A Comparison of Solution and Membrane-Bound DNA x DNA Hybridization, as Used to Infer Phylogeny

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Abstract. A new method of membrane-bound DNA x DNA hybridization was devised to accommodate the study of small quantities of DNA obtained from museum specimens for phylogeny reconstruction. Membrane-bound, single-stranded target genomic DNAs were competitively hybridized with a total genomic DNA probe to form hybrid duplexes required for the DNA dissociation experiments. We compared the thermal elution profiles derived from dissociating duplexes made with probes of whole genomic, single-copy, and repetitive DNA, as well as solution DNA x DNA hybridization using sc tracer. Quantitatively, pairwise indices of genetic distance derived from duplexes made with genomic probes depended entirely on hybridization of repetitive sequences, but a parallel set of experiments using repetitive and sc probes produced qualitatively similar results. The indices of genetic distance generated by the membrane-bound hybrids form an internally consistent, resolved tree which is in agreement with the solution DNA x DNA hybridization trials and traditional views of the phylogeny of the taxa under study.

Key words: DNA x DNA hybridization — Southern hybridization — Repetitive DNA — Ancient DNA

Introduction

While preparing for a comparative DNA study of the phylogeny of the avian family of finfoots (Gruiformes: Heliornithidae) we were faced with a problem—two species were rare and unavailable as fresh specimens. For these species, we isolated DNA from museum specimens about 30 years old (Houde and Braun 1988). The methods that have been commonly used to characterize old or ancient DNA are generally limited to sequencing of short amplified or cloned sequences of single genes (e.g., Higuchi et al. 1984; Pääbo 1985). Ancient DNA has never been used for DNA x DNA hybridization, which is otherwise an effective method of assaying genetic similarity and estimating phylogenies (Springer and Krajewski 1989; Bledsoe and Raikow 1990; Bledsoe and Sheldon 1990; Sheldon and Kinnaird 1993). In performing Southern hybridization (Southern 1975) with the museum DNA, we were led to suspect that the Southern method could be modified for use with small samples of degraded DNA to provide data similar to that produced by solution DNA x DNA hybridization. With this in mind, we developed a new technique of membrane-bound DNA x DNA hybridization similar to the methods of Johnson (1981) and Wren et al. (1989) except that we measured duplex DNA on a membrane substrate rather than dissociated DNA in solution. This difference enabled us to hybridize several samples competitively and to distinguish between homoduplexes and each of the heteroduplexes.

To study the properties and effectiveness of the membrane-bound method, we prepared hybrids using probes made from total genomic DNA, the single-copy fraction, and the repetitive fraction. We compared the results of these experiments with one another and with traditional solution DNA x DNA hybridization experiments (e.g.,
Britten et al. (1974) using the same DNA samples. These experiments provided insight into the nature of single-copy and repetitive DNA hybridization, the effect of sample degradation on DNA hybridization results, and the systematic theory of DNA hybridization.

Systematic Issues

We were interested in reconsidering the phylogenetic relationships of three groups of gruiform birds—finfoots (Helornithidae), cranes (Gruidae), and Limpkin (Aramidae). The finfoots present an interesting evolutionary problem because there are three species with a pantropical distribution: the Masked or Asiatic Finfoot (Heliopais personata), the African Finfoot (Podica segenalis), and the Neotropical Sungrebe (Heliornis fulica). Heliopais and Podica are the two taxa whose DNA was only available from museum specimens. The distribution of finfoots might reflect an ancient origin and isolation by seafloor spreading. Whether they do hinges on their infrafamilial phylogenetic relationships and on their relationships to the Neotropical Limpkin (Aramus guarauna). Traditionally, Aramus has been placed with the cranes, near or within the Gruidae (e.g., Olson 1985), but Sibley and Ahlquist (1990) suggest that it might be the sister of Heliornis.

Materials and Methods

First Experimental Set: Membrane Hybridization with Genomic Probes. Genomic DNA was isolated from fresh specimens of Sandhill Crane (Grus canadensis), Limpkin (Aramus guarauna), Sungrebe (Heliornis fulica), and Cardinal (Cardinalis cardinalis). Genomic DNA was isolated from museum specimens of African Finfoot (Podica segenalis) and Masked Finfoot (Heliopais personata), both about 30 years old, as described by Houde and Braun (1988). Tissues consisted of frozen red blood cells (Grus), frozen muscle and liver (Aramus, Cardinalis), muscle and liver stored in 70% EtOH (Heliornis and Heliopais), dried muscle (Podica), and dried skin and muscle preserved with arsenic (Heliopais). In each case, 1-2 g of tissue was suspended in 10-15 ml solution of 100 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% SDS. Cohesive tissues were quick frozen in liquid nitrogen and ground with mortar and pestle. Protein was digested with 200 μg/ml proteinase K overnight at 37°C. Alcohol-preserved muscle and liver of Heliopais was digested for 4 additional days, with continuous inversion on a rotary deck and with fresh proteinase K added every 24 h. An equal volume of Tris-buffered phenol was thoroughly mixed by gentle inversion and then separated by centrifugation at ca. 3,000 rpm at room temperature (RT) for 10 min. The aqueous phase was removed and similarly extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). NaCl concentration was raised to 1 M and 2.5 vol cold EtOH were added to precipitate the nucleic acids. DNA was collected by spooling (fresh specimens) or centrifugation (museum specimens). Pellets were washed with 70% EtOH, vacuum dried, and resuspended in 0.5 ml TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). RNase was added to a concentration of 100 μg/ml and incubated for 1 h at 37°C. Some samples were further purified by CsCl density centrifugation and additional RNase digestion until the ratio of optical density at 260/280 nm equaled 1.80 ± 0.02.

DNA concentration was determined by optical density at RT. Concentration was adjusted to 200 μg/ml with TE and remeasured. One milliliter of DNA was sonicated with a Branson sonifier cell disrupter 200 for 8 min at power setting 3, 50% duty cycle in an Eppendorf microfuge tube set in an ice bath. DNA concentration was remeasured and adjusted to 2 μg/ml. The ratio of optical density at 260/280 nm was generally 0.8-0.91 higher than before sonication.

DNA obtained from museum specimens was not sonified, but instead gel purified to remove strands shorter and longer than those produced by sonification of high-molecular-weight DNA. Three to 4 mg Podica and Heliopais DNA was electrophoresed on separate 1.5% agarose gels at 90 V for 1 h alongside sized Grus DNA and molecular weight markers. The gel was stained with ethidium bromide and examined on an ultraviolet transilluminator. DNA corresponding in weight to the Grus DNA was excised and electroeluted with a Schleicher and Schuell Elutrap at 200 V overnight at RT. DNA was washed with TE and concentrated in Centricon 30 tubes which were centrifuged at 2,000 rpm for at least 1 h at RT. NaCl was added to 1 M. Equal volumes of n-butanol were mixed with the DNA and allowed to separate to remove residual EthBr. Two-and-one-half volumes of 70% EthOH was added and DNA was allowed to precipitate overnight at -20°C. Precipitates were centrifuged for 15 min at 7,000 rpm at 4°C. Pellets were washed with 70% EthOH, vacuum dried, and resuspended in 500 mM NaCl, 50 mM MOPS pH 7.0. DNA was further purified with Qiagen Pack 100 ion-exchange columns. Impurities were eluted with 750 mM NaCl, 50 mM MOPS, 15% EtOH pH 7.0 and DNA was eluted with 1.5 M NaCl, 50 mM MOPS, 15% EtOH pH 7.5 (Qiagen instructions). DNA was precipitated with 0.5 vol isopropanol chilled on ice for 15 min and then centrifuged at 7,000 rpm for 15 min. Pellets were washed with 70% EthOH, vacuum dried, and resuspended in TE to a concentration of 2 μg/ml.

Each DNA specimen (500 ng) was electrophoresed for 1 h at 90 V on a 1.5% agarose gel alongside 123-bp ladder molecular-weight marker and HindIII-digested bacteriophage lambda DNA. Gels were stained with EthBr and photographed on a UV light box (Fig. 1). DNA preparations were selected for uniformity in size (200-550 bp) and staining intensity.

DNA specimens were diluted 50% with deionized formamide to 1
μg/ml, denatured at 95°C for 5 min, and then placed on ice. Uncharged nylon hybridization membranes (N04-HY000-10, 0.45 μm, Micron Separations, Inc.) and 3MM Whatman paper were preincubated to fit a 8 × 12-well dot-blot manifold (MiniCell II Schleicher and Schuell). A numbered grid was drawn with pencil onto each membrane to match the dot-blot manifold. Membranes were presoaked for 10 min in 50 mM TBE (45 mM Tris-borate, 1 mM EDTA) and then stacked on top of two briefly soaked pieces of Whatman paper in the dot-blot manifold. One hundred nanograms DNA in 100 μl was loaded into each well of the dot-blot manifold and applied to the membrane with aspiration. DNA of each species was applied in a row such that the order of all six species (two separate specimens of Heliorbis were used) was repeated in 12 columns. DNA was crosslinked to the membrane with shortwave ultraviolet light (Church and Gilbert 1984). Fourteen identical membranes were prepared for hybridization.

In a preliminary set of experiments using only Gras, Aramus, Heliorbis, and Cardinalis we blotted 90, 95, 100, and 105 ng of target DNA of each species in the same hybridization experiment to determine whether uncertainty of target DNA concentration or pipetting errors up to 15% could affect the thermal elution curves. As this had no effect on relative thermal stability and the positions of the thermal elution curves, it will not be discussed further.

Each of the same sized-DNA specimens that were crosslinked to membranes were used also as probes—except Heliorbis, which was probe with a different species that was not sized. Sheared genomic DNA was labeled by random priming using one radiolabeled nucleotide (alpha-32P-dCTP 3,000 Ci/mM; Feinberg and Vogelstein 1984) to specific activities (cpm/25 ng/ml) of 1.5–4.5 × 10^7. Probe DNAs were not resized by gel electrophoresis subsequent to the labeling reaction.

Membranes were sealed in food-storage bags (2 membranes/bag, back to back) and incubated at 60°C for 10 min in 60 μl prehybridization solution (0.25 mM Na2HPO4, 7H2O pH 7.2, 1% crystalline bovine serum albumin, 1 mM EDTA, 7% SDS). One milliliter radiolabeled probe was injected into each bag and resealed. Several membranes in bags were incubated at a time in an immobile vertical position in a model 2067 Forma Scientific CHIP bath and circulator. Temperature of the bath was lowered incrementally over a period of 5 days. Reactions using Aramus, Heliorbis #1, and Podice as probes (filters 1–6) were incubated at 75°C 10 min, 65°C 6 h, 60°C 14 h, 55°C 12.5 h, 50°C 11.5 h, 45°C 13 h, 40°C 11 h, 35°C 24 h, 30°C 9 h, 25°C 3 h, and 20°C 12 h. Reactions using Gras, Heliorbis #2, Heliorbis, and Cardinalis as probes (filters 9–16) were incubated at 60°C 17.5 h, 55°C 8 h, 50°C 16 h, 45°C 10.5 h, 40°C 13 h, 35°C 12 h, 30°C 13.3 h, 25°C 10.3 h, and 20°C 15 h.

Two membranes each were added to 400 μl wash solution (40 mM Na2HPO4 7H2O pH 7.2, 1 mM EDTA, 1% SDS) precooled to 20°C in resealable plastic food-storage bags. Four bags at a time were suspended in the bath and circulator. Temperature was raised in 5°C increments from 20°C to 75°C. After 20-min incubation at each temperature, a strip of each membrane containing one row of eight DNA dot blots (different species) was cut off and air dried. Temperature was raised 5°C and the process was repeated until all 12 rows were removed. Approximately 15 min was required for each temperature increase. The filters were reassembled and autoradiographed for 1 h. Each membrane was cut into 96 pieces along the numbered premarked grid such that each piece of membrane contained exactly one hybridized dot blot.

Each hybrid DNA blot was denatured with 1 ml 1 M HCl in a 7-ml polystyrene scintillation vial for 20 min. Three milliliters liquid scintillation fluid (DuPont Formula 989) was added to each vial and shaken vigorously. Each sample was counted for 2 min (the limiting statistical parameter, sigma 2 set to 0.5% = 200K disintegrations, or 2–3 orders of magnitude higher than the recording range) in a liquid scintillation counter.

Scintillation counts (high-energy beta emissions) were log-transformed and replicate data (one datum from both filters in each pair) were averaged. Individual values that were 20% greater than the average of the two were discarded to eliminate erroneously high values that resulted from equipment malfunction (i.e., occasional electrical discharges from the refrigeration unit of our Tracer scintillation counter that caused samples to phosphoresce). In a single case (Heliorbis #2 probe × Gras, 20°C) out of 1,556 data, we discarded the lower of two such values. Thermal elution curves were plotted and their cubic regression lines were calculated with Sigmaplot version 3.1 (Jandel Scientific). We found that thermal elution curves typically exhibited an inflection and that residual values were substantially smaller when third-order rather than when second-order regressions were used. Tm' was calculated as the temperature intercept of the mean of the dissociation curve's maximum and minimum (log counts per minute [log cpm]; program by A. Houde in Turbo Pascal available upon request). Data were log-transformed to improve separation of heteroduplex curves at intermediate melting temperatures. Delta Tm' is the difference between Tm' of a homoduplex hybrid and Tm' of a heteroduplex hybrid probed with the same species in the same experimental set. The complete distance matrix was folded by averaging reciprocal delta Tm's. The folded delta Tm's were fitted to trees using the Cavalli-Sforza and Edwards (1967) option of the ‘Fitch’ program in PHYLIP 3.4 (Felsenstein 1989). Cardinalis was designated as the outgroup. The integrated area beneath each thermal elution curve was calculated (program by A. Houde in Turbo Pascal available upon request). Normalized integral of reassociation (NIR) was computed as the integral of a heterologous thermal elution curve divided by the integral of the homologous curve of the same experimental set, times 100.

Second Experimental Set: Membrane Hybridizations with Single-Copy and Repetitive Probes. Genomic DNA of Gras canadensis, Aramus guarauna, and Heliorbis falcus was blotted to six separate membranes as described above, with a replicate set of DNA dots to fill six rows of the dot-blot manifold. The two museum specimens, Podice and Heliorbis, were not used because quantities of their DNA were limiting and the question of their relationship was addressed adequately in the first experimental set. New isolates of genomic DNA of Aramus guarauna and Heliorbis falcus #1 were prepared, as described above. Genomic DNA of each of these three species, including the new preparations, was separated to C0 1000 into single-copy (sc) and repetitive fractions by A. Cacock. DNA was sheared to an average length of 800 bp. Twenty-five nanograms of each of the sc and repetitive fractions of each species were radiolabeled to specific activities of 2.7–8.0 × 10^7 cpm, as described above, except that sc DNA was not denatured by boiling prior to labeling. Each filter was individually hybridized in 15–20 ml of prehybridization solution and incubated at 65°C 19 h, 60°C 22 h, and at each successively lower 5°C increment to 20°C for 12 h. Thermal elution was performed as described above, but filters were not allowed to air dry, nor were they autoradiographed. Each hybrid DNA blot was added directly to 4 ml of scintillation cocktail, shaken vigorously, and counted twice for 2 min. Data were plotted and delta Tm' was calculated by the methods described above.

Third Experimental Set: Solution Hybridization with Single-Copy Tracers. Solution DNA hybrids were prepared using the method of Britten et al. (1974) modified by Sibele and Al queries (e.g., 1981, 1990), Sheldon et al. (1992), and Sheldon and Kinmarney (1993). Genomic DNA of Gras canadensis, Baleaerica pavo (Black Crowned Crane), Aramus guarauna, Heliorbis falcus, and Gallus gallus (domestic chicken) was sonified into ca. 500 base-pair fragments. Sc DNA was prepared by reassociating 200 μg of DNA to C0 1000 at 50°C in 0.48 M sodium phosphate buffer. Probe DNA was oligolabeled with tritium. Hybrids were formed in 0.48 M sodium phosphate buffer by reassociating 0.002 μg (20,000–50,000 dpm) of probe and ca. 10–20 μg target DNA to C0 > 22,000. They were dissociated in lots of 35 in a thermal elution device similar to that of Sibley and Alqueries (1981) and Kirsch et al. (1990). Thermal fractions were taken from 60°C–90°C at 2.5°C increments by pumping 4 ml of 0.12 M sodium phosphate buffer...
through columns consisting of 1 ml hydroxyapatite in 5-ml syringe barrels and collecting the eluate in 20-ml scintillation vials. Scintillation cocktail (15 ml) was added, and the vials were shaken and counted.

Three replicate hybrids were prepared for each pairwise comparison. Because of mechanical problems, we produced somewhat fewer hybrids than this, but achieved at least one measurement per cell in the 5 × 5 matrix, except Aramus × Gallus.

Tm’s of dissociation distributions were calculated as described in Sheldon and Bledsoe (1989). Delta Tm’s were computed as the differences between the average homoduplex Tm and individual heteroduplex Tm’s. Trees were estimated using the Cavalli-Sforza and Edwards (1967) option of the “Fitch” program in PHYLIP 3.4 (Felsenstein 1989), with Gallus designated as the outgroup.

Results

First Experimental Set

Thermal elution profiles are presented in Fig. 2. Tmid and delta Tmid values are summarized in Tables 1 and 2. Only one phylogenetic reconstruction (Fig. 3) is supported by the complete dataset, and its accords well with traditional classifications of these birds (e.g., Wetmore 1960). All of the members of the traditionally recognized family Heliornithidae are thermodynamically most stable when hybridized to one another. Heliornis and Heliopais

Fig. 2. Thermal elution profiles of the first membrane-hybridization experimental set. A Grus probe; B Aramus probe; C Heliornis #1 probe; D Heliornis #2 probe; E Podica probe; F Heliopais probe; and G Cardinalis probe. Open circle Grus target; closed circle Aramus target; open triangle point up Heliornis #1 target; closed triangle point up Heliornis #2 target; open square Podica target; closed square Heliopais target; and open triangle point down Cardinalis target. X axis is temperature (°C); y axis is log cpm.

Fig. 3. Phylogenetic reconstruction based on the first membrane-bound experimental set, with Cardinalis specified as an outgroup. Upper values at nodes indicate the range of all delta Tmid’s in the folded matrix of the first experimental set. Lower values at nodes indicate the average delta Tm in the folded matrix of the third experimental set, in those cases in which comparisons were made.
Table 1. Tmid’s of the first experimental set

<table>
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<tr>
<th></th>
<th>GRU</th>
<th>ARA</th>
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<th>HR2</th>
<th>POD</th>
<th>HEP</th>
<th>CAR</th>
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<td>62.8</td>
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</tbody>
</table>

a Taxa in column headings are probes; those in row headings are targets. Abbreviations: GRU Grus, ARA Aramus, HR1 Heliornis #1, HR2 Heliornis #2, POD Podica, HEP Heliopais, and CAR Cardinalis

Table 2. Folded matrix of delta Tmid’s of the first experimental set

<table>
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a Taxa in column headings are probes; those in row headings are targets. Abbreviations as in Table 1

Examples of thermal elution profiles are presented in Fig. 5. Tm and delta Tm values are summarized in Tables 6 and 7. The solution hybridization experiments support the same branching topology as the previous experimental sets, although Heliopais and Podica were not included in this set and Gallus (order Galliformes) was used instead of Cardinalis as the outgroup. Delta Tm values are smaller than delta Tmid’s of the first experimental set and those of the repetitive probes in the second experimental set. Delta Tm values are greater than delta Tmid’s of the second experimental set in which sc probes were used.

Discussion

Retroviral vs Single-Copy Sequences in DNA Hybridization

Membrane-bound (mb) DNA hybridization of whole genomic and repetitive DNA clearly provides information useful for phylogenetic inference. The distances produced are internally consistent and concordant with traditional classification of these birds based on morphology and solution sc DNA hybridization. Ironically, repetitive DNA appears to provide most of the phylogenetic signal in this system. (See Methodological Considerations.) Such a result is surprising given that repetitive DNA is commonly removed from genomes before hybridization on the rationale that it overly complicates the system (e.g., Britten et al. 1974). While such complication may be expected, in general the inclusion of homologous repetitive elements in DNA hybrids should yield useful phylogenetic information. Not only will sequence similarities diverge in time, but so will sequence copy numbers. Thus, there are two levels of similarity assayed by hybridization. Consistent with this notion, Schluze
and Lee (1986) obtained similar results for hybrids made with SC and middle-order repetitive DNA, and others (Hoyer et al. 1964; Britten and Davidson 1971; Doolittle 1985) have noted greater similarity of repetitive fractions of genomes of closely related than distantly related species.

There are problems inherent in repetitive DNA hybridization that we had to overcome. The inadvertent inclusion of paralogous sequences jeopardizes phylogenetic inference because nonhomologous DNA reassociates and contributes to measures of genetic distance (Sar-

Fig. 4. Thermal elution profiles of the second membrane-hybridization experimental set. Left (A,C,E): fractioned single-copy DNA probes; right (B,D,F): fractioned repetitive DNA probes; top to bottom: Grus probes (A,B), Aramus probes (C,D), and Heliornis probes (E,F). Open circle Grus target, closed circle Aramus target, open triangle point-up Heliornis target. Note the spread between the curves of the right, but not the left, plots. X axis is temperature (°C); y axis is log counts per minute.

Table 4. Delta Tmid’s of the second experimental set using fractioned single-copy DNA probes

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* Taxa in column headings are probes; those in row headings are targets. Abbreviations as in Table 1.

Table 5. Delta Tmid’s of the second experimental set using fractioned repetitive DNA probes

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* Taxa in column headings are probes; those in row headings are targets. Abbreviations as in Table 1.

Fig. 5. Representative solution DNA hybridization dissociation curves in which Balearia pavonina is radiolabeled. A. Standard frequency distribution used to estimate modal dissociation values. B Cumulative frequency distribution used to estimate mean dissociation values (Tm). Closed square, Grus canadensis; open square, Balearia pavonina; closed triangle point down, Aramus guarauna; open triangle point down, Heliornis fulica; closed circle, Gallus gallus.

ich et al. 1989; Schmid and Marks 1990). Also, if few highly similar sequences occur in high copy numbers, they may exaggerate genetic similarity of otherwise-diverged species. Finally, concerted evolution (Dover et al. 1981; Doolittle 1985), which may slow or speed the
Table 6. Tm's of the third experimental set*

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<td>86.2</td>
</tr>
</tbody>
</table>

* Taxa in column headings are tracers (probes); those in row headings are drivers (targets). Abbreviations as in Table 1 and BAL Baleine, GAL Gallus. *= no data

Table 7. Delta Tm's of the solution DNA x DNA hybridization experimental set*

<table>
<thead>
<tr>
<th></th>
<th>GRU</th>
<th>BAL</th>
<th>ARA</th>
<th>HR1</th>
<th>GAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRU</td>
<td>—</td>
<td>3.1</td>
<td>6.6</td>
<td>11.6</td>
<td>15.9</td>
</tr>
<tr>
<td>BAL</td>
<td>3.8</td>
<td>—</td>
<td>6.5</td>
<td>11.8</td>
<td>16.2</td>
</tr>
<tr>
<td>ARA</td>
<td>5.4</td>
<td>5.6</td>
<td>—</td>
<td>11.8</td>
<td>16.3</td>
</tr>
<tr>
<td>HR1</td>
<td>13.4</td>
<td>12.8</td>
<td>13.5</td>
<td>—</td>
<td>16.9</td>
</tr>
<tr>
<td>GAL</td>
<td>15.4</td>
<td>14.9</td>
<td>*</td>
<td>13.5</td>
<td>—</td>
</tr>
</tbody>
</table>

* Taxa in column headings are tracers (probes); those in row headings are drivers (targets). Abbreviations as in Tables 1 and 7. *= no data

divergence of repetitive but not single-copy elements, could distort estimates of genetic distance. Why these potential pitfalls have not completely obfuscated the phylogenetic signal in our data appears to be the result of a combination of factors, including (1) our method of forming hybrids and the phenomenon of self-hybridization, (2) the insensitivity of our frequency distribution and hybrid indices (particularly Tm high) to reassociated paralogous and high-copy-number sequences, and (3) the lack of effect from concerted evolution on DNA hybridization estimates of phylogenies. All are discussed below.

Self-Hybridization

For heteroduplex hybrids, radiolabeled probe DNA tends to reassociate more readily with itself than with driver DNA. This occurs because probe sequences can find identical partners among themselves. When repetitive DNA is included in the probe DNA, the rate of self-hybridization increases dramatically because of the increased probability of repetitive sequences finding a partner (e.g., Britten et al. 1974).

There are two main ramifications of self-hybridization. In solution hybridization, self-hybridization introduces error into the system because self-hybrids increase the measured melting temperature in heteroduplex hybrids. Furthermore, both strands in each homoduplex are labeled and so counted as two heteroduplexes. To alleviate this problem in solution hybridization, probe DNAs are introduced in small amounts relative to driver DNAs and repetitive DNAs are removed from the probe. In mb DNA hybridization, self-hybridization does not present a problem because self-hybrids are not measured since they form and remain in solution. Only hybrids that occur between the solution-based probe and the membrane-bound target are measured. More importantly, high-copy-number repetitive DNA is probably quickly eliminated from the probe by self-hybridization in solution before cross-hybridization on the filters. Thus, the effects of anomalous amounts of repetitive DNA in the system are minimized.

Frequency Distribution

The elution curve of solution hybridization is often expressed as a cumulative frequency distribution (i.e., to calculate Tm, T50H, and NPR, but not Tmode). Each elute of ss DNA is added to the sum of all those eluted at lower temperatures. Thus, noise introduced into the curve from poorly matched sequences at low temperatures affects the entire curve. Furthermore, Tm and T50H are calculated from the entire curve, including the "low-temperature foot" if present. The mb DNA method does neither.

The elution curve of mb DNA hybridization resembles the inverse of the solution hybridization curve because the y-axis represents raw (log) counts of ds DNA rather than percent counts of ss DNA (Fig. 6). The frequency distribution can be thought of as "subtractive" instead of additive. The waning population of ds DNA is monitored as increasingly well-matched duplexes are removed by melting. Eluates are not measured, so se-
sequences that melt have no additive effect on the curve at any higher temperatures. The curve can be thought of as additive from high to low temperatures (i.e., reverse orientation); the addition of increasingly mismatched sequences builds on a frequency distribution of better-matched sequences—not the addition of increasingly well-matched sequences to mismatched sequences, as in solution hybridization. However, the mb hybridization elution curve is not literally cumulative because ds DNA at each temperature is measured from different DNA samples on the membrane substrate and it is not normalized.

mb DNA Hybrid Indices

The indices we used to characterize the stability (Tmid) and reassociation (NIR) of the mb DNA hybrids are different than those normally employed in solution DNA hybridization (Tm, T50H, Tmode, and NPR). The choice of these indices was the result mainly of methodological differences between mb DNA and solution DNA hybridization. These indices have proved largely insensitive to noise in the data, such as might be introduced by repetitive DNA.

Tmid Index

Tmid is the temperature (x) intercept of the mean of only two points, the maximum and minimum (y values) of the fitted curve. Tmid will be only as high as stable heteroduplexes remain on the membrane, so only the elevation of the curve to the right of Tmid (high temp.) affects Tmid. For example, if two curves have the same maximum and minimum values, but one is convex down and the other is convex up, then the convex-up curve will exhibit a higher Tmid. This is true even if the curve that is convex down has a higher maximum count value and, thus, a higher average number of counts (Fig. 7). In so doing, Tmid distinguishes sequence-level similarity (copy number; reassociation) from site-level similarity (divergence; melting). High levels of reassociation of repeated sequences of low similarity (i.e., paralogous sequences), if anything, actually lower Tmid by raising the average of the minimum and maximum y values, drawing the temperature intercept to the left. Thus, we observe that hybrids involving museum DNA, in which levels of homoduplex reassociation are lower than those of heteroduplexes, still have higher homoduplex than heteroduplex Tmid’s.

Since each point in the curve is an absolute value of counts, rather than a percentage of the total number of counts in the curve, the counts are independent of one another, and noisy data in one part of the curve does not affect the rest of the curve. Tm and T50H, on the other hand, are mean values computed from cumulative distributions, and phylogenetically uninformative noise on either side of Tm or T50H shifts these values accordingly and distorts estimates of genetic distance (Sarich et al. 1989; Schmid and Marks 1990).

A common form of repetitive DNA distortion in solution DNA hybridization is a low-temperature peak resulting from the dissociation of highly diverged paralogous sequences (e.g., Brownell 1983; Sarich et al. 1989; Schmid and Marks 1990). In our mb hybridization curves, the reassociation of such diverged repeats would be expressed as higher-than-normal initial (maximum) count values followed by a sudden drop in counts. As noted, such low-end perturbations can only affect Tmid in one way: an anomalous reduction in counts caused by the dissociation of a single sequence in high copy number would lead to a dip in the curve that would exaggerate distance rather than similarity. Moreover, its effects will be minimized by the least-squares fitting of the curve.

NIR Index

The usual method of quantifying initial hybridization is the normalized percentage of reassociation (NPR or NPH), but we found this unacceptable in our experiments using museum DNA. Museum DNA typically exhibits much reduced NPR that is not correlated with thermal stability of the duplexes that form (i.e., genetic distance). In other words, truly closely related organisms could yield at the same time low NPR and small genetic distance (e.g., delta Tmid) when one of the DNA samples in a duplex is degraded.

NIR is the integrated area under each heteroduplex elution curve normalized to the area under the homoduplex curve. It combines information from two sources, not unlike the commonly used T50H index. The first source is the initial amount of hybridization (NPH or NPR), and the second is the slope of the curve (thermal stability of duplexes). To the extent that the two are positively correlated (i.e., inversely correlated with genetic distance), as they normally are, they will be mutually reinforcing. Conversely, to the extent that they are negatively correlated or uncorrelated, NIR will represent a compromise of their input. Such is the case in experiments that employ degraded DNA, like that we obtained.
from museum specimens. NIR will be better correlated with Tmid than would NPR under these circumstances because NIR and Tmid both include a measure of thermal stability. This is not to say that either NIR or Tmid is used to calculate the other; they are not. Rather than picking a single point on the curve to serve as a distance metric (e.g., Tm, Tmode, T50H, NPR, etc.), NIR is a function of the entire curve.

NIR is expected to be proportional to NPR because the larger the number of counts representing duplexed DNA, the higher the curve and the larger its integral. NIR is so large relative to the variances in the curve that might be caused by repetitive DNA that it is unlikely to be noticeably perturbed by those variances.

Concerted Evolution

Repetitive sequences may evolve very rapidly through amplification en masse or evolution in concert (Dover et al. 1981; Doolittle 1985). As a result, rates of evolution of repetitive elements may not be time dependent. However, evolution need not be clocklike for DNA hybridization to estimate phylogenies accurately (e.g., Sheldon 1987; Bledsoe 1987; Springer and Krajewski 1989). DNA hybridization measures the sum of differences that have occurred between two taxa since they diverged from a common ancestor. When several taxa are compared and an outgroup is specified, phylogenetic hierarchy is provided by the pattern of unique differences along the terminal branches of a tree and shared differences on the subterminal branches. These unique differences may accumulate at any rate (reviewed in Bledsoe and Sheldon 1990; Sheldon and Kinnarney 1993).

Methodological Considerations

Disparity Between mb and Solution DNA

Hybridization Distances

By reducing stringency of annealing conditions throughout the incubation period, we attempted to drive renaturation rapidly to completion to obtain a full spectrum of similar and diverged hybrids. We began incubation at the same conditions of high stringency and duration typically used in Southern hybridization under which only very similar sequences can hybridize. As temperature was lowered over a 5-day period, more and more base-pair mismatch was tolerated, allowing more divergent sequences to anneal with one another.

It is important to appreciate that Tmid's between our experimental sets are not comparable to one another or to Tm's in our studies, because we did not maintain criterion throughout the annealing. Tm is affected by criterion of both reassociation and dissociation (Bonner et al. 1973; Britten et al. 1974), and Tmid would presumably be similarly affected. We also used different salt concentrations and polar solvents in the first two experimental sets than are normally used in solution DNA hybridization. Thus, we could generate Tmid's of greater or lesser magnitude by altering the criterion of either reassociation or dissociation. Such alterations, however, do not affect the relative magnitude of Tmid's within the same experimental set.

In retrospect, our strategy of lowering incubation temperature may have been unnecessary for the reassociation of repetitive elements, and it was counterproductive to the reassociation of sc elements. The low sc DNA delta Tmid values in the second experimental set demonstrated clearly that the sc DNA did not contribute quantitatively in the genomic hybridization experiments (i.e., the first experimental set). The reaction conditions of DNA concentration that we employed allowed sufficient time for repetitive elements to hybridize at high stringency, but the sc elements were simply not in sufficiently high concentration to permit substantial hybridization of well-complemented single strands of sc sequences before the annealing temperature was lowered. At temperatures low enough to permit reassociation of sc sequences, so much base mismatch was permitted that the Tmid of homoduplex curves was similar to those of heteroduplex curves.

Hypothetically, the sc probes in our second experimental set could have misleadingly suggested the same phylogenetic relationship as the repetitive probes simply because repetitive elements in the target DNA drove the reaction. The third experimental set showed that this was not the case.

Disparity of Reciprocal Distances

Differences in Tmid's of homoduplexes (range 57.2–65.0°C) contributed largely to asymmetry of delta Tmid's. Asymmetry also could be influenced by differences between taxa in genome complexity or size, in proportions of single copy to repetitive fractions, and in numbers of kinds of genes. While there is no evidence that proportions of sc and repetitive elements differ dramatically between most bird groups (but see Eden et al. 1978), few groups have been studied in detail. Cranes, the only Gruiformes yet examined, were found to possess about the same proportions of sc and repetitive DNA as other birds (Krajewski 1989), but individual satellites may differ in abundance by more than an order of magnitude compared with other birds (Madsen et al. 1994 and references therein).

We suspect that differences in homoduplex Tmid's probably reflect differences in average sequence length between preparations. For example, homoduplex Tmid's of Heliornis #1 and #2 differed by 3.2°C. If the length of probe DNAs varied because of differential nicking of template DNAs, then this would not be evident in EtBr-stained electrophoretic gels of ds DNA, but once denatured, the strands would fall apart into shorter sequences.
The radiolabel might also be expected to degrade the probe DNA over time, and since no two probes could be labeled to exactly the same specific activity, degradation might vary.

Effects of Degraded DNA on Hybridization and Phylogenetic Inference

Reciprocal comparisons involving Helioptas and Heliornis #2 exhibited asymmetry. When used as targets, their NIP values were depressed. When used as probes, NIP of some or all heteroduplex hybrids was elevated above that of homoduplexes. These phenomena are illustrative of the properties of degraded DNA in hybridization.

When a degraded ingroup is used as a probe, relative distances from it may be more reliable than reciprocals because all pairwise hybrids will be similarly affected. Furthermore, the radiolabeling reaction will be most efficient at polymerizing from intact templates, thus enzymatically selecting superior substrates for hybridization among the pool of degraded DNA.

Could the apparent closer relationship of Helioptas and Heliornis to one another than to Podica be artifactual? Probably not. Helioptas and Heliornis #2 were consistently poorer targets for hybridization than Podica in all outgroup comparisons. It seems unlikely that they could form more thermostable hybrids with each other than with Podica if either were, in actuality, more closely related to Podica.

Similarly, the monophyly of Heliornis-Heliornis-Podica could not be an artifact resulting from the use of DNA derived from museum specimens. Sequence identity between a museum DNA and any other DNA can only be reduced by degradation (e.g., by depurination or crosslinking). If degradation of museum DNAs (Podica and Helioptas) had an effect in this study, then this would have falsely suggested a closer relationship among the species obtained as fresh specimens (i.e., Heliornis, Aramus, and Grus). This was quite the opposite of what we observed: Heliornis grouped with Podica and Heliornis, not with Aramus and Grus.

Perhaps the potentially misleading effect of poor templates did not complicate phylogenetic reconstruction in this study only because the branch nodes in these Grufforms are sufficiently disparate.

Competitive Hybridization

There may be competition among targets for hybridization with probe DNA, because several target DNAs are incubated in the same hybridization solution. This may be useful in ordering taxa from most closely related to least related. The choice and number of taxa used in a given experimental set may affect percent of reassociation but would not be expected to alter thermal stability as measured by Tmed.

Conclusion

We introduce a new method of DNA hybridization that employs a membrane substrate for duplex formation like the Southern technique but that produces a frequency distribution of sequence thermostability analogous to that of solution DNA hybridization. The method differs from solution DNA hybridization because stability of double-stranded hybrids rather than instability of single-stranded eluates is quantified in melting trials. Our method may be useful in the study of so-called "ancient DNA" as it may utilize unfractoned genomic isolates, making more frugal use of limited resources. It also appears to be relatively insensitive to the effects of DNA degradation and reassociation of repeated elements that would normally complicate solution DNA hybridization. While the sequences assayed in our system are predominantly repeated elements, the phylogenetic reconstruction of our test organisms using the new method agrees completely with that produced from a parallel set of experiments using solution DNA hybridization of the single-copy fraction. Thus, the repeated fraction appears to be phylogenetically informative.

Acknowledgments. We thank Adelgis Caccone for fractioning the DNA for the second experimental set and Anne E. Houde for writing the Tmid and NIR computer programs. For specimens we are grateful to G.M. Gee, M. Louette, S.A. Neshitt, S.L. Olson, E. Ortiz, C. Pickett, M. Romero, and R.L. Zusi. This research was supported in part by a NSF postdoctoral fellowship (BSR-8700162) to P.H.

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Kirsch JAW, Ganje RJ, Oleson KG, Hoffman DW, Bledsoe AH (1990) TED, an improved thermal elution device for the simultaneous hydroxyapatite chromatography of solution DNA/DNA hybrids. Biotechniques 8:505–506