events should be rare at these dinucleotide repeats. In humans, an extensive body of literature on microsatellite stability has emerged in recent years. Thus far, only a single report describes length mutations (two) at dinucleotide microsatellite loci (Kwiatkowski et al. 1992). These mutations were found in a sample of 4,480 meioses leading to an estimated mutation rate of 4.5×10^{-4} per meiosis. This figure should however be related to numerous reports which have failed to detect any spontaneous mutation to new length alleles. For example, completely stable inheritance has been found in samples of a few hundred to thousands of meioses, as investigated by Dracopoli and Meisler (1990), Love et al. (1990; mouse data), Kwiatkowski et al. (1991), Morral et al. (1991), Oudet et al. (1991), Petersen et al. (1990), Richards et al. (1991), Small et al. (1991), Todd et al. (1991; mouse data), Weber et al. (1991), Decker et al. (1992), Edwards et al. (1992), Fornage et al. (1992), Froguel et al. (1992), Goto et al. (1992), Hazan et al. (1992), and Wilkie et al. (1992). Moreover, documented data sets with 20 to 100 stable meioses exceed 100 (for instance, see recent issues of *Nucleic Acids Research*). Altogether, there may be in the order of 50,000 human microsatellite meioses published and still only two mutations have been found (i.e. mutation rate of about 10^{-4} to 10^{-5}). Clearly, this gives evidence for a significantly more stable situation than the high mutation rate characterizing minisatellite loci (Jeffreys et al. 1988; reported rates of 1.1×10^{-2} to 2×10^{-3} in birds, Burke and Bruford 1987, Westneat 1990). Furthermore, observations of very strong linkage disequilibrium between microsatellite loci and adjacent polymorphic markers are likely to reflect stability even on an evolutionary time scale (Chehab et al. 1991, Oudet et al. 1991).

Although it remains to be shown, using pedigree material with parentage confirmed by DNA fingerprinting, that avian microsatellites are as stable as in humans, it is difficult from a molecular point of view to see why they would behave in a different manner. Thus, it is likely that avian microsatellites will serve as effective tools for the detection of extrapair parentage.

Practical aspects of microsatellites in parentage testing.—With regard to parentage testing, an analysis of the polymorphism at one microsatellite locus will not of course be as effective as

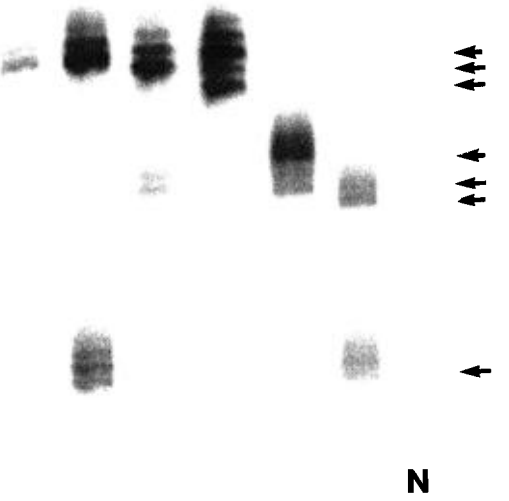

Fig. 4. PCR amplification of STG1 microsatellite locus in six unrelated Barn Swallows using DNA prepared from single feathers. N is a negative control with PCR master mix, but without added DNA. Arrows refer to interpreted alleles.

hybridization with one multilocus DNA fingerprinting probe. However, several microsatellite loci can be analyzed simultaneously in multiplex PCR reactions and also separated in the same gel (Luty et al. 1990, Edwards et al. 1991, Ellegren et al. 1992, Hazan et al. 1992). Applying Jamieson's (1965) formulae for exclusion power, a combination of five microsatellite loci each having a heterozygosity of about 0.8 yields an efficiency of 0.98 to 0.99 for detection of false paternity (given known maternity). The same power is reached with only three loci having a polymorphism corresponding to the Barn Swallow STG1 locus. The efficiency given by this combination of markers would imply that, if there is a match between the alleles of an offspring and its putative father, paternity will be incorrectly assigned in 1 or 2 cases of 100. However, as soon as the alleles of an offspring and either of its putative parents do not match, an illegitimate mating can almost certainly be concluded due to the apparent stability of the systems. To properly distinguish between extrapair fertilization and intraspecific brood parasitism in cases when offspring lack alleles from their putative father but display alleles present in their putative mother, probability estimates established on the basis of allele frequencies in the population need to be considered.

If the extreme variability produced by DNA fingerprinting can be dispensed with, locus-specific systems offer the general advantage of a simpler genetic interpretation. This is further accentuated in microsatellite analysis since: (a) alleles can be distinguished (which may not be the case in agarose electrophoresis of large minisatellite alleles of similar size; Devlin et al. 1991) and defined by size, thus allowing computerization and the exchange of data or collaborative studies between laboratories; (b) recent advances with "sequencing instruments" and software designed to registrate microsatellite polymorphisms permit an automated and nonradioactive assay for these markers (Edwards et al. 1991); and (c) even with manual handling the time schedule from DNA preparation to result need not be longer than 24 h.

An obvious limitation, however, to the practical value of microsatellite polymorphisms is that primers designed for one species will only amplify a corresponding locus in closely related species. This has been shown for some mammals (Moore et al. 1991), including some whales (Schlötterer et al. 1991). In the latter case, microsatellite-flanking primers could amplify heterologous sequences in species thought to have been separated for 20 million years. The Barn Swallow STG4 primers also amplified a polymorphic locus in the House Martin and the Bank Swallow (estimated split 5–10 million years ago; Sibley and Ahlquist 1990), showing that amplification of microsatellites in closely related species holds true for birds as well.

General considerations of PCR analysis.—PCR is most efficiently performed with sequences shorter than 1 to 2 kb. Thus, microsatellites are more adaptable to PCR than minisatellites due to their smaller size. Regarding the minute template-DNA requirements, I used a DNA amount approximately corresponding to a blood volume of 0.01 μl for each PCR reaction. Using conventional blood-sampling techniques, this would imply that the DNA amount will not limit the possibility of performing repeated DNA analyses. Moreover, the demonstration that feathers may be used for DNA typing adds a new perspective to the sampling routines in genetic studies of birds. First, collecting feathers is obviously easier than collecting blood. Second, since feathers may be stored at ambient temperature before DNA preparation, there is no need for freezing facilities when sampling or transporting specimens. Third, feather analysis allows samples to be collected even when birds are absent. For example, feathers collected at nests or from areas where birds have been molting are likely to give necessary DNA yields. Since about 1% of the preparations from single remiges and rectrices was sufficient for PCR analysis, smaller feathers (e.g. coverts or body feathers) also would probably be suitable for analysis.

One promising possibility with feathers as a DNA source and PCR as a hypersensitive detection method is to DNA type specimens of birds in museums. Although old DNA generally is degraded to some extent, relatively short microsatellite regions may be intact (cf. Hagelberg et al. 1991). Using the microsatellite loci described in this study, I have typed more than 100-year-old Barn Swallows and Pied Flycatchers stored as museum skins (Ellegren 1991b, 1992). The obvious benefit of such an approach is that the genetic variability may be monitored over time. This can have major significance when designing conservation strategies following, for example, an extensive population bottleneck. Furthermore, this approach would allow the genetic analysis of extinct species (cf. Thomas et al. 1989).

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