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Application of DNA-Markers to Study the Ecology and Evolution of Raptors

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ABSTRACT

Several molecular techniques have been developed recently which allow the study of ecology and evolution of birds in much greater detail and precision than ever before. Depending on the level of relatedness ("evolutionary window"), different markers can be applied: For paternity studies (mating systems), DNA fingerprinting with multilocus-probes (minisatellites, oligonucleotides), singlelocus probes (singlecopy genes), and PCR methods such as RAPD (Randomly amplified polymorphic DNA) or microsatellite-PCR are the methods of choice. The same methods can be used to identify individual birds; e.g. as an unequivocal marker for endangered or valuable animals kept in an aviary. For evolutionary and taxonomic studies, sequences of marker genes allow the reconstruction of phylogeographic and phylogenetic relationships between species, genera and families of birds. If population differences are to be analysed, then the mitochondrial D-loop region can be an appropriate marker. For relationships between species and genera, usually mitochondrial genes, such as the cytochrome b gene, are excellent markers. Higher order relationships are often difficult to assess and more conservative genes (e.g., rDNA) or DNA-DNA hybridisation would be more appropriate than mitochondrial DNA.

INTRODUCTION

It is not the intention of this article to cover all aspects of molecular markers or to provide recipes how to perform the various molecular techniques in the laboratory. Books which contain this information are Erlich (1989), Innis *et al.* (1990), Sambrook *et al.* (1989), Hillis *et al.* (1996), Hoelzel (1992), McPherson *et al.* (1991), Burke *et al.* (1991a), Zimmer *et al.*, (1993), Avise (1994), Wink & Wehrle (1994) and Weising *et al.* (1995). Rather, this article will focus on a

limited selection of techniques (which have been successfully employed in the author's laboratory), and intends to briefly explain their principles and to illustrate them with a few examples.

Principles of genetic variation

The genetic information is encoded by the sequence of the four DNA bases adenine (A), thymine (T), guanine (G) and cytosine (C). DNA exists as complementary double strands, in which A pairs with T and G with C via hydrogen bonding. Genes are transcribed into mRNA which is translated into protein in the ribosomes. Animal genomes contain large regions (up to 98% of total DNA) of non-coding DNA, which are not translated into proteins. Among this "silent" DNA are repetitive DNA sequence elements (including mini- and microsatellites), pseudogenes or genes which had a function during early evolution but are now "switched off".

Prior to any cell division the DNA has to be copied, so each cell obtains a genome with identical DNA sequences. In analogy, a similar DNA replication occurs during meiosis. Since polymerases have proof reading mechanisms copy errors are extremely rare so that all members of a taxon should theoretically have more or less identical genomes. Environmental radiation (X-ray, UV, radioactivity), thermal stress or chemical reactions can modify individual nucleotides (e.g., depurination, deamination, reduction or oxidation). Since the complementary DNA strand usually still contains the correct information, most of these modifications are repaired by special "repair enzymes". Although the DNA polymerases hardly make a mistake during replication and the repair enzymes find most mistakes, nevertheless, mutations occur which are transferred to the daughter cell or if present in sperm or egg cells to the next generation. If these point mutations take place in non-coding DNA regions, they do not influence the fitness of the individual; i.e. these mutations will be transferred to the next cell or next generation and will accumulate over long time periods. The situation differs if mutations occur in genes encoding proteins. If such a mutation will change the genetic code, a new amino acid will be incorporated in the respective protein. Depending on the protein and the site of amino acid exchange, such a mutation can lead to defective or inactive proteins. Bearers of these traits have a reduced fitness and will be eliminated soon; therefore, these mutations will not accumulate but disappear through selection. If the amino acid exchange takes place at a less important site of the protein, these mutations may be maintained and transferred to the next generations. The most common situation are point mutations confined to the third codon position. Since the genetic code is redundant, a nucleotide substitution in the third codon position does not change the amino acid: therefore, this mutation is neutral from the point of view of fitness and selection. As a consequence, most nucleotide substitutions will occur in non-coding DNA regions and third codon positions

of coding genes; theoretically each individual should have its unique set of point mutations. Besides point mutations, a series of other mutations can occur in the genome, such as deletions, inversions or insertion of DNA sequences. Again, if these events take place in non-coding DNA regions, these mutations should have no influence on the fitness.

Usually, animals are diploid organisms with two alleles for each gene locus, one derived from the father, the other from the mother. During meiosis, a recombination of chromosomes takes place leading to a new and stochastic arrangement of the original paternal and maternal haploid genome. Because of mutational events (mentioned in the previous paragraph) and this recombination, each individual has unique DNA sequences, although the phenotype can be identical or at least very similar for individuals of the same population.

Depending on the "molecular marker" employed (Table 1), the genetic variability can be used to characterize and identify individuals, populations, species or phylogenetic relationships. Since non-coding DNA regions show more mutational variation, they are often used to identify individuals or to analyse the paternity within families. Mutations in protein coding genes are less common, but still present: In mitochondrial genes these mutations are up to 10 times more

Table. 1 Molecular methods to study genetic and phylogenetic relationships

Hierarchical level ("evolutionary window")	*Method of choice*
Identification of individuals	DNA Fingerprinting Microsatellite PCR
Parentage analysis	DNA Fingerprinting multi-/single locus probes RAPD Microsatellite PCR
Population genetics	Isoenzyme electrophoresis RFLP (mtDNA, PCR-products) SSCP and related methods Microsatellite-PCR Nucleotide sequences (D-loop)
Analysis of closely related species	Protein electrophoresis RAPD RFLP Nucleotide sequences (mtDNA)
Phylogeny reconstructions	DNA-DNA-Hybridisation Amino acid sequences Nucleotide sequences: mtDNA (< 20 mio years) rDNA (deep branches)

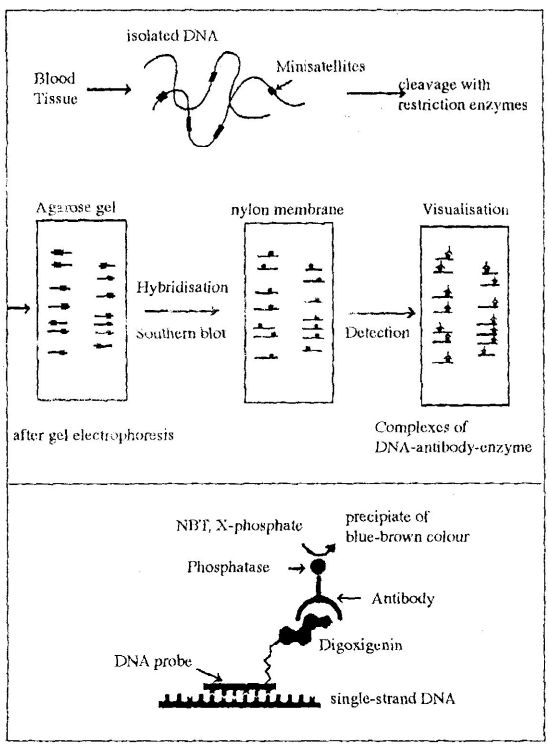
abundant than in nuclear genes. DNA sequences which reflect these mutations can be employed to reconstruct the evolutionary past, i.e. the phylogeny or phylogeography of species.

MOLECULAR METHODS TO STUDY GENETIC RELATIONSHIPS

DNA-FINGERPRINTING WITH DNA PROBES

Although many birds have monogamous pairbonds (Birkhead & Møller 1992) social monogamy sometimes differs from genetic monogamy; i.e. even in a truely monogamous species not all young in a nest are necessarily fathered by the male which cares for them (Birkhead & Møller 1992). This phenomenon was already suspected in birds seen to perform an "extra-pair copulation" (EPC) as this behaviour was called. Until recently it was quite difficult to prove that such an extra-pair copulation may lead to an "extra-pair fertilization" (EPF). The situation changed substantially when A. Jeffrey introduced the method of

Figure 1. Schematic illustration of DNA-fingerprinting with oligonucleotide probes and immunological detection.



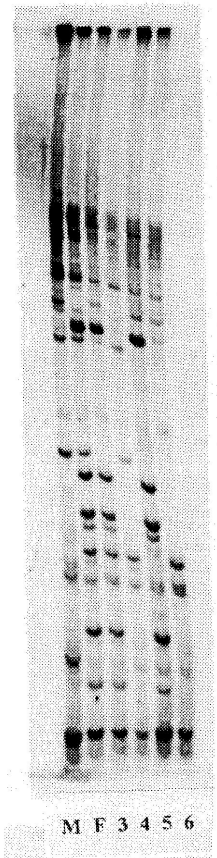


Figure 2. Illustration of a DNA fingerprint of *Falco peregrinus*

DNA was isolated from blood, digested with Hinf I and subjected to agarose gel electrophoresis. Hybridisation was directly carried out in the dried gel using a ^{32}P-labelled $(CAC)_6$ probe. Detection through autoradiography. Lane 1 and 2 prepresent male and female, lane 3 to 6 young falcons which were claimed by their owner to have been bred from 1 and 2; the fingerprint provides evidence that the 1 and 2 are probably the real parents of the young.

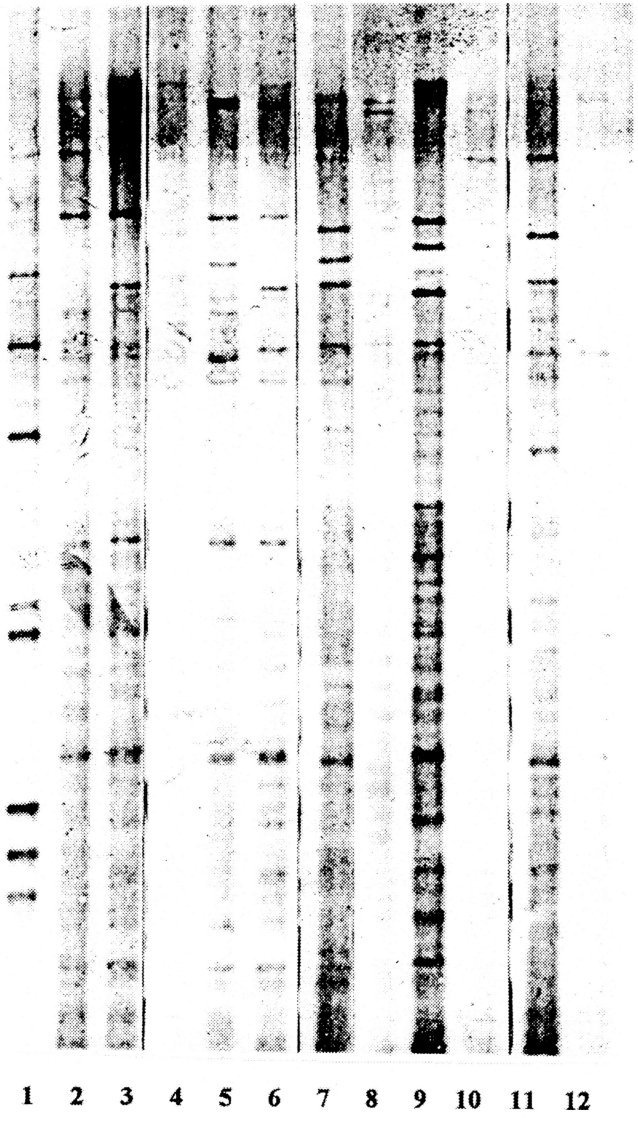
DNA-fingerprinting with DNA multilocus-probes in 1985 (Jeffrey *et al.* 1985 a,b). These DNA probes detect repetitive DNA (so-called "minisatellites") which is widely distributed in the genome of animals and plants (see above). The size and positition of these repeats are specific for each individual and provide a pattern of DNA bands, that can be regarded as a "fingerprint" (Figs. 1-3). Since a young obtains half its genome from the mother and the other half from the father, it also inherits the respective simple repeats; the analysis of the position of these simple repeats allows the identification of individuals and also their parentage. Theoretically, a young should share 50% of its minisatellite positions with those of its mother and the other 50% with its father (Avise 1994; Burke *et al.* 1991b; Hoelzel 1992; Weising *et al.* 1995).

In Figure 1 the basic procedure of DNA fingerprinting with DNA probes is schematically illustrated. After isolation of total DNA from blood or tissue samples, the DNA is cleaved by a restriction endonuclease, such as Hinf I, which produces DNA fragments of reproducible length. These DNA fragments

are then separated by size on an agarose gel by electrophoresis. After electrophoresis the DNA is transferred from the gel onto a nylon membrane (so-called "Southern blot") to which it is bound by heat treatment. The presence of minisatellites is determined with aid of multilocus DNA probes (which can

Figure 3. Illustration of a DNA fingerprint from *Torgos tracheliotus negevensis*

Birds are kept in captivity for a breeding programme (O. Hatzofe, Israel); aim of the study was to detect the degree of relatedness between birds to prevent inbreeding. Detection with immunological methods as outlined in Fig. 1.; Lane 1: size marker; lanes 2 to 12 are individual vultures



vary for different groups of birds). Two types of multilocus probes are distinguished: either long stretches of DNA (so-called "Jeffreys-probes") (Jeffreys *et al.* 1985a,b) or small oligonucleotides such as $(CAC)_5$ (Schäfer *et al.* 1988a,b). Under appropriate conditions the DNA probes hybridize with the single-stranded minisatellite DNA on the membrane. After eliminating unbound or unspecifically bound probes, the hybrid DNA must be visualized: Several procedures are commonly used:

1. If the DNA probe was labelled with radioactive phosphate (^{32}P-ATP), then the radioactive DNA bands can be detected by autoradiography (Fig. 2).
2. If radioactivity is to be avoided then immunological procedures can be employed, as explained in Fig. 1. In this case the oligonucleotide probe is coupled to the cardiac glycoside digoxigenin and digoxigenin-bearing hybrids can be detected by digoxigenin-specific antibodies. The antibodies are coupled to the enzyme alkaline phosphatase (Fig.1). If a chromogenic substrate such as 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) is added, then the phosphate groupe is hydrolyzed and a blue indol derivative is formed. The colour reaction is enhanced through nitrobluetetrazoliumchloride (NBT) which is reduced to a blue precipitate (Figs. 1, 3). Instead of a chromogenic substrate, also chemiluminescent substrates can be used (such as Lumigen PPD).

With all these methods, complex DNA profiles ("DNA fingerprints") are produced (Figs. 2,3) which can be analysed statistically (calculation of band-sharing coefficients; BSC). If birds are related such as in mother-child relationships, then the BSC is 0.5 or higher. If unrelated birds are compared this value is below 0.2. Whereas DNA-fingerprinting with oligonucleotide or Jeffreys probes produce complex band patterns, single-locus probes reveal much simpler patterns which are often much easier to interpret.

During the last decade a number of DNA fingerprint studies with birds have shown that EPFs do occur in socially monogamous species, such as in *Passer domesticus* (Burke *et al.* 1991b), *Merops apiaster* (Jones *et al.* 1991), *Prunella modularis* (Burke *et al.* 1989), *Taeniopygia guttata* (Birkhead *et al.* 1990), *Haematopus ostralegus* (Burke *et al.* 1991b), *Actitis macularia* (Oring *et al.* 1992), *Campyrhynchus nuchalis* (Rabenold *et al.* 1990), *Parus caeruleus* (Kempenaers *et al.* 1992), *Progne subis* (Morton *et al.* 1990), *Ficedula hypoleuca* (Lifjeld *et al.* 1991), *Passerina cyanea* (Westneat 1990), *Agelaius phoeniceus* (Gibbs *et al.* 1990), *Acrocephalus paludicola* (Schulze-Hagen *et al.* 1993), *Phalacrocorax aristotelis* (Graves *et al.* 1992), *Puffinus tenuirostris* (Austin *et al.* 1993) and *Emberiza schoeniclus* (Dixon *et al.* 1994). In other species, even in those living colonially and thus having theoretically a good chance for EPCs and EPFs, no EPF could be determined, such as in *Fulmarus glacialis* (Hunter *et al.* 1992), *Calonectris diomedea* (Swatschek *et al.* 1994), *Phylloscopus trochilus* (Gyllensten *et al.* 1990),

and *Phylloscopus sibilatrix* (Gyllensten *et al.* 1990).

So far, only few studies dealt with raptors. In colonially living Eleonora's Falcon (*Falco eleonorae*) EPFs could not be discovered (Swatschek *et al.* 1993, 1994). No or very low EPF rates were seen in other falcons such as the Merlin (*Falco columbarius*) (Sohdi 1991), the Lesser Kestrel (*Falco naumanni*) and the American Kestrel (*Falco sparverius*). Although only few species have been analysed so far, the results suggest that EPFs seem to be absent or rare in birds of prey.

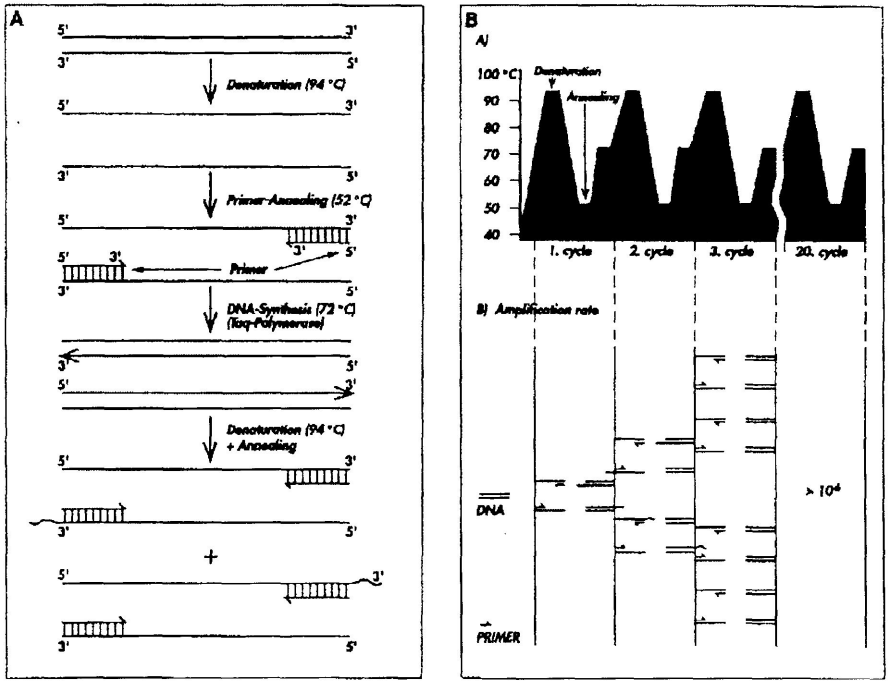
Besides paternity studies DNA fingerprinting can be employed to solve a large number of biological and forensic problems (see Avise 1994); for example, to determine the identity or non-identity of a raptor, or whether a bird kept in aviary was removed from the wild or bred in captivity (Fig.2). DNA fingerprinting has been frequently used in court trials as a testimony for criminal offences recently and was allowed as evidence for illegal trading or capturing of raptors and other birds. In breeding programmes, an interbreeding of closely related birds is not advisable. DNA fingerprinting can be a helpful method to identify birds which are not closely related, e.g., that show a higher degree of heterozygosity. Fig. 3 shows a DNA fingerprinting study with *Torgos tracheliotus* (carried out in the author's laboratory) that were kept in a breeding programme in Israel (O. Hatzofe, pers. comm.). The corresponding BSC clearly point out which birds are closely related.

PCR-METHODS

The development of the polymerase chain reaction (short PCR) has far-reaching implications for most fields of biology and medicine. Because of its speed and sensitivity the method has already revolutionized modern sciences, since PCR allows the rapid and specific amplification of any piece of DNA to such a degree that this DNA can be further processed by electrophoresis, sequencing or cloning (Erlich, 1989; Innis *et al.* 1990; Zimmermann *et al.* 1992; McPherson *et al.* 1991; Mullis *et al.* 1994; Wink & Wehrle 1994; Avise 1994).

The principles of PCR are schematically explained in Fig. 4: Total DNA is heated to >94 ¡C so that both strands become separated ("melting" or "denaturation" of DNA). If PCR primers are present (these are small pieces [usually 15 to 30 bases long] of single-stranded DNA) and the temperature is lowered to < 50°C, an annealing of PCR primers occurs with homologous regions of the target DNA (for which they have been tailored). Then the temperature is increased to 72°C at which Taq-polymerase from the thermophilic microorganism, *Thermus aquaticus*, has its reaction optimum. Starting at the respective PCR primers the corresponding DNA strands are copied. The reaction is terminated after 30 to 60 seconds by heating the vial to 94°C. Then a new cycle of annealing and synthesis follows, which is repeated *ca.* 30 times. Since the amount of amplified PCR product is

Figure 4. Schematic illustration of the polymerase chain reaction (PCR)
A. Principle of DNA dublication; B. principie of amplification.



doubled every cycle, about 109 copies of a piece of DNA are theoretically obtained after 30 cycles (Fig.4 B). Since primers or nucleotides become depleted during the last cycles and the Taq polymerase loses its activity, the reaction is no longer exponential in the end. Even 10^8 copies of DNA are enough to perform further analysis, such as electrophoresis, restriction analysis or sequencing.

The crucial element of PCR is the sequence of the PCR primers. The better their homology to the nucleotide sequence of the target DNA, the more efficient and specific the following PCR. This is especially important when PCR is used for diagnostics or the amplification of marker genes. A prerequisite is that the sequence of the target gene must be known.

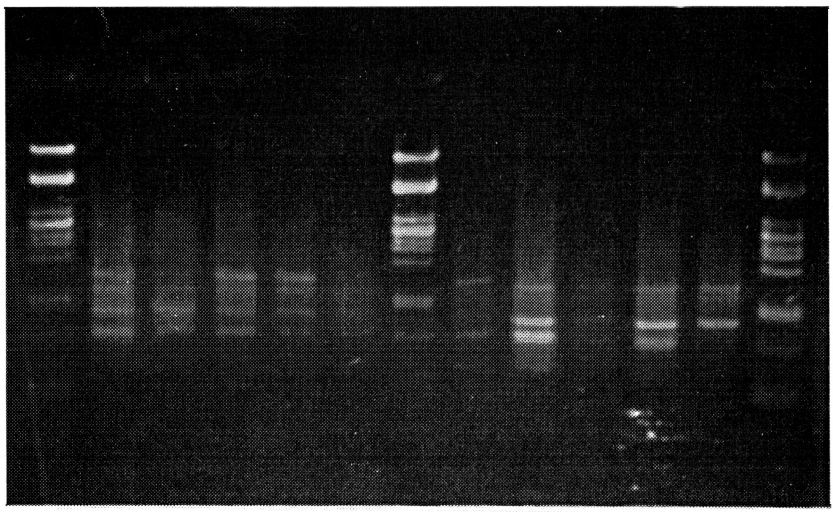
PCR will also revolutionize genetic studies of raptors and other birds and can be used to elucidate close genetic relationships, such as paternity (Table 1 and 2) as well as to reconstruct the phylogeny of genera, families or even phyla of organisms. In the following, a distinction is being made for PCR applications which will produce "fingerprints" and those which will lead to nucleotide sequences.

RAPD-PCR

In a few applications, PCR employs a single short primer of 10 bases length which does not bind to a specific gene but to several targets; the technique of RAPD (pronounced "rapid"; randomly amplified polymorphic DNA) produces

Figure 5. RAPD-PCR: Example from *Acrocephalus paludicola* from Poland

Lanes 1 and 14, size markers; Lane 2 female1, lanes 3 to 6 young of female 1; Lane 13 female 2, lanes 9 to 12 young of female 2.
PCR products are separated by agarose gel electrophoresis; visualisation of the ethidium bromide stained gel under UV.



complex patterns of DNA bands (Fig. 5), similar to the situation seen in DNA-fingerprinting (see above). Interpretation of these patterns follows a similar logic as in DNA fingerprinting (Hoelzel 1992; Avise 1994). RAPD-PCR has become important for several areas of population genetics (Tab. 1 and 2). Since the amount of target DNA or PCR primer concentrations and the annealing temperature influence the number of bands in the resulting fingerprint, this method requires a rigorous standardization and protocol to obtain reproducible results. Since this method demands much less DNA (about 10 -20 ng/analysis) than DNA fingerprinting with DNA probes, RAPD-PCR may be a method of choice if DNA supply is limited.

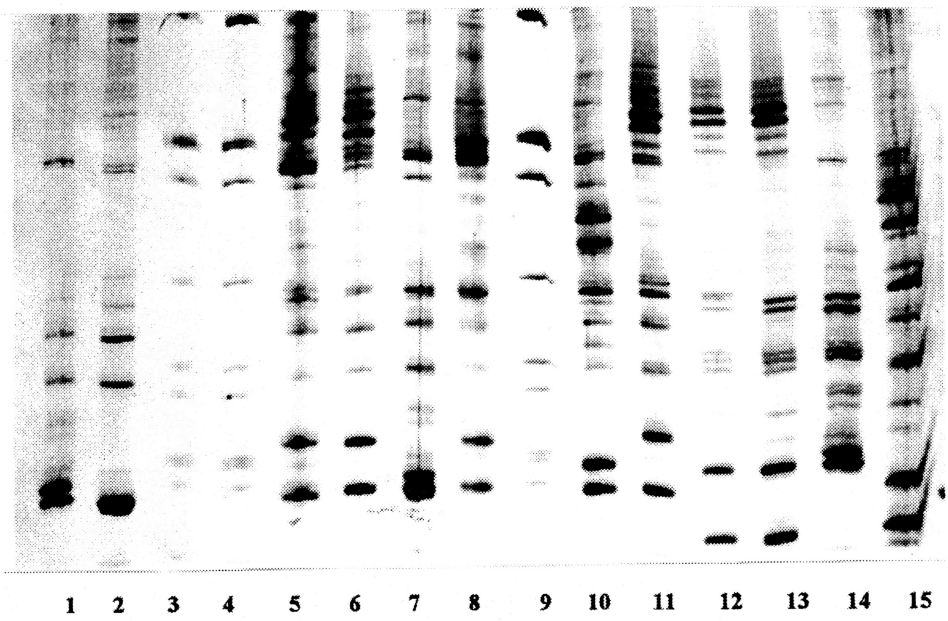
MICROSATELLITE-PCR (MS-PCR)

Paternity studies, identification of individuals

Repetitive DNA sequences are usually bordered by specific sequences in each species. If PCR primers are constructed accordingly (Hoelzel 1992), profiles of repetitive DNA sequences, such as "microsatellites" (their sequence is similar to the oligonucleotide probes used in DNA fingerprinting) are generated (Fig. 6). The interpretation of these DNA patterns (which are best resolved by polyacrylamid electrophoresis) follows a similar philosophy as "classic" DNA fingerprints (see

Figure 6. Microsatellite PCR: Example from *Acrocephalus paludicola* from Poland

Lanes 3,4 and 9 DNA size marker; lane 1 female 1, lane 2, 5 to 8 young from female 1, lane 10 female 2, lane 11 to 15 young from female 2.
PCR Products were separated by polyacrylamide electrophoresis; DNA bands were stained with silver nitrate.

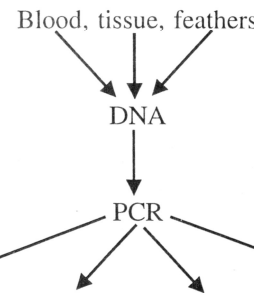


above). The disadvantage is, that MS-primers have to be developed for each group of organisms; strategies to find their sequences are outlined in Hoelzel (1992) and Hillis *et al.* (1996). The advantage of MS-PCR is its high degree of resolution and reproducibility and the fact that only small amounts (< 20 ng) of DNA is required for PCR. If DNA supply is limited (i.e. in hairs or feathers), than MS-PCR is a method of choice for a study of paternity and population genetics (Tab. 1 and 2). MS-PCR will probably replace many applications in which DNA fingerprinting with DNA probes are used today.

Molecular sexing

We have recently developed a modification of MS-PCR, by using "nested" PCR primers which amplify inner parts of micro- and minisatellites. These PCR primers are derived from oligonucleotide probes used for DNA fingerprinting. Only a single primer is employed as in RAPD-PCR. As shown in Fig. 7, complex fingerprints are generated. We have employed this strategy for molecular sexing of bird species, in which both species cannot be distinguished morphologically. For *Crex crex* we found a DNA band which was sex specific (Fig. 7); using this primer we could determine the sex of more than 300 birds of unknown sex (Wink

Table 2. PCR as a key technique in the study of the molecular ecology, genetics and evolution of birds

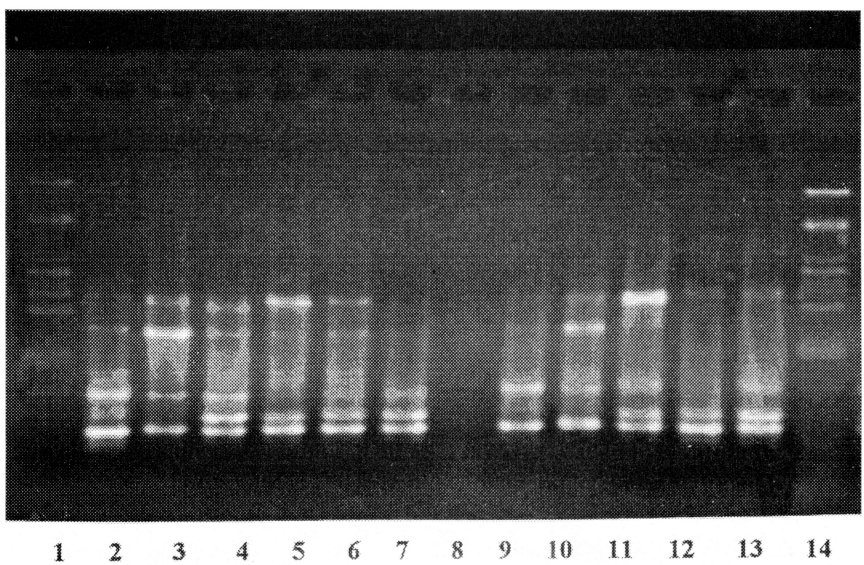


	RAPD-PCR	MS-PCR	SSCP	SEQUENCING
Identification of individuals	**	**	-	-
Paternity analysis	*	**	-	-
Molecular sexing	*	**	-	-
Population genetics	**	**	**	*
Phylogeny reconstructions	*	*	-	**

*= application possible; ** appropriate method; RAPD=Random amplified polymorphic DNA; MS-PCR= Microsatellite-PCR, SSCP=Single-strand conformation polymorphism; method to detect point mutations

Figure 7. Molecular sexing of *Crex crex* by MS-PCR

Lanes 1 and 14= molecular weight standard; lanes 2,3,9,10 females (sex was determined experimentally), lanes 4-7, 11-13 males; lane 8 = blank; the sex-specific DNA band is indicated by an arrow.



& Schäffer, unpublished). Currently, we use this method to sex vultures, bustards and other birds. This finding corresponds to earlier experiments from J. Epplen, who detected sex-specific DNA bands with oligonucleotide probes.

ANALYSIS OF NUCLEOTIDE SEQUENCES

If PCR primers are employed which amplify a single gene or DNA sequence only (Tab. 3), so that only a single PCR product is obtained, DNA sequencing is another option. DNA sequences are well-suited to study questions of speciation, to reconstruct the phylogeny or phylogeography of a group of taxa (Tab. 1 and 2). Sequencing of PCR products has been simplified recently, in that PCR products can be employed for didesoxy nucleotide sequencing after Sanger *et al.* (1977) without further purification (Hanke & Wink 1994) in that non-incorporated nucleotides and PCR primers are enzymatically degraded prior to sequencing.

Marker genes employed for sequencing are not those encoding proteins responsible for the morphology of a species, but rather enzymes or rRNA (Hillis *et al.* 1996, Soltis *et al.* 1992, Avise 1994) (Fig. 8). Problems of adaptation and convergence, which can obscure the morphological analysis, are much reduced when using sequences of marker genes. For animals, mitochondrial genes are

Figure 8. Shematic ilustration of mitochondrial genes and nuclear rDNA

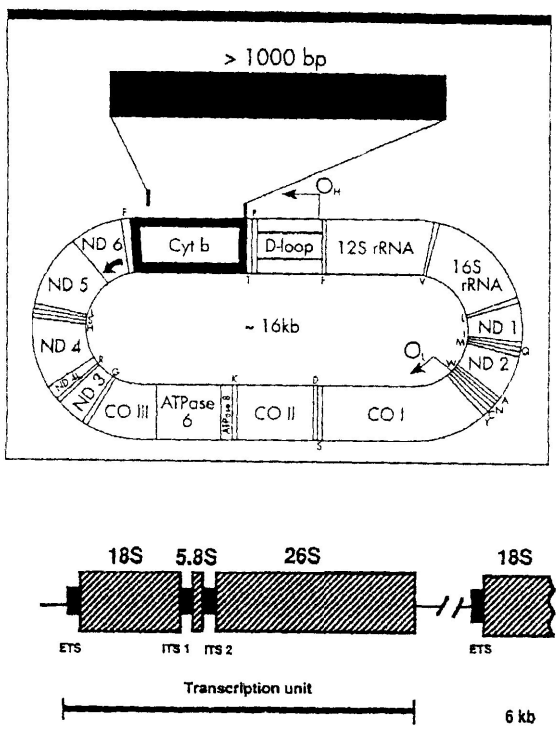


Table 3: Primer sequences (5' - 3') used for PCR and direct sequencing of cytochrome b (modified from Kocher et al. 1989). Positions in the chicken mitochondrial genome corresponding to the 3'-end of each primer are given in parentheses. L = L-strand, H = H-strand.

PCR-amplification:									
mt-A	(L-14970):	CAA	CAT	CTC	AGC	ATG	ATG	AAA	CTT	CG
mt-Fr	(H-16086):	TCA	GTT	TTT	GGT	TTA	CAA	GAC	CAA	TG
Sequencing:										
mt-B	(H-15318):	TCA	AAA	TGA	TAT	TTG	TCC	TC		
mt-G	(L-15155):	CAT	CCT	TCT	TCT	TCA	TCT	GCA	TCT	AC
mt-C	(L-15291):	CTA	CCA	TGA	GGA	CAA	ATA	TCA	TTC	
mt-D	(L-15546):	ATC	CCA	TTC	CAC	CCC	TAC	TAC	CCC	
mt-H	(L-15699):	CAA	ACC	AGA	ATG	ATA	CTT	C		

often chosen, since they have a higher variability than nuclear DNA and deletions, inversions and insertions are of minor importance. Among these mitochondrial genes, the cytochrome b gene (Fig. 8) has often been employed for studies on bird phylogeny (Baker 1992; Birt *et al.* 1992; Cooper *et al.* 1992; Edwards & Wilson 1990; Edwards *et al.* 1991; Friesen *et al.* 1993; Hedges & Sibley 1994; Helm-Bychowsky & Cracraft 1993; Kocher *et al.* 1989; Kornegay *et al.* 1993; Meyer 1994; Richman & Price 1992; Shields & Helm-Bychowsky 1988; Smith *et al.* 1991; Taberlet *et al.* 1992; Van Wagner & Baker 1990). Even more variation can be found in the mitochondrial D-loop region (Fig. 8), which has been used to define the structure of the Dunlin complex (*Calidris alpina*) (Wenink 1994). To analyse deeper branches of a phylogeny, more conserved sequences are often more appropriate such as nuclear ribosomal genes (Fig.8), e.g., 18S and 28S rRNA. The untranscribed spacers between the 18S-5.8-28S rRNA (so-called ITS-regions) are more variable and often useful at the intrageneric level.

In protein encoding genes nucleotide substitutions between closely related species are usually found in the third codon position (see above) (Tab. 4) and are transitions (exchange from A to G or C to T) whereas transversions (exchange from A and G to C or T or vice versa) are rather found between taxa which have diverged a longer time before. In general, the number of nucleotide substitutions and divergence times are correlated ("molecular clock concept"). Since the speed of this clock varies between genes and between groups of organisms, any calibration (e.g., 2% nucleotide substitutions equals 1 million years of divergence for mitochondrial genes; Shields & Wilson 1987; Wilson *et al.* 1987) must be applied with much caution, but it can tell us the corresponding order of magnitude.

Sequence data are aligned (Tab. 4) and evaluated with specific computer programmes. Two different mathematical approaches are often employed (Stewart

Table 4. Alignment of DNA sequences of a part of the cytochrome b gene of some falcon species

.= nucleotide identical to that in the first line; ? = undetermined (after Seibold, 1994)

	111	111	111	111	111	111	122	222	222	222	222	222	222	222	222	222	222	222	222	222
	888	888	888	999	999	999	900	000	000	001	111	111	111	222	222	222	233	333	333	334
	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890
Gallus gallus	ATC	TTC	CTT	CAC	ATC	GGA	CGA	GGC	CTA	TAC	TAC	GGC	TCC	TAC	CTC	TAC	AAG	GAA	ACC	TGA
F.peregrinus	...	.A.	A.A	...	..T	..G	...	...	A.T	...	..	...	..T	..T	T.G	..T	..A	...	..	..G
F.mexicanus	..T	.A.	A.A	...	..T	...	...	...	A.T	...	..	...	..T	...	..G	...	..A	...	...	...
F.chicquera	...	.A.	A.G	...	..T	...	...	...	A.C	...	...	...	..G	..T	..A	...	..A	...	..	...
F.biarmicus	...	.A.	..?	...	..T	...	?..	...	A.C	...	..	A..	...	...	..A	...	...	..	...	...
F.jugger	...	.A.	..A	...	..?	...	..	...	A.C	...	...	A..	...	...	..A	...	...	..	...	...
F.cherrug	..T	.A.	..G	...	...	...	T..	...	A.C	...	...	A..	...	...	?.G	...	...	...	..T	...
F.rusticolus	..T	.A.	..G	...	...	..	T..	...	A.C	...	...	A..	...	...	A.G	...	...	...	..T	
F.eleonorae	..T	.A.	A.A	...	..T	...	...	...	A.C	...	..T	...	..T	...	..G	...	..A	...	...	...
F.concolor	..T	.A.	A.A	...	...	...	...	...	A.C	...	...	...	..T	..	..G	...	..A	..G	...	...
F.subbuteo	..T	.A.	A.A	...	..T	...	...	...	A.C	...	..T	...	..T	...	..G	...	..A	..G	...	...
F.naumanni	...	.A.	A.A	...	...	...	...	...	A.C	...	...	..T	...	...	..G	...	..A	...	..A	...
F.tinnunculus	..T	.A.	A.G	...	...	...	..?	...	A.C	...	...	...	...	..T	..G	...	..A	...	..A	...
F.vespertinus	...	.A.	A.A	...	...	...	...	...	A.C	...	...	...	...	...	..G	...	..A	...	...	...
F.columbarius	...	.A.	A.A	...	...	...	...	..?	A.C	...	...	...	..T	...	...	...	..A	...	...	

1993):

1. Maximum Parsimony (MP) is a character state method. It follows in essence a cladistic philosophy and only informative characters are used. Both exact (for small data sets) and heuristic algorithms (for large data sets) are available which produce either clado- or phylograms (in phylograms, branch lengths are proportional to the number of nucleotide substitutions). Data can be corrected for biased transversion/transition ratios and for uneven codon usage, although these manipulations are controversial. Bootstrap analyses provide some evidence for the stability of each furcation, although the interpretation of bootstrap values is still controversial (Brown 1994). A detailed description of MP can be found in the handbook accompanying the computer programme PAUP (Version 3.1) (Swofford 1993).

2. Neighbour joining (NJ) is a "distance matrix" method which evaluates all characters and groups the taxa according to their distances (e.g. number of nucleotide substitutions) to each other. This method can analyse even large data sets effectively. Character weightings and bootstrap procedures are as in MP. A good implementation is given by the computer programmes PHYLIP (Felsenstein 1985) and MEGA (Kumar *et al.* 1993).

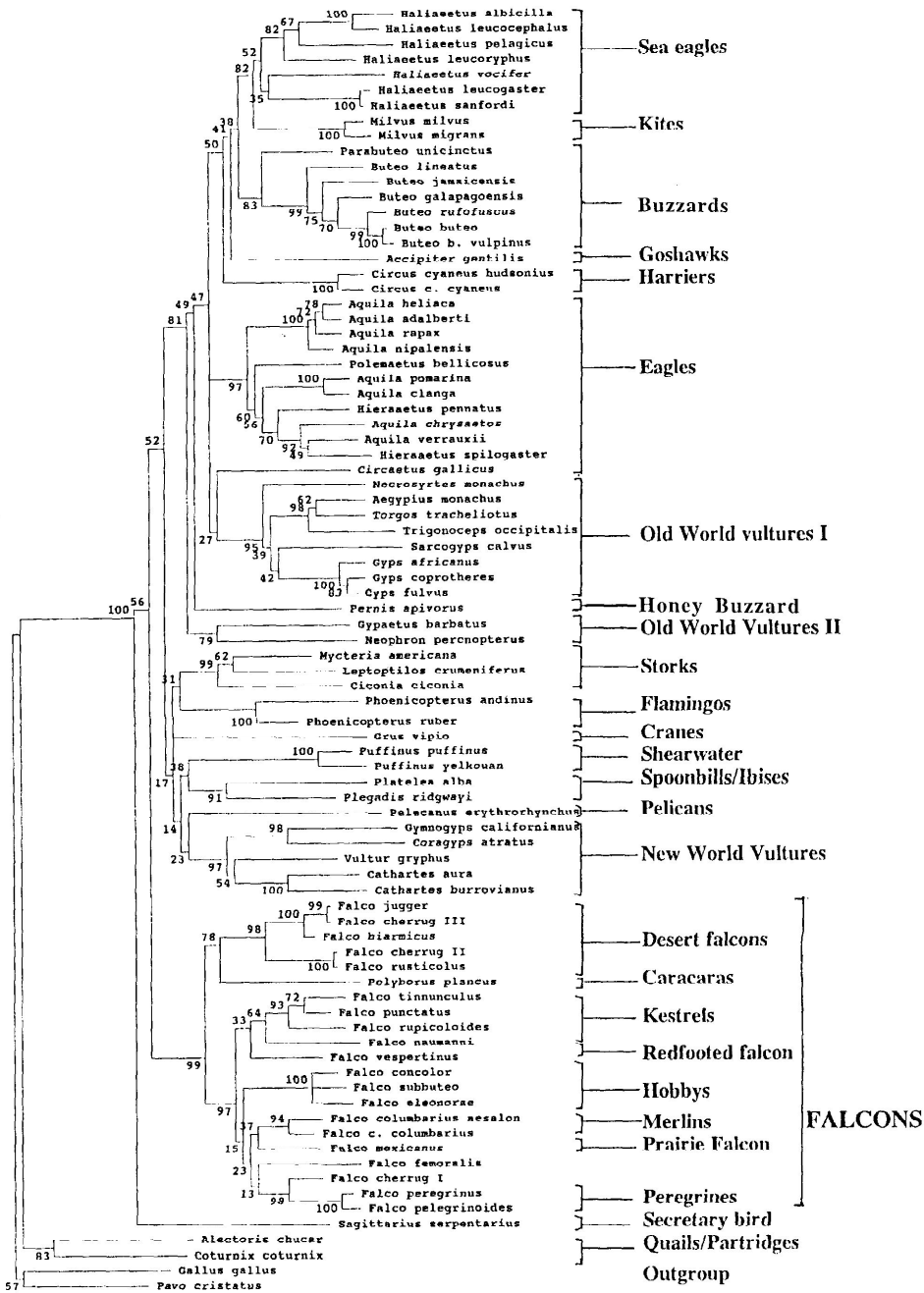
These sequence data can be used to reconstruct the evolutionary past of organisms and have been applied already to analyse the phylogeny of diurnal and nocturnal raptors by Avise *et al.* (1994), Heidrich & Wink (1994,1996), Heidrich *et al.* (1995a,b; 1996), Seibold *et al.* (1993, 1996), Wink (1994, 1995), Wink *et al.* (1993a,b; 1994; 1995; 1996a,b). As an example, Fig. 9 shows the phylogram of diurnal raptors, indicating that the families Accipitridae, Sagittariidae, Cathartidae and Falconidae are not closely related but appear to represent convergent evolutionary lines (for a more detailed discussion see Wink 1995; Wink *et al.* 1995, 1996). We have also used sequences of the cytochrome b gene, to analyzed phylogenetic relationships of other bird groups, such as shearwaters (Wink *et al.* 1993 a,b; Heidrich *et al.* 1996), gulls (Wink *et al.* 1994; Heidrich *et al.* 1996), skuas (Blechschmidt *et al.* 1993), Houbara (Gaucher *et al.* 1996), chiffchaffs (Helbig *et al.* 1993, 1995, 1996) and turdids (Wittmann *et al.* 1995).

CONCLUSIONS

DNA fingerprinting with single- and multilocus probes, but also PCR techniques, such as RAPD and microsatellite PCR provide means to identify individual animals and to establish mating systems (Burke *et al.* 1991a,b). Recently, the identification of individual domestic and endangered animals has been achieved using tiny transponders. Since these transponders can be removed by microsurgery and replaced by new transponders (i.e., this method is not absolutely safe), DNA markers could complement the transponder technique, since a genetic "fingerprint"

Figure 9. Reconstruction of the phylogeny of diurnal raptors inferred from nucleotide sequences of the cytochrome b gene (>1000 bp) (after Seibold, 1994; and Wink 1995)

Analysis by NJ and Jukes Cantor distance algorithm; values represent bootstrap values of 500 replications;



cannot be manipulated in the same way (Wink 1996). It would suffice to store a blood sample of a valuable animal that has been marked by a transponder, and to determine the DNA fingerprint only when needed. Using this strategy the transponder technique would become more or less safe and reliable.

DNA sequence analysis is a very powerful tool for taxonomy and evolutionary studies and allows the reconstruction of phylogenies and corresponding phylogeographies (Avise 1994). It is not a substitute for rigorous morphological, ethological or vocal studies, but a complement to them (Sibley 1994).

Besides the techniques mentioned in this review, many other useful molecular techniques are also available (Avise 1994; Hoelzel 1992, Hillis *et al.* 1996). Because many innovations are due in molecular biology in the near future, it is important to be prepared for them. Nearly all these techniques will be based on DNA analysis and since erythrocytes of birds contain a nucleus, the most convenient way to obtain bird DNA is from blood. Other sources can be muscle tissue (from dead birds stored at - 20 °C or in ethanol) or feathers (not always reliable). Samples can be either stored in EDTA buffer (10% EDTA, 0.5 % NaF, 0.5% thymol, 1% Tris, pH 7.5) or in ethanol (final concentration should be > 70%). Heparin should be avoided as an anticoagulant, since many enzymes are inhibited by heparin.

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