SHORT COMMUNICATIONS

Museum Collections as a Source of DNA for Studies of Avian Phylogeny

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The use of molecular tools in systematics is now widespread and the only reason some taxa have not yet been studied is that they are rare and unavailable. Recent studies of mammals (Higuchi et al. 1984, Pääbo 1985, Doran et al. 1986) demonstrated that long-dead specimens are a potential source of DNA.

We undertook this study as part of a larger project on the evolutionary relationships of the finfoots (Gruiformes: Heliornithidae). The DNA of the Sungrebe (*Heliornis fulica*) has been compared with some other Gruiformes by solution hybridization (Sibley and Ahlquist 1985). The other 2 members of the family, the African Finfoot (*Podica senegalensis*) and the Asiatic Finfoot (*Heliopais personata*), were unavailable as fresh specimens and were not compared with *Heliornis* in that study.

Although DNA obtained from fresh specimens is always preferred to DNA from museum specimens, DNA is an extremely stable molecule that may survive for hundreds of years under appropriate conditions. This DNA may be partly degraded or contaminated with bacterial DNA, but it is useful for a variety of analyses, including restriction endonuclease mapping, Southern hybridization, nucleotide sequence comparisons, and solution hybridization.

Tests for preservatives.—We tested two specimens of *Heliopais* preserved in alcohol for the presence of unbound formalin by the method of Waller and McAllister (1986). Two dry specimens, one *Podica* and one *Heliopais*, were tested for the presence of arsenic by the method of Hawks and Williams (1986). The *Podica* specimen was also tested for the presence of other preservatives by infrared spectrophotometry and gas chromatography/mass spectrometry at the Smithsonian Institution's Conservation Analytical Laboratory.

DNA isolation.—We extracted DNA from a study skin of *Heliopais personata* (Smithsonian Institution: USNM 534558), from 2 specimens of *H. personata* (Smithsonian Institution: USNM 509508, 509509) preserved in 70% ethanol, and from a dry roughed-out carcass of *Podica senegalensis* (Musée Royal de l'Afrique Centrale, Belgium: 92569), which was catalogued as a skeletal specimen. Tissues of the dry *Heliopais* specimen included 2.0 g of dry skin and scales of the wing and foot, remiges including some in sheaths, and muscles and tendons of the distal appendages. Tissues of the wet *Heliopais* specimens included 3.0 g from USNM 509508 and 3.6 g from USNM 509509 of liver, kidney, pectoral muscle, and subcutaneous fat. Tissues of the *Podica* specimen included 1.2 g of various dry muscles, lung, kidney, and testes. The tissues of the dry specimens were dispersed with a scalpel in 5 ml of extraction buffer solution (0.1 M EDTA [ethylene diaminetetraacetic acid], 0.01 M Tris [trishydroxymethylaminomethane], 0.1 M NaCl, 0.5% SDS [sodium dodecyl sulfate] detergent; all adjusted to pH 8.1 with hydrochloric acid after mixing). The tissues of the wet specimens were dispersed in 2 ml of extraction buffer. Once homogeneous, the volumes of the tissue in all of the solutions were raised to 15 ml by addition of extraction buffer. Proteinase K was added to a final concentration of 200 μg/ml and incubated overnight at 50°C for protein digestion. Ten ml of phenol (saturated with 10 mM Tris buffer pH 8.0) was thoroughly mixed with the samples and then centrifuged at 3,000 rpm for 10 min. The aqueous phase was removed and extracted twice with 10 ml of PCI (phenol : chloroform : isoamyl alcohol in a ratio of 25:24:1). The aqueous phase of the second PCI extraction was extracted with 10 ml of CI (chloroform : isoamyl alcohol in a ratio of 24:1). Each nonaqueous supernatant was extracted a second time with 10 ml TE (10 mM Tris, 0.1 mM EDTA) to recover residual DNA and this was added to final volume of DNA in solution. NaCl concentration was raised to 0.2 M to facilitate precipitation of DNA. Two and one-half volumes of cold ethanol were added to the DNA solutions and precipitation was allowed to proceed overnight at −20°C. The samples were then centrifuged for 30 min at 9,000 rpm at −5°C. The DNA pellets were air dried, washed twice with 15 ml of 70% ethanol, and centrifuged at 9,000 rpm for 10 min at −5°C. Pellets were resuspended in 1 ml TE. The quantity of DNA recovered was determined from its optical density at 260 and 280 nanometers or by spotting 1 μl of the sample alongside 1 μl spots of DNA solutions of known concentration on an agarose plate containing 1 μg/ml of ethidium bromide, and visually comparing the fluorescence of the spots under short wave ultraviolet light.

DNA was extracted by similar protocols from fresh tissues (e.g. red blood cells, heart, and liver) of Sandhill Crane (*Grus canadensis*), Cardinal (*Cardinalis cardinalis*), Alligator (*Alligator mississippiensis*), and a lymphoid tumor from a domestic cat (*Felis domesticus*). These samples were purified further by RNase digestion or cesium chloride density gradient centrifugation or both.

Southern transfer.—We separated DNA samples on

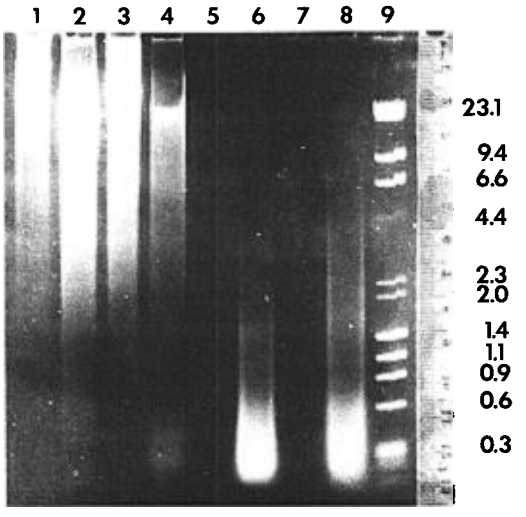

Fig. 1. Electrophoresis of Eco RI digested DNA (10 μg/sample except *Podica*) in an agarose gel. Lane 1: *Grus*; lane 2: *Alligator*; lane 3: *Felis*; lane 4: *Cardinalis*; lane 5: blank; lane 6: 1 μg undigested *Podica* DNA; lane 7: blank; lane 8: *Heliopais*; and lane 9: Molecular weight markers consisting of Hind III-digested DNA of bacteriophage lambda and Hae III-digested DNA of bacteriophage φX174. Units of calibration are kilobases.

vertical electrophoretic gels of 1% agarose at 60 V for 3 h in a Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0). This procedure separates fragments according to molecular mass, which can be estimated by inclusion of appropriate markers in the gel. Two gels were loaded with DNA obtained from the dry museum specimens of *Podica* and *Heliopais*, and the DNA of *Grus, Alligator, Felis, Cardinalis*, and markers (Fig. 1). Ten micrograms (μg) of DNA were digested with Eco RI and loaded into each well, except in the first gel which would be probed with *Podica* DNA 1 μg of undigested *Podica* DNA was loaded. In a second gel which would be probed with *Grus* DNA 1 μg of *Grus* DNA was loaded and the *Heliopais* DNA was undigested. DNA from the wet *Heliopais* specimens were electrophoresed separately. The gels were stained with ethidium bromide and photographed on an ultraviolet light box with a metric ruler alongside the molecular weight markers. The DNA in the gels was denatured by a 15-min immersion in a solution of 0.4 M NaOH and 0.8 M NaCl, and neutralized for 15 min in a solution of 0.5 M Tris-HCl and 1.5 M NaCl, both at room temp. The DNA from each gel was transferred onto a nitrocellulose filter overnight with 10× SSC (1.5 M NaCl, 0.15 M sodium citrate) (Southern 1975). The filters were baked, washed in distilled H_2O, and soaked overnight in a prehybridization solution containing 50% formamide, 200 μg/ml sheared, denatured salmon sperm DNA, 1 M NaCl, 10 mM EDTA, 0.1% sarkosyl (sodiumlaurylsarcosine), 0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% bovine serum albumin, and 50 mM PIPES (piperazinediethanesulfonic acid), pH 6.5. DNA for use as hybridization probes were radiolabeled with ^{32}P by nick translation (Rigby et al. 1977) to specific activities in excess of 2×10^8 counts per minute per microgram of DNA (cpm/μg). Probe DNA was denatured by adding 1/10 volume of 2 N NaOH, mixed directly with the prehybridization solution, sealed in a plastic bag, and incubated overnight at 37°C. The first filter was probed with *Podica* DNA (1.6×10^7 cpm) in 10 ml of hybridization solution. The second filter was probed with *Grus* DNA (1.5×10^7 cpm) in 10 ml of hybridization solution. Both probes were derived from the same *Podica* and *Grus* DNA specimens that were electrophoresed and transferred to the filters. After hybridization the filters were washed twice for 30 minutes each at 37°C in a solution of 2× SSC and 1% sarkosyl. Stringency was then raised by washing the filters for 1 h at 37°C and then again at 50°C in a solution of 0.1× SSC and 0.1% sarkosyl, after which they were wrapped in cellophane and autoradiographed overnight with Kodak XAR film. Detailed descriptions of the rationale, methodology, and usefulness of Southern transfer techniques can be found in Southern (1975) and Maniatis et al. (1982).

Both of the wet *Heliopais* specimens tested negative for unbound formalin. The dry *Heliopais* specimen tested positive for arsenic. The *Podica* specimen showed no indication of treatment with preservatives of any kind. The infrared and mass spectral analyses of the *Podica* specimen indicated that it consisted only of naturally occurring compounds.

We recovered approximately 240 μg of DNA from 2.0 g of dry tissue of the arsenic-treated study skin of *Heliopais personata*. We recovered 410 μg DNA from 1.2 g of dry tissue of the untreated carcass of *Podica senegalensis*. These quantities represent about 5–10% and 10–20%, respectively, of what might be expected from fresh tissue. The ratio of optical densities at 260 and 280 nm of the *Podica* sample was 1.8, indicating that the DNA was relatively free of RNA and protein contamination. Although the yield of DNA was more than twice as great from the *Podica* specimen as from the *Heliopais* specimen, the *Podica* DNA was further degraded than the *Heliopais* DNA. The majority of DNA in both samples was only 200–300 bases long (Fig. 1), but fragments up to 6 kb of *Podica* and fragments at least 9 kb, possibly even 20 kb, of *Heliopais* were identified as avian DNA by hybridization with our probes (Fig. 2).

The yield of DNA from the wet *Heliopais* specimens was much lower than from the dry specimens. The largest DNA fragment we obtained from the wet specimens was about 200 bases, which is too small to bind to nitrocellulose filters for Southern transfer.

In the blot probed with the putative *Podica* DNA, the strongest hybridization occurred between the probe and itself in lane 6 (Fig. 2). The probe hybridized with nearly equal intensity to the putative *He-*

liopais DNA, with marked less intensity to *Grus*, and still less with *Cardinalis*. Hybridization with the *Alligator* sample was very weak. There was virtually no hybridization between the *Podica* probe and the *Felis* or the phage molecular weight markers.

In the blot probed with *Grus* DNA, the strongest hybridization reactions occurred between the probe and itself (lane 1). The *Grus* probe hybridized about equally with *Podica*, *Heliopais*, and *Cardinalis*. It hybridized very weakly with *Alligator*, and virtually no hybridization occurred between the probe and *Felis* or the phage markers.

Ideally, various chemicals used to preserve museum specimens should be surveyed to determine their different effects on DNA. With such knowledge one could better judge which specimens might be expected to contain DNA in the best condition. This is an important consideration because part of the specimen must be destroyed to extract the DNA. It is likely that a specimen will be sampled primarily because it is rare and otherwise unavailable. Such specimens should not be damaged unnecessarily.

Once DNA has been successfully extracted from non-fresh tissue, the most important questions are whether this DNA is endogenous and to what extent it is degraded. We believe the DNA recovered from the museum specimens and identified by Southern transfer was definitely the DNA of *Podica senegalensis* and *Heliopais personata*. The relative intensity of hybridization accurately reflected the expected phylogenetic relationships of these organisms. The crane DNA probe hybridized extensively with the single stranded avian DNA on the filter, but less so with the alligator, and not at all with the cat or phage DNA. The *Podica* DNA probe hybridized most avidly with itself and the other putative heliornithid. Like the crane probe, it hybridized to the other avian, but not the nonavian, DNA specimens. Bacterial DNA, a likely contaminant, would have hybridized with equal intensity to all the vertebrate DNA. The fact that the *Podica* sample could be labelled to a high specific activity by nick translation indicates that this DNA was a good substrate for both DNase I and DNA polymerase I, and is thus biologically viable.

The amount and condition of DNA we obtained from the two alcohol specimens of *Heliopais* were inadequate for use in phylogenetic studies. We do not know the cause of this degradation. It is possible that endogenous nucleases remain for longer periods in fluid-preserved specimens than in dried specimens. Alternatively, it is possible that these particular specimens were treated previously with formalin, which would have destroyed the DNA, even though they tested negatively for unbound formalin.

Any method of detecting or measuring homology of two or more organisms is potentially useful for investigating phylogenetics and genetics. Molecular approaches to systematics are varied; each technique possesses its own strengths and weaknesses for a given systematic problem. A major advantage of studying DNA is that it is an extremely stable molecule and that samples from rare or extinct organisms may be available from museum specimens.

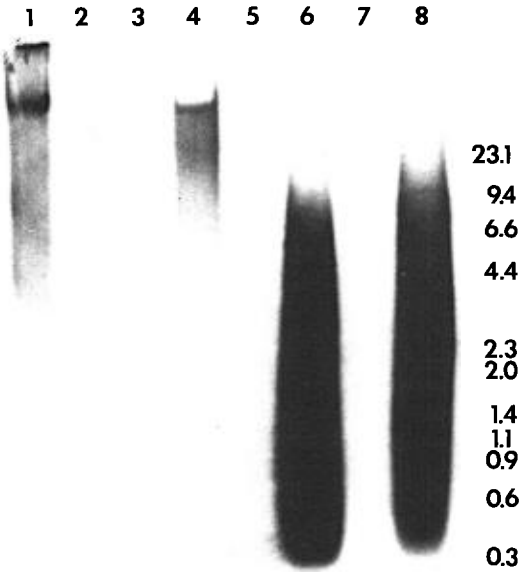

Fig. 2. Southern hybridization of the DNA in Fig. 1 with *Podica* DNA as a probe. Intensity of hybridization is strongest to *Podica* (lane 6) and *Heliopais* (lane 8), then *Grus* (lane 1), less to *Cardinalis* (lane 4), and weak to *Alligator* (lane 2). There was no hybridization to *Felis* or the phage markers, so neither of these is visible. Units of calibration are kilobases.

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Note added in proof—Digestion with Nuclease SI, which selectively destroys ssDNA, demonstrated that the majority of DNA we recovered from the museum specimens was ssDNA, as estimated by 1.0% agarose gel electrophoresis (3 h, 60 V). This ssDNA was converted into Nuclease SI-resistant dsDNA by treatment with high concentrations of Large Fragment *E. coli* DNA Polymerase I (10 units/μg, 20 h, 15°C) and Avian Myeloblastosis Virus Reverse Transcriptase (10 units/μg, 1 h, 42°C) without the addition of oligonucleotide primers.

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Application of Synaptonemal Complex Techniques for Determination of Diploid Number and Chromosomal Morphology in Birds

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Avian karyological studies are often complicated by the large numbers of microchromosomes which characterize the chromosomal complements of most bird species (Shields 1982, 1983). The exact number of microchromosomes can be difficult to ascertain, as they are often covered by the macrochromosomes or lost during preparation of mitotic material for cytological analysis. In addition, the small size of the microchromosomes makes it difficult or impossible to determine the position of their centromeres. Consequently, many reports of bird karyotypes present modal or approximate diploid numbers (e.g. Biederman et al. 1980), or emphasize the morphology of the macrochromosomes (e.g. Bartlett and Threlfall 1987). We discuss a technique for accurate determination of the diploid number and chromosomal morphology of birds by electron microscopic analysis of silver-stained synaptonemal complexes.

Fig. 1. Giemsa stained somatic karyotype prepared from hypotonically-treated bone marrow of a female *Colinus virginianus*. The diploid number is clearly 82; however, the morphology of the smaller chromosomes cannot be determined from this type of preparation.

The synaptonemal complex (SC) is a ribbonlike proteinaceous structure which forms the longitudinal axis of pachytene bivalents during meiotic prophase I. Each meiotic chromosome forms an axis which, upon pairing with its homolog, becomes one of the lateral elements of the SC. Staining with silver differentiates preferentially the two lateral elements of the SC from their associated chromatin and surrounding nucleoplasm, thereby providing simple linear representations of chromosomal behavior and orientation during pachynema. In addition, the positions of the centromeres of the meiotic chromosomes are often discernible as dark or thickened regions on the lateral elements of the SCs in early pachytene nuclei.

The technique for obtaining pachytene nuclei from the testes of adult male birds (in breeding condition) was modified from one described for mammalian testicular material (Moses 1977). We used plastic-coated microscope slides to pick up the spermatocyte nuclei after spreading on the convex surface of a 0.5% (w/v) NaCl solution. The slides were prepared by dipping them into a Coplin jar that contained a 0.6% (w/v) solution of plastic (Falcon Optilux petri dish) in chloroform. After air-drying, the edges of the plastic coat