Systematics

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INTRODUCTION

Systematics is the branch of biology that deals with the classification of living organisms, describing their diversity and interrelationships. It can be divided into three parts:

- **Taxonomy** is the description and naming of new taxa (a taxon is any specifically defined group of organisms). Taxonomic groups are used to categorize similar taxa for identification, such as field guides. Taxa do not necessarily mirror evolutionary relationships. Taxonomists have not agreed universally on a single species concept (Mayr 1969, Sibley and Ahlquist 1990). The oldest is the *Typological or morphological species concept*. This concept combines a group of organisms into a single species if they conform sufficiently to certain fixed properties or differ anatomically from other populations of organisms. For many years, the *Biological species concept* was favored in ornithology. According to this species concept, a species consists of "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups." In the *Phylogenetic* or *Evolutionary species concept*, a species comprises a group of organisms that shares an ancestor and can be separated from others by distinctive characters. This species concept describes lineages that maintain their integrity with respect to other lineages through both time and space. At some point in the progress of such groups, members may diverge from one another: when such a divergence becomes sufficiently clear, the two populations are regarded as separate species. See Otte and Endler (1989) for additional details regarding various species concepts.

- **Classification** is the organization of information about diversity that arranges it into a convenient hierarchical system of classification, such as the Linnaean system.

- **Phylogenetics** is the field of biology concerned with identifying and understanding the evolutionary relationships among the many different kinds of life on earth. It is the basis for evolutionary systematics. Phylogeny is the determination of the ancestral relationships of organisms, and the group's evolutionary history.

Classification of plants and animals is a basic discipline of biology, and the *Systema Naturae* of Linné in 1753 was a landmark in this field. Traditionally, systematists and taxonomists have used morphological and anatomical characters to define species and subspecies. More recently, they also have used behavior, vocaliza-

tions, and biochemistry. The new era of molecular biology has provided a broad set of genetic tools that complement existing methods.

It is likely that biologists will soon establish an improved taxonomy and classification of most orders of the living world that is based on phylogenetic relationships and not solely on similarity. Many morphological characters can be formed by convergent evolution, and anatomical similarity alone can result in misleading classifications. Genetic characters, which are more numerous overall than the former, can help to clarify systematics. Today a dream of Charles Darwin may become a reality. In 1857 Darwin wrote to his friend Thomas H. Huxley: *"In regard to classification, & all the endless disputes about the 'natural system' which no two authors define in the same way, I believe it ought, in accordance with my heterodox notions, to be simply genealogical. The time will come I believe, though I shall not live to see it, when we shall have fairly true genealogical trees of each kingdom of nature..."*

In this chapter, I introduce the methods used in taxonomy, classification, phylogeny, and systematics and then discuss in detail the newer DNA methods.

PRINCIPAL METHODS

Comparative Nonmolecular Characters

An array of details can be recorded about an organism, and each detail can be used as a character for comparison with the same homologous character (i.e., a character inherited from a common ancestor) in other organisms. These characters can be tabulated and analyzed by cladistics, a method that groups organisms on the basis of common ancestry into clades that represent monophyletic groups (cf. Wiley 1981, Wiley et al. 1991). The intrinsic characters below have been used in systematic studies. The list is not exhaustive, nor will it ever be as innovations continue to extend the range of characters that can be documented and improvements are made in assessing the usefulness of characters in classification.

Measurable characters. A comprehensive set of measurements that can be taken on live, freshly killed or dried museum specimens of raptors has been described by Biggs et al. (1978). Those measurements found to be practical in extensive field and museum work are described and illustrated in Figs. 1 and 2.

Workers should practice such taking such measurements in general before doing so on the organisms they are studying, and should repeat all measurements to determine the extent of both intra- and inter-recorder variability. When comparing measurements from fresh and dry specimens, shrinkage in the latter should be accounted for. Special attention should be given to recording body mass, body temperature and neural (brain) mass. Brain mass values can be important when behavioral and sociological data are compared. Scaling

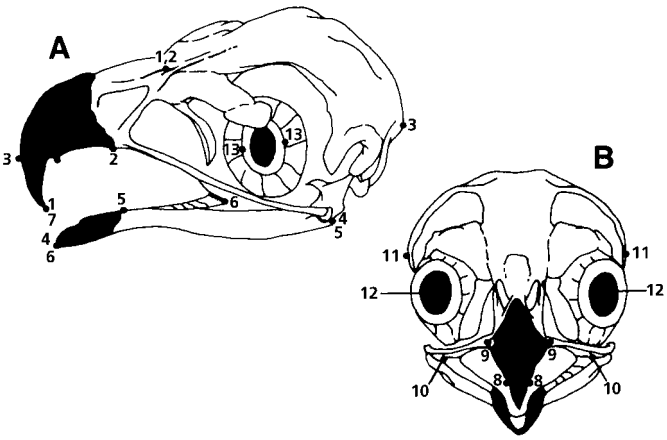


Figure 1. Views of a raptor skull. A — dorsolateral, and B — anterior, with measuring points taken by calipers and indicated by numbers and detailed below.

1. Bill chord — from the suture at the bill-skull junction to the tip of the mandible.

2. Bill depth — from center of the suture at the bill-skull junction to the junction of the cutting edge of the upper mandible and cere (or skin) along the gape.

3. Skull length — from the center back of the skull to the front edge of the upper mandible with the calipers held parallel to the plane of the top of the head.

4. Jaw length — from the posterior point of the ramus of the lower jaw to the tip of the lower mandible.

5. Jaw-bill length — from the posterior point of the ramus of the lower jaw to the junction of the cutting edge of the dorsal surface of the lower mandible and the skin forming the edge of the gape.

6. Gape length — from the back of the fold of the gape, with the mouth almost closed, to the tip of the lower mandible.

7. Tooth depth (for those species with tomial teeth) — from the tip of the upper mandible to the tip of the longest tomial tooth.

8. Tooth width (for those species with tomial teeth) — between the tips of the tomial teeth.

9. Bill width — between the junctions of the cutting edges of the upper mandible and the cere (or skin) on each side of the gape.

10. Gape width — between the back points or fold of the gape when the mouth is closed.

11. Skull width — between the widest points of the skull behind the eyes, with calipers vertical to the plane of the top of the head.

12. Eye spacing — as the width between the centers of the eyes, with the calipers as close to the surface of the eyes and the recorder's eyes as far away as possible.

13. Eye diameter — between the outer edges of the colored (iris) area of the eye, corresponding to the inner edge of the ring of sclerical ossicles. *Figure originally from Kemp (1987).*

Figure 2. Diagrammatic layout of a raptor with measuring points taken by various methods and indicated by numbers and detailed below.

14. Wing length — Taken with a wing rule, from the front of the folded wrist to the tip of the longest primary, with the feather flattened and checking that it is not affected by molt.

15. Secondary length — Taken with wing rule, from the front of the folded wrist to the tip of the outermost secondary, with the feather flattened and checking that it is not affected by molt.

16. Alula length — Taken with a wing rule, from the proximal side of the protuberance on the carpometcarpus to which the alula is attached, to the tip of the longest alula feather, with the feather flattened and checking that it is not affected by molt.

17. — **Ulna length index** — Taken with a wing rule, from the front of the folded wrist to the inner surface of the elbow joint (inner surface of the distal humerus head).

18. — **Humerus length index** — Taken with a wing rule, from the outer edge of the elbow joint (posterior surface of proximal ulna head) to the anterior edge of the distal end of the coracoid (forming a point at the anterior edge of the shoulder).

19. — **Femur length index** — Taken with calipers, from the top of the exterior proximal crest of the femur to the anterior center of the tibiotarsal-tarsometatarsal joint.

20. Tibiotarsal length — Taken with calipers, from the anterior center of the tibiotarsal-tarsometatarsal joint.

21. Tarsometatarsal length — Taken with calipers, from the posterior center of the tibiotarsal-tarsometatarsal joint to the dorsal base of the center toe (point is located by flexion of the toe).

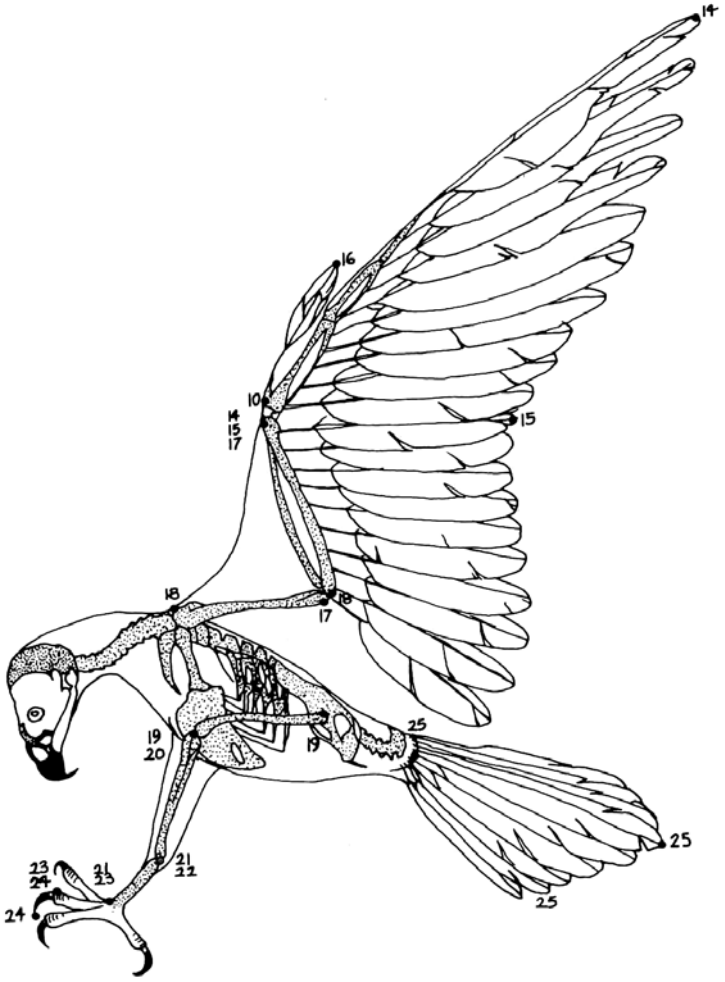
22. Foot volume — Recorded by displacement of water when immersing the foot and tarsus up to the tibiotarsal-tarsometatarsal joint.

23. Toe lengths — Taken with calipers, along the dorsal surface of each straightened toe, from the junction with the tarsometatarsus (found by flexion of the toe) to the claw-skin junction.

24. Claw chords — Taken with calipers, from the dorsal surface of the claw at the junction with the skin to the tip of the claw.

25. Tail lengths — Taken with a wing rule, from the feather-skin junction of the central pair of rectrices to their tips (center tail length), and to the tip of one of the outer pair of rectrices (outer tail length), with the feather flattened and checking that it is not affected by molt.

Figure originally from Kemp (1987)



relationships based on these parameters have great potential in predicting a wide range of attributes for homoeotherms, particularly those concerning life-history indexes, growth, and energetics (Calder 1983, 1984).

Other important parameters include mass and wing area. Mass should be recorded as accurately as possible (preferably with electronic balances), and the accuracy of the weighing instrument should be noted. Adjustments to the mass, to account for differences in body condition or the presence of food in the crop or stomach, can be attempted. Wing area can be drawn directly onto scaled, gridded paper or photographed together with an appropriate scale. To ensure comparable measurements, the wing should be extended with the leading edge forming a straight line perpendicular to the body. Tracing should include secondaries and tertials.

Anatomical characters. Anatomical characters incorporate any external or internal structural attributes of an organism. External characters include plumage color, structure, and form; soft-body-part colors and extent; bill and foot form; and the sizes and proportions of these characters. Internal characters most often refer to skeletal or myological attributes as well as details of organ systems. A comprehensive introduction to raptor morphology is provided by Jollie (1976, 1977a,b). Obviously, characters that differ with age and sex must be described separately for each class. This is a regular requirement for raptors, in which juvenile and sub-adult plumages are common and sexes differ at least in size if not in other characters. Age and sex differences posed many taxonomic problems to earlier workers (e.g., Finch-Davies 1919). Molecular methods now are avail-

able to sex individuals not only of "difficult" species, but also individuals at early stages of development (see below).

In addition to anatomical characters in full grown specimens, the ontogeny of these characters at various stages (embryological and post-hatching) often provides important systematic insight. Embryology rarely has been explored in raptor studies (Desai and Malhotra 1980, Bird et al. 1984), even though it may shed light on evolution of such important characters as fused phalanges (Olson 1982). Post-hatching development is regularly recorded and used in suggesting relationships (e.g., of *Circaetus* and *Terathopius* eagles [Brown and Amadon 1968] that have been confirmed by DNA sequence data [Wink and Sauer-Gürth 2000]).

The morphology of chromosomes (karyology) is an established technique whose application to birds has been reviewed (Shields 1982). Only the basic chromosome number and size has been determined for some raptors (e.g., Belterman and de Boer 1984, de Boer and Sinoo 1984, Schmutz et al. 1993). Studies of centromere position and arm proportions, or more advanced studies of chromatin banding within chromosome arms, are rare or lacking (Harris and Walters 1982, Shields 1983, Bed'hom et al. 2003).

Behavioral characters. Detailed ethograms have not been recorded for any species of raptor, although Walter (1983) has suggested a method for tackling this problem. The use of communication characters (primarily visual and vocal in raptors and in most other birds) has been advocated for assessing differences among species. These characters can be documented and analyzed using tape recorders, cameras and video equipment. Basic display patterns, such as pendulum flights by eagles, have been used in systematic studies (e.g., Brown and Amadon 1968), but detailed recording and analysis of these characters have yet to be achieved for most species. Maintenance patterns, such as scratching and stretching postures, too, deserve further attention. Locomotion, feeding and hunting patterns, with special attention given to any ritualization of these behaviors, also warrant study.

Molecular Characters

Biochemical characters. Biochemical characters involve either estimation or direct documentation of protein or nucleic-acid structural diversity. Such characters involve collection and storage of suitable tissues, followed by some form of laboratory analysis. Simpler techniques are most relevant to comparisons among closely related species or populations within species where larger sample sizes are available. More complex techniques may be applied to an array of species, from which only a few samples of each are available.

Historically, the first molecules to be analyzed were proteins. Amino-acid sequences were used to infer overall phylogenetic relationships. Microcomplement fixation and allozyme analysis were employed at the species and subspecies level (Prager et al. 1974, Brush 1979, Avise 1994). Except for allozyme analysis, the resolution of the protein methods was low and the analysis time-consuming. Because DNA methods are faster and more informative, protein methods, including allozyme analysis, largely have been replaced by them (see below).

Sample Preparation and Storage

The deposition of voucher specimens is strongly advocated to ensure the results of any study against subsequent changes in the systematic or taxonomic status of the organism involved. Ideally, such specimens should store as diverse a set of characters of the organism as possible. Specimens can include an entire carcass (providing information on plumage, skeletal, myological, and general anatomy), an entire clutch of eggs (preferably containing embryos), any slides of chromosome karyotypes, preserved tissues, DNA preparations, images (film or video) of nests, behavior (such as displays), and developmental stages, and tape recordings of vocalizations. Specimens also may include tissues preserved for later biochemical studies. All specimens should be deposited with an institution whose charter includes maintenance of material for perpetuity, such as an established museum.

The fresher the tissue is for analysis, the better. Freshly killed birds should be rapidly dissected and the appropriate tissues used immediately or stored appropriately to prevent degradation of proteins and DNA. Sometimes, tissues are quick-frozen in liquid nitrogen and stored at temperatures at -80°C, or in liquid nitrogen. Some techniques require less demanding storage conditions. One should first decide on the analytical procedures to be employed and then determine the optimum collection and storage system for that technique.

For DNA studies and, in particular, intra-species studies, the more samples (5 to 10 is a minimum) per species, the better. For the detection of gene flow between populations, a higher number of samples is

required (sometimes up to several hundred). A comprehensive and complete sampling (e.g., covering all members of a genus, family or order, or within-species samples from all major populations) is one of the main keys to a successful study.

In birds, blood tissue provides high-quality DNA and is better than feathers or feces. Museum skins sometimes can be a source for DNA, but DNA therein often is highly degraded. Blood (50 to 200 µl are sufficient) and tissues should be stored in 70% ethanol or, if possible, an ethylenediaminetetraacetic acid (EDTA) buffer containing 10% EDTA, 1% sodium lauryl sulfate (SDS), 0.5% NaF, 0.5% thymol in 100 mM Tris, pH 7.4 (Arctander 1988). Using the EDTA buffer, blood tissue can be stored for long periods at ambient temperatures without refrigeration. Because polymerase chain reaction (PCR) methods can pick up tiny amounts of contaminations, separate needles and pipette tips must be used for each bird.

DNA METHODS

The principles of the major DNA methods (Table 1) useful for studying the systematics of raptors are described briefly below.

DNA as a Logbook of Life

The field of DNA analysis is developing rapidly, and new techniques are being devised constantly (Hoelzel 1992, Avise 1994, Hillis et al. 1996, Mindell 1997, Karp et al. 1998, Hall 2001, Storch et al. 2001, Frankham et al. 2002, Beebe and Rowe 2004). For many groups of organisms, genetic data already are available that help identify an individual to a species (sometimes called *DNA-barcoding*). Molecular methods can answer questions in population genetics, such as immigration and dispersal rates and gene flow, and assess the connectivity between the breeding and wintering grounds of species.

Because DNA methods and DNA markers are important tools in systematics and taxonomy, the following paragraphs review some of the important background information on DNA (Griffiths et al. 1999, Klug and Cummings 1999, Alberts et al. 2002).

The genetic information of all organisms is encoded in DNA. DNA is built from four nucleotides: adenine (A), guanine (G), thymine (T) and cytosine (C). The genetic message is fixed in the specific sequences of A, T, G and C. DNA consists of two complementary strands organized as a double helix. In the nucleus of eukaryotic cells (i.e., cells with compartmentalized internal structure), linear DNA is arranged in separate

Table 1. Important methods of molecular biology that are useful in evolutionary and phylogeographic studies.

Method[a]	DNA Type	Adequate for Studying
Sequencing	mtDNA[a], ncDNA[a]	Phylogeny, taxonomy, phylogeography
STR-Analysis[a]	Microsatellites	Population genetics, tracing of individuals, paternity, and pedigree
SNP-Analysis[a]	Point mutations in all genes	Population genetics, tracing of individuals, paternity, and pedigree
AFLP[a]	Nuclear genome	Population genetics, gene mapping
ISSR[a]	Nuclear genome	Phylogeny, population genetics, hybridizations, gene mapping
DNA fingerprinting	Satellite DNA (VNTR, STR)	Paternity, tracing of individuals
Sexing	Sex chromosome	Molecular sexing

[a]STR = short tandem repeats; SNP = single nucleotide polymorphisms; VNTR = variable number tandem repeats; mtDNA = mitochondrial DNA, ncDNA = nuclear DNA; AFLP = amplified fragment length polymorphisms; ISSR = inter-simple sequence repeats.

chromosomes. At one time or another, all cells of the body, except gametes, have a double set of chromosomes and are termed diploid.

Eukaryotic cells carry DNA in the nuclear genome (ncDNA), but also in their mitochondria (mtDNA). Algae and plants have a third genome in form of chloroplast DNA (cpDNA). Mitochondrial DNA, which is a circular molecule similar to the DNA found in bacteria, was derived from endosymbiotic bacteria that were taken up by the ancestral eukaryotic cell some 1.4 billion years ago. The mitochondrial genome of animals consists of 16,000 to 19,000 base pairs (bp) and contains 13 genes that code for enzymes involved in the respiratory chain, 22 genes for transfer RNA (tRNA) and two genes for ribosomal RNA (rRNA) (Table 2). Mitochondria have a short stretch of non-coding DNA, the D-loop or control region (or origin of replication), that is four to six times more variable than protein-coding genes such as cytochrome b. A typical animal cell has between 100 and 1000 mitochondria, each of which harbors 5 to 10 copies of mtDNA. This makes mtDNA an especially frequent molecule in cells, although it accounts for only 1% of all cellular DNA. MtDNA, therefore, provides an important source of genetic material for DNA studies. MtDNA is inherited maternally and can be regarded as clonal in nature (Avise 1994, Hillis et al. 1996, Mindell 1997, Karp et al. 1998, Hall 2001, Storch et al. 2001).

The nuclear genome of birds and other vertebrates typically consists of more than one billion base pairs. Only 25% of the genome represents genes and gene-related sequences used by the organism. About 2% of the DNA actually encodes proteins. Seventy-five percent of the genome consists of extragenic DNA, with highly repetitive sections, such as long interspersed nuclear elements (LINE), short interspersed nuclear elements (SINE), and mini- and microsatellite DNA. In animal genomes, there are abundant sequences that consist of almost identical elements of 15 to 100 bases that are tandemly repeated 5 to 50 times (i.e., so-called mini-satellite DNA or VNTR — *variable number tandem repeats*). Mini-satellites show many point mutations and vary in the lengths of their repetitive elements in each DNA locus. Another abundant repetitive element consists of tandem repeats of two (sometimes up to five) nucleotides, such as (GC)n or (CA)n, that are repeated 10 to 50 times (so-called STR; *short tandem repeats*, or microsatellites).

The genome of vertebrates has more than 20,000 distinct loci of STR sequences that usually consist of polymorphic alleles. The repetitive elements are highly variable in length, a phenomenon that is caused by uneven crossing-over during meiotic recombination and slippage of DNA polymerase during replication. VNTR- and STR-loci are hot spots of evolution and are inherited co-dominantly. The chance that two individu-

Table 2. Composition of mitochondrial DNA.

DNA	Number of Elements	Substitution Rate
16S rRNA	1	Low
12S rRNA	1	Low
tRNA	22	Low
Cytochrome b	1	Medium
Cytochrome oxidase (CO), subunits I-III	3	Medium
NADH dehydrogenase (ND), subunits I-VII	7	Medium
ATP synthase, subunits a, b	2	Medium
D-loop (ori)	1	High

als have identical sets of VNTR and STR profiles is less than one in a million. Therefore, these genetic elements are ideal markers when a high degree of genetic resolution is required (Avise 1994, Hillis et al. 1996, Mindell 1997, Karp et al. 1998, Hall 2001, Storch et al. 2001).

DNA has several repair and copy-reading enzymes that help conserve its molecular structure. Even so, mutations do occur. Point mutations and chromosomal recombination are abundant, and the genomes of any two individuals of the same species are not identical. About one million single nucleotide differences are common among individuals of the same species. Mutations can be regarded as evolutionary landmarks that are transmitted to later generations, and as such can be used to trace the origin of any organism.

The total number of mutations to the original sequence increases with time. This is the base for the molecular-clock concept (Zuckerkandl and Pauling 1965), which is helpful in many areas of evolutionary research. Molecular clocks are not as precise as physical clocks and are better thought of as defining relative time windows. Molecular clocks based on amino-acid exchanges are nearly linear with time, whereas nucleotide clocks are linear only initially. Over time the latter level out because of multiple substitutions that occur during longer divergence times. Many protein-coding mitochondrial genes have an estimated divergence rate of 2% substitutions per million years (Wilson et al. 1987, Tarr and Fleischer 1993), and may be reliable up to about five million years, after which divergences will be underestimated because of the plateau effect caused by multiple substitutions. Older events can be evaluated using non-synonymous substitutions or amino acid changes that are linear over a much longer time period. Mutation rates differ between coding and non-coding DNA regions. Mutations in synonymous codon positions do not influence the fitness of the organism. Consequently, they are not a target of selection processes (i.e., they are neutral mutations and show higher apparent mutation rates). Mutation rates also differ between nuclear and mitochondrial genes; protein coding mtDNA evolves 10 to 20 times faster than protein coding nuclear genes.

The genome can be regarded as the "logbook of life" in which previous evolutionary events are fixed in terms of mutations. In vertebrates, an estimated 100,000 to 10 million nucleotide differences exist among individuals belonging to the same species. Differences between closely related species are in the range of 10 to 100 million nucleotide substitutions. Presently, it is not possible to detect all genetic polymorphisms. Instead DNA markers are analyzed as representatives for the whole genome. Depending on the biological question to be answered, different methods have been developed that are appropriate to study a given problem (Table 1).

Before the availability of rapid DNA sequencing, *DNA-DNA hybridization* was a widely used tool in molecular systematics. Sibley and Ahlquist (1990) used this method to formulate phylogenetic relationships in birds (Sibley and Monroe 1990). Many workers have since criticized DNA-DNA hybridization on methodological grounds (see Ericson et al. 2006). Today DNA-DNA hybridization can best be regarded as a historical method that has been replaced by a wide set of more versatile techniques based on PCR and DNA sequencing.

DNA Sequencing

The analysis of nucleotide sequences of marker genes is a powerful method for reconstructing the phylogeny of organisms (Hillis et al. 1996, Mindell 1997, Karp et al. 1998, Griffiths et al. 1999, Hall 2001, Storch et al. 2001, Frankham et al. 2002, Beebe and Rowe 2004). These methods are now being employed by researchers studying all parts of the living kingdom with one aim being to assemble the tree of life. Raptors are only a small group in this effort, but some progress already has been made (see below). Phylogenetic information is fundamental for taxonomy and systematics as they allow establishment of a natural, genealogical classification. The general procedure to produce and analyze DNA sequences in a phylogenetic and phylogeographic context is outlined in Fig. 3. Many researchers send their sequences to public databases such as GENBANK (National Institutes of Health) or EMBL (European Bioinformatics Institute). For a detailed description of methods and concepts see Hillis et al. (1996), Mindell (1997), Karp et al. (1998), Hall (2001), Storch et al. (2001), Frankham et al. (2002) and Beebe and Rowe (2004).

Studies of the phylogeny of raptors typically focus on the nucleotide sequences of conserved marker genes, such as protein coding mtDNA (Table 2) and of coding ncDNA (such as RAG1) (Griffiths et al. 2004) or non-coding DNA (such as intron regions of protein coding genes, including LDH, and ODC-6). Species that evolved several million years ago exhibit enough divergence among geographically separated lineages to permit useful analyses. In these cases, sequencing of mitochondrial genes, such as cytochrome b, ND or CO (Table 2) often helps to identify the mitochondrial line-

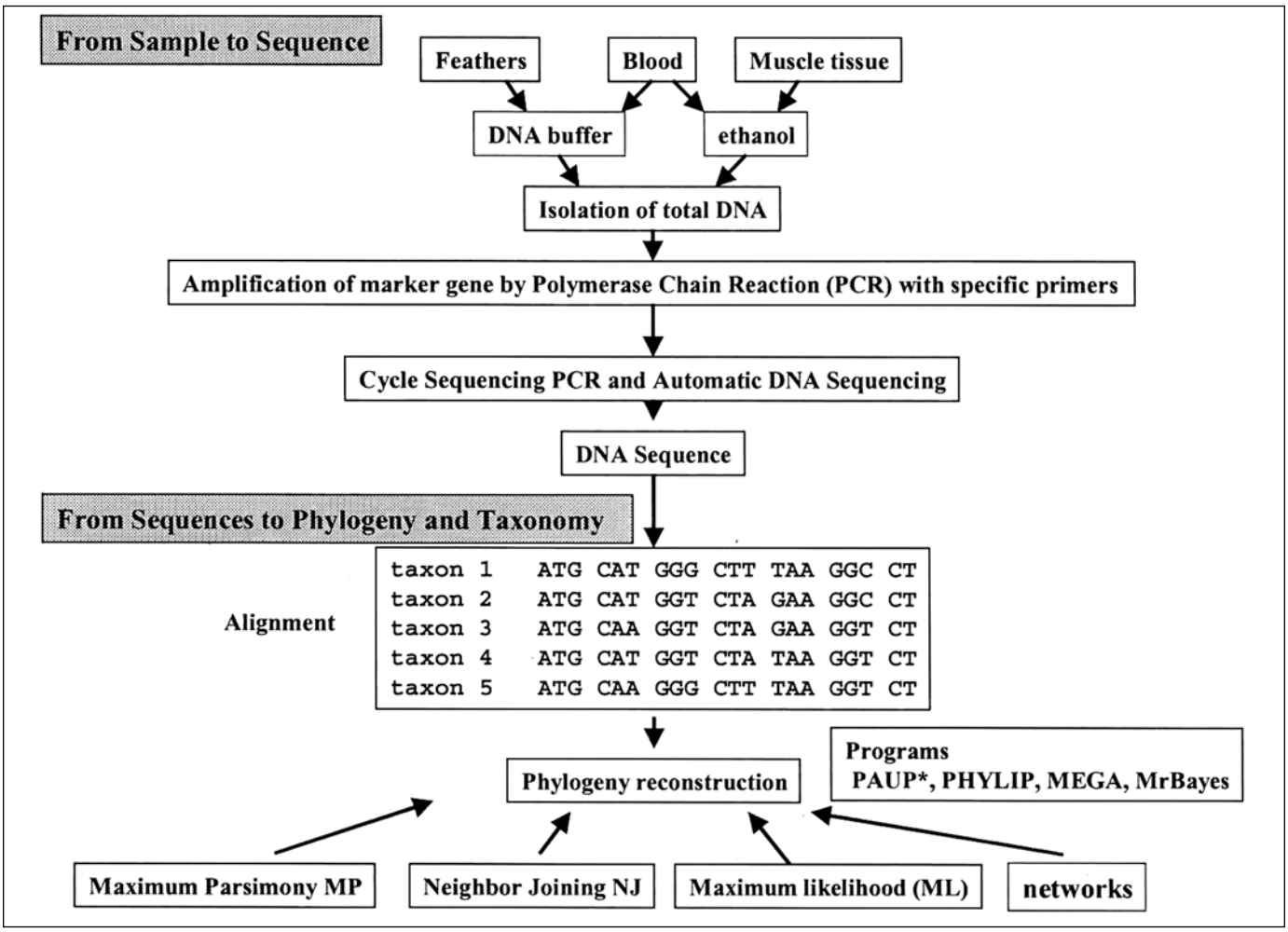


Figure 3. From sample to DNA sequence and phylogeny.

ages of groups (so-called haplotype). Because the mitochondrial D-loop is more variable, this stretch of DNA may provide an even higher resolution, but, because of its variability, it can sometimes be difficult to amplify and sequence the D-loop region by PCR.

Haplotypes of individual raptors caught during migration or on the wintering grounds sometimes can be used to determine the bird's geographic origin. In a best-case scenario, the breeding populations have distinctive haplotypes with little gene flow between lineages (Fig. 4a). If the haplotype of the migrating bird matches that of a breeding population, its origin can be inferred with some certainty (Fig. 4c). In a worst-case scenario (Fig. 4b), populations have several haplotypes and share them with neighboring populations, suggesting considerable gene flow among them. In this case, an intelligent guess is possible only if the haplotype of a migrant has to be allocated.

In relatively new species, intraspecies-sequence variation is quite small, and it can be difficult to establish an informative genetic population map using this variation. When this happens, DNA methods with a higher resolution are required.

Application of DNA sequences to study taxonomy and systematics of raptors. Diurnal raptors have been grouped into five families; Accipitridae, Pandionidae, Sagittariidae, Falconidae, and Catharthidae, and placed in the order Falconiformes (del Hoyo et al. 1994) or the infraorders Falconides and Ciconiides (Sibley and Monroe 1990). Whether Falconiformes is a monophyletic group remains an open question that many researchers are currently attempting to resolve. However, morphological and molecular data (based on several nuclear and mitochondrial genes) provide evidence that at least Falconidae do not share direct ancestry with Accipitridae, Sagittariidae, Pandionidae, and Cathartidae (Wink 1995, Wink et al. 1998b, Fain and Houde 2004, and Ericson et al. 2006).

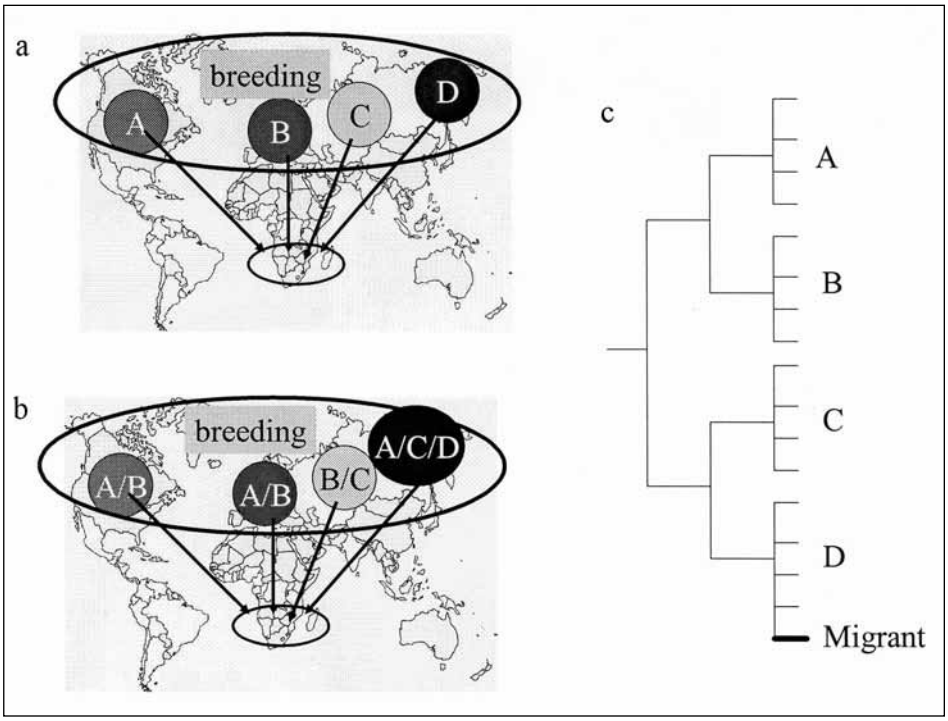


Figure 4. Distribution of haplotypes in geographically distinctive populations of a species that breeds in the northern hemisphere but winters in southern Africa.

Best-case scenario (a): all populations have unique haplotypes (C); therefore, a bird found in the wintering quarters (here with the haplotype D) unambiguously can be attributed to its breeding population D (C).

Realistic scenario (b): individual populations have more than one haplotype and share them with neighboring populations. A bird with the haplotype A can thus have originated from North America, Europe or East Asia.

(c) Cladogram of haplotypes found in scenario A.

DNA sequences have become an important tool for taxonomy and evolutionary studies including owls and diurnal raptors. Recent work in this area includes that of Wink (1995), Seibold and Helbig (1995a,b, 1996), Wink et al. (1996), Griffiths (1997), Matsuda et al. (1998), Wink and Heidrich (1999), Haring et al. (1999, 2001), Wink and Sauer-Gürth (2000, 2004), Groombridge et al. (2002), Riesing et al. (2003), Hendrickson et al. (2003), Godoy et al. (2004), Griffiths et al. (2004), Kruckenhauser et al. (2004), Pearlstine (2004), Roques et al. (2004), Roulin and Wink (2004), Gamauf et al. (2005), Helbig et al. (2005), Lerner and Mindell (2005), and Nittinger et al. (2005).

PCR Methods (Fingerprinting)

The analysis of genetic differences within a species demands methods that have a high degree of resolution. Sequences of mtDNA are sometimes uninformative at an intraspecific level. Because mtDNA is inherited maternally, hybridization and introgression can mask an unambiguous allocation of individuals to species, lineages and populations. To overcome these problems, molecular markers of ncDNA that are inherited by both sexes and that have a higher degree of resolution are more appropriate. These methods involve the amplification of polymorphic DNA markers by PCR and their separation by high-resolution gel electrophoresis (often on agarose, better on polyacrylamide gels) or by capillary electrophoresis (using a DNA sequencer).

DNA fingerprinting with VNTR or oligonucleotide probes has been employed to trace individuals for paternity and pedigree studies (Hoelzel 1992, Swatschek et al. 1993, 1994; Karp et al. 1998); it also can be applied to estimate adult mortality rates (Wink et al. 1999). Classical fingerprinting often has been replaced by microsatellite analysis (see below), that is more reliable and can be better automated.

Microsatellite (STR) Analysis

Each raptor has two alleles for each locus: one derived from the father, the other from the mother (Fig. 5). These alleles can be identical (homozygote) or not (heterozygote). As mentioned above, a vertebrate genome may contain more than 20,000 microsatellite loci that are characterized by 10- to 20- fold repeats of short-sequence elements, such as CA, TA, GACA, etc. The alleles of these loci show a high degree of length polymorphism. For each polymorphic STR locus, several alleles exist that differ in the number of tandem repeats; thus, they can be distinguished by size.

Because the sequences that flank microsatellite loci vary between species, special efforts are needed to iden-

tify sequences that can be used to amplify the STR loci. Several protocols have been published on generating species-specific STR sequences. A typical STR analysis is schematically illustrated in Fig. 5. A single locus provides information for two alleles; usually more than 8 to 10 polymorphic loci are needed to identify an individual unambiguously. For pedigree and population studies, 10 or more polymorphic STR loci are required. To reduce the number of PCR and sequencing runs, it is useful to establish a multiplex PCR system that allows the parallel analysis of several loci in one run.

Allele frequencies can be determined to characterize populations. If unique alleles can be identified within a population, they can help to assign an unknown individual to such a population. If unique alleles are not available, allele distributions are tabulated and allele frequencies calculated for any locus and population.

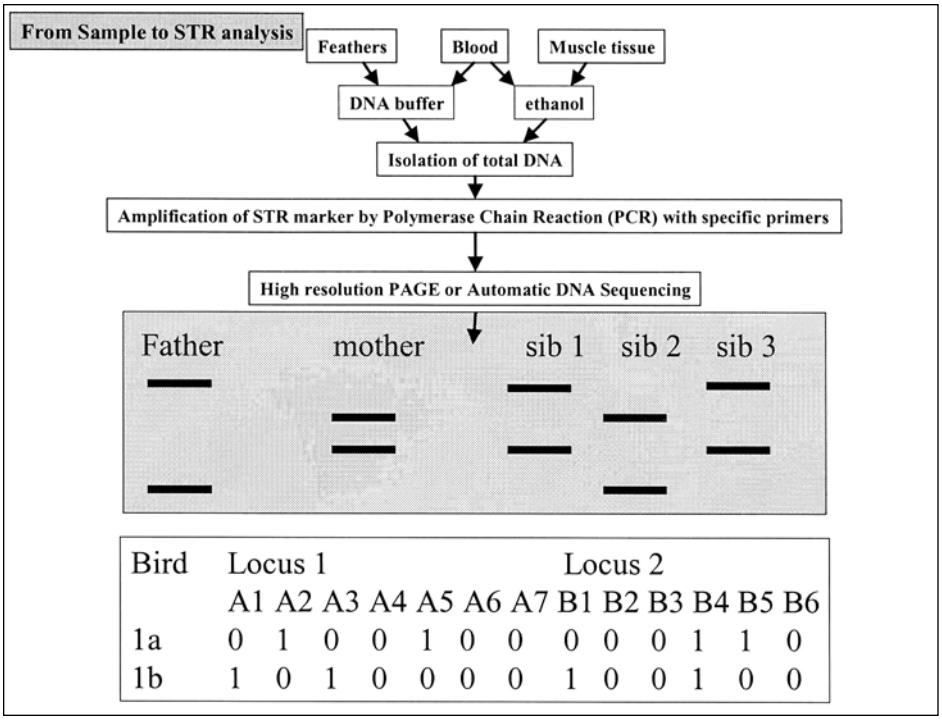
The presence and absence of alleles can be recorded in a 1/0 matrix and evaluated by cluster analysis (e.g., unweighted pair-group method with arithmetic means [UPGMA], neighbor-joining) and other programs (such as STRUCTURE or GENELAND). The result is a phenogram as shown in Fig. 4c. Individuals with similar patterns are clustered together in a clade. Examples for the use of STR-markers are found in Gautschi et al. (2000, 2003a,b), Nesje and Roed (2000a), Nesje et al. (2000), Nichols et al. (2001), Martinez-Cruz et al. (2002, 2004), Mira et al. (2002), Hille et al. (2003), Kretzmann et al. (2003), Sonsthagen et al. (2004), Topinka and May (2004), Busch et al. (2005), and Wink et al. (2006).

Single Nucleotide Polymorphisms (SNP)

Information from single-nucleotide polymorphisms (SNPs) can be used to build a genetic map of populations, so long as at least 30 loci are determined for each individual. SNPs are analyzed in a similar way as STR data (i.e., via a 0/1 matrix) and have a similar resolution power (Lopez-Herraez et al. 2005). Because SNP marker systems have yet to be established for individual species, they are not available for raptors at this time. However, because SNP analysis can be automated via DNA chips and mass spectrometry, this method is likely to become an important tool in the future.

Amplified Fragment Length Polymorphism (AFLP) and Inter-Simple Sequence Repeats (ISSR)

If information on PCR primers of microsatellites is not available, genomic-fingerprint methods including amplified fragment length polymorphism (AFLP) and inter-simple sequence repeats (ISSR) provide an alternative.



Bird	Locus 1							Locus 2					
	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	B4	B5	B6
1a	0	1	0	0	1	0	0	0	0	0	1	1	0
1b	1	0	1	0	0	0	0	1	0	0	1	0	0

Figure 5. Illustration of the inheritance of STR markers. Microsatellite PCR products are analyzed by polyacrylamide gel electrophoresis (PAGE). The lower box illustrates a 1/0 matrix that can be constructed from STR data. It can be analyzed by phenetic methods that produce phenograms (similar to the cladogram shown in Fig. 4[c]).

AFLP combines restriction-length analysis with PCR, making it a convenient and powerful tool. In the first step, DNA is digested by two restriction enzymes, MseI and PstI, which produce sticky ends. These sticky ends are ligated with oligonucleotide adaptors that recognize the restriction site and which also carry a PCR recognition sequence. Using specific PCR primers for the MseI and PstI adaptors, PCR fragments can be generated that relate to restriction fragments. These can then be separated by high-resolution polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis (Fig. 6). The result is a complex fingerprint that can be detailed in a 0/1 matrix and analyzed by cluster methods. AFLP loci are inherited co-dominantly. Examples for the application of AFLP analysis can be found in de Knijff et al. (2001) and Irwin et al. (2005).

ISSR produces fingerprints similar to AFLP. The procedure involves fewer experimental steps than AFLP and, therefore, is easier to carry out. ISSR uses a single PCR primer, whose sequence is identical to common microsatellite motives, such as (CA)10. Because such loci are widely present in genomes and occur in both orientations, a single primer is enough to amplify between 10 and 80 loci (i.e., DNA stretches between adjacent microsatellite loci) simultaneously. Because the PCR products differ in size they need to be analyzed by high resolution PAGE or capillary electrophoresis (Fig. 7). The ISSR loci are inherited co-dominantly and, since some of them are polymorphic, they provide information on the genomic makeup of an individual. In practice, several ISSR primers are used, so that several hundred loci are available for analysis. The advantage of ISSR is that the primers work universally in most animals and plants. There is no need to define PCR primers for an individual species, such as in microsatellite analysis. The results are plotted in a 1/0 matrix and evaluated by cluster analysis (e.g., UPGMA) that places individuals together based on the similarity of their ISSR band patterns.

ISSR can reveal population specific DNA bands, which can be useful to trace back individual birds to individual populations (Wink et al. 2002). Beause ISSR loci are inherited by both sexes, this method also allows the analysis of hybrids and of sex (Wink et al. 1998a, 2000). ISSR markers also can be used to infer phylogenies of closely related taxa, such as genera (Wink et al. 2002, Treutlein et al. 2003a,b).

Molecular Sexing

Another useful molecular method for raptor systematic work is molecular sexing. This technique allows the sexing of birds, which can be difficult in monomorphic species outside the breeding season and in nestlings. In

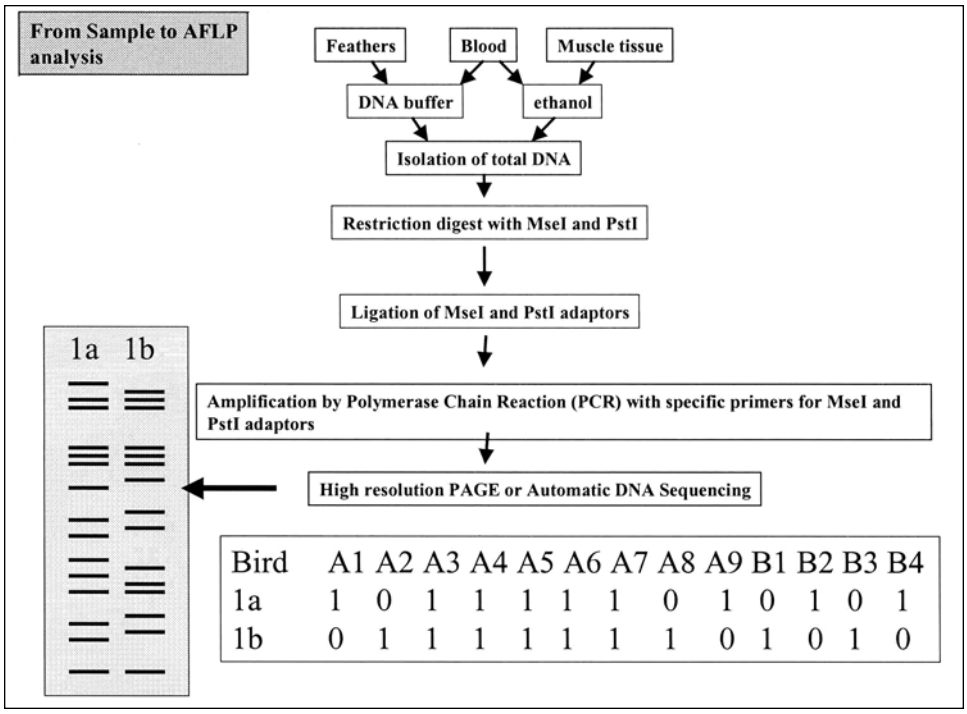


Figure 6. Illustration of the AFLP method.

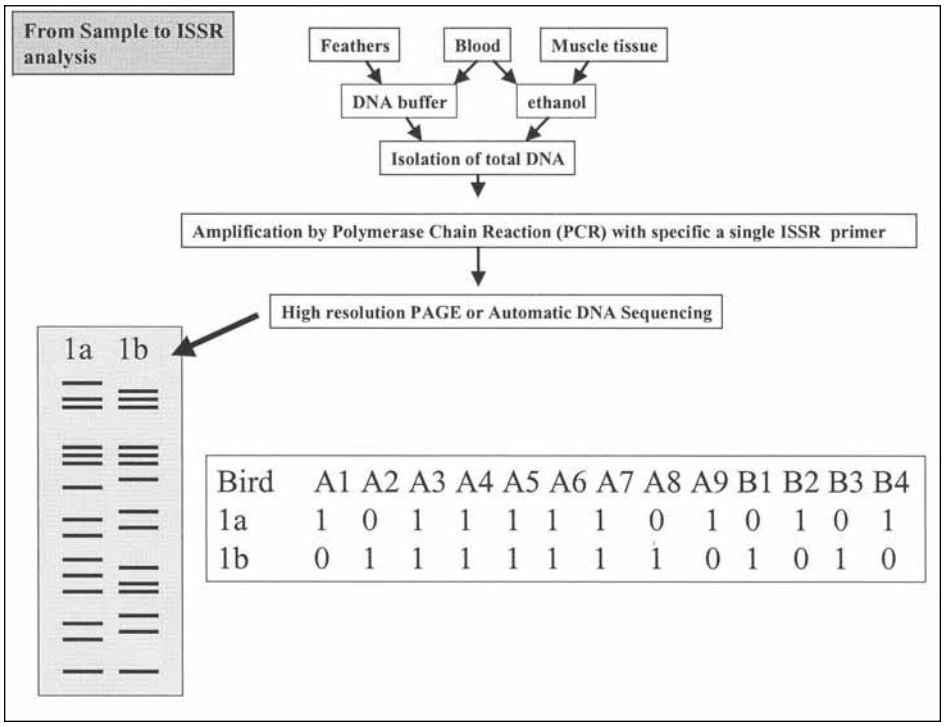


Figure 7. Illustration of the ISSR method.

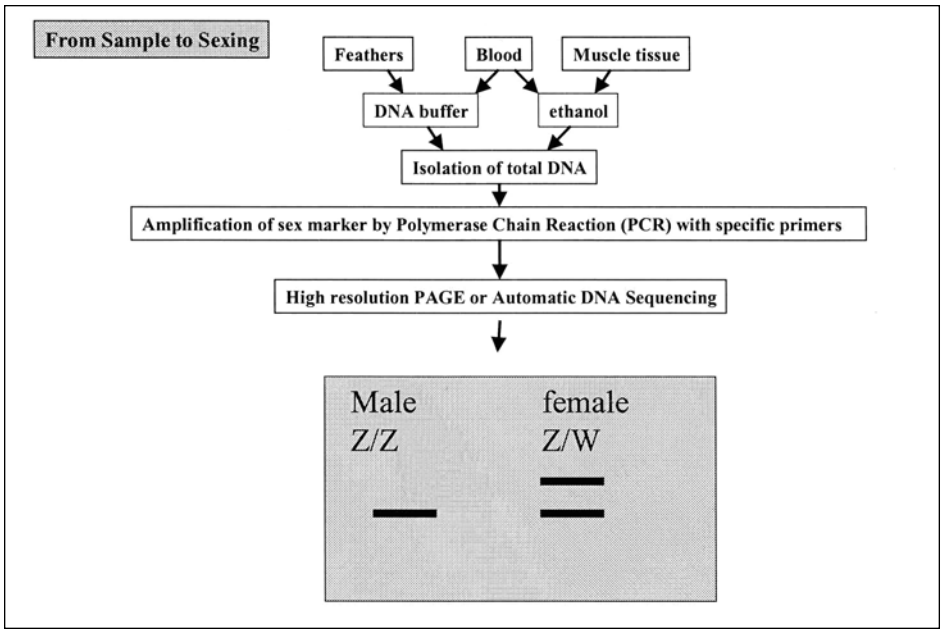


Figure 8. From sample to molecular sexing.

birds, sex chromosomes are in the opposite order as in mammals: females have heterogametic ZW chromosomes, whereas males are homogametic WW. PCR methods have been developed that target introns of the CHD gene present on the sex chromosomes. Because the alleles differ in size, two PCR products can be obtained in females as opposed to one in males (Fig. 8). Using high resolution PAGE, molecular sexing has been successful with all species of birds that have been examined to date (Kahn et al. 1998, Morrison and Maltbie 1999, Höfle et al. 2000, Nesje and Roed 2000b, Becker and Wink 2003, Ristow and Wink 2004, Ristow et al. 2004).

Consequences for Taxonomy and Systematics

According to the rules of cladistics, only monophyletic groups that are derived from a common ancestor should form taxonomic units such as genera, tribes, or families. Because convergent and adaptive characters have been used in traditional taxonomy, not all current taxonomic units are monophyletic. Molecular phylogenies that are less prone to convergence offer the opportunity to detect para- and polyphyletic groups. Examples of the latter found within raptors are vultures and eagles. It had been recognized earlier that New World vultures differ from Old World vultures. Within the Old World vultures, two major and unrelated clades could be determined unambiguously by DNA methods (Wink 1995). Thus, vultures are a polyphyletic assemblage that are adapted to a special lifestyle and have convergently developed certain characters. Eagles of the genus *Aquila* are paraphyletic in that eagles of the genus *Hieraaetus* and *Lophaetus* are not included in the genus despite ancestral relationships to it (Wink et al. 1996, Wink 2000, Wink and Sauer-Gürth 2000, 2004; Helbig et al. 2005). As a consequence, molecular phylogenies will help redefine monophyletic taxa; but this may lead to a change in genus names.

Another point of possible concern is that cryptic species (superficially identical sibling species) have been overlooked because of similar anatomy. Cryptic species appear to be more common in nocturnal than diurnal raptors (Olsen et al. 2002). The use of DNA barcoding will probably result in identifying new species of raptors and owls in the future.

Molecular data also can be used to determine the systematic status of species or subspecies. If subspecies show a high degree of both morphological and genetic differentiation, it is reasonable to treat them as "good" species (Helbig et al. 2002). Recent examples of taxa researchers have suggested should be split into two or more species include: Asian Imperial Eagle (*A. heliaca*) and Spanish Imperial Eagle (*A. adalberti*), and Bonelli's Eagle (*Hieraaetus fasciatus*) and African Hawk-Eagle (*H. spilogaster*) (Wink et al. 1996, Cardia et al. 2000, Wink 2000, Wink and Sauer-Gürth 2000, 2004; Helbig et al. 2005). These changes already have been widely accepted.

SUMMARY

The systematics of raptors has been studied in many ways. Molecular methods in use in evolutionary research also can be applied to taxonomy, phylogenetics, phylogeography, and the population genetics of raptors. These new methods are powerful tools that can supplement information obtained from morphology, geography, behavior, vocalizations, breeding biology, banding, telemetry, and isotope analysis.

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