I. INTRODUCTION

The mid- to late nineteenth century was the heyday of comparative vertebrate taxonomy (e.g., Garrod, 1874; Gadow, 1892). Many subfamilial relationships were self-evident even before the Darwinian revolution. At higher taxonomic ranks,

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however, there is a discontinuity in morphological similarity that still obscures the relationships of many families and orders of birds (e.g., compare Cracraft, 1981; Olson, 1985; Sibley and Ahlquist, 1990).

Gruiformes present many unsolved mysteries of systematics and biogeography. They include many highly diverged, depauperate or monotypic families scattered patchily the world over. Most previous attempts to resolve their phylogeny have yielded conflicting results.

Molecular data hold great promise for resolving problem phylogenies. The 12S rDNA is used here to address gruiform phylogeny because it includes both evolutionarily labile and conserved regions, hence it is believed to have a broad window of resolution for addressing recent and very ancient divergences (Kocher et al., 1989; Mindell and Honeycutt, 1990; Simon et al., 1994). We achieve some resolution of gruiform phylogeny and contribute some basic description of the evolution of the 12S rDNA gene. Complementary analysis of morphological characters is treated elsewhere (Houde, in preparation).

A. The Birds

Gruiformes traditionally include rails, coots, and gallinules (Rallidae, 120 species worldwide; e.g., Gallinula, Laterallus, Rallus), rotatoles (Mesitornithidae, 3 species, Madagascar, e.g., Mesitornis), hemipodes (Turnicidae, 15 species, from Africa through southern Eurasia to Australia, e.g., Turtix), the Australian plains-wanderer3 (Pedionomidae, 1 species, Australia, not used in this study), finfoots and surugrebe (Heliornithidae, 3 species, pantropical, e.g., Heliornis, Podica), the kagu (Rhynochetidae, 1 species, New Caledonia, i.e., Rhynochetos), the sunbittern (Eurypygidae, 1 species, South America, i.e., Eurypyga), trumpeters (Psophiidae, 3 species, South America, i.e., Psophia), seriemas (Cariamidae, 2 species, South America, e.g., Carianas), the limpkin (Aramidae, 1 species, Neotropics, i.e., Anamus), cranes (Gruidae, 14 species, cosmopolitan except South America, i.e., Anthropoides (= Grus), Blearica, Grus), and bustards (Otididae, 23 species, from Africa through southern Eurasia, to Australia, e.g., Ardeotis (= Choriotes)) (Sibley and Monroe, 1990). We use the familial nomenclature of Wetmore (1960).

At the morphological extremes of this assemblage are the small quail like hemipodes and the large long-legged cranes. Seriema, trumpeters, kagu, and limpkin are lanky and superficially crane-like, with stubby tails (except seriema). Seriema, trumpeters, sunbittern, finfoots, kagu, and some rails are forest dwellers. Hemipodes, bustards, and cranes inhabit prairies or steppes, although cranes also inhabit wetlands. Limpkin, finfoots, and rails prefer watery habitats.

Phylogenetic Issues

Most of what is commonly believed about the relationships of Gruiformes can be traced to descriptive comparative anatomy and phyletic inferences made more than

3Lowercase lettering for proper names of birds has been used throughout this chapter to conform with the editorial policy of this book.
FIGURE 5.1 Relationships of Gruiformes and others according to Olson's (1985) phenetic assessment of morphology and paleontology (tree interpreted from text only). Branch lengths are not proportional to distance.

A century ago (e.g., Fürbringer, 1888; Gadow, 1892). Within the past three decades, however, new issues in gruiform phylogeny and systematics have been raised and old ones rekindled.

Olson worked mainly with rails (Olson, 1973, 1975, 1977, 1985), maintaining that trumpeters are sister to rails and resurrecting an old idea that the extinct flightless *Aptornis* (= *Aptorhynchus*) from New Zealand is closer to kagu than to rails (Beddard, 1898 vs Brodkorb, 1967). As strong proponents of gruiform polyphyly (see Fig. 5.1), Olson and Steadman (1981) asserted that Australian plains-wanderer and bustards are Charadriiformes, not Gruiformes (Olson, 1985). Olson further suggested that sunbittern and/or roatelos are relicts of an ancient assemblage that includes herons (Olson, 1979, 1985). Olson considered ibises to be intermediate between Charadriiformes and Gruiformes. Olson and Steadman marshaled an eclectic assortment of (mostly) osteological traits in support of their hypotheses.

Cracraft erected a group, "Psophii," to which limpkin, then cranes are sisters, respectively (Cracraft, 1981, 1982; see Fig. 5.2). In it, trumpeters and seriemas form a sister clade to sunbittern and kagu plus *Aptornis*. The effort by Cracraft was the first attempt at a cladistic analysis of gruiform osteology, but no formal analysis was available for his 26 characters. Cracraft, like Olson, advocated a closer relationship between *Aptornis* and kagu than between kagu and sunbittern.

Sibley and colleagues came the closest to treating all traditionally recognized Gruiformes in a single analysis (Sibley and Ahlquist, 1985, 1990; see Fig. 5.3). They corroborated the treatment by Steadman and Olson of plains-wanderer. However, their more contemporary, noncommittal reconstructions were to replace the nearly fully resolved dichotomous trees of their earlier works (Sibley et al., 1993). In their treatise, *Phylogeny and Classification of Birds*, hemipodes were placed as sister to all neognathous birds except fowl and waterfowl. They separated rails from all other Gruiformes at the subordinal rank "Ralli" (Sibley and Ahlquist, 1990). Roatelos were unstudied but also placed in their own suborder. Last, the suborder "Grues" consisted of a ladderized tree beginning apically with cranes, then limpkin plus sun
Grebe, trumpeters, seriema or kagu or both, bustards, and then finally sunbittern at the base. It will be of interest below that in one figure they illustrated a sister relationship of bustards and seriema, even though in others they did not (1990: Fig. 335 vs Fig. 363). They stridently advocated a close sister relationship for limpkin and sun grebe, but not for kagu and sunbittern. However, both sungrebe and kagu were removed from their 1993 publication. In it, the only interfamilial relationship they advocated was between trumpeters and cranes. They found Gruiformes and Charadriiformes to be broadly indivisible (i.e., possibly not mutually monophyletic).

**FIGURE 5.2** Relationships of Gruiformes and others according to Cracraft's (1982) cladistic analysis of morphological characters. Note the fundamental dichotomy of Gruiformes into suborders Ralli and Grues. Branch lengths are not proportional to distance.

**FIGURE 5.3** Relationships of Gruiformes and others according to Sibley and Ahlquist's (1985: 1990) reconstruction from DNA hybridization. Note the exclusion of hemipodes from Gruiformes and the fundamental dichotomy of Gruiformes into suborders Ralli and Grui. Branch lengths are not proportional to distance.
Houde argued that the treatment by Sibley and colleagues of the Neotropical sungrebe and limpkin had profound implications for interpretation of character polarity and biogeography (Houde, 1994; Houde et al., 1995). Sibley and Ahlquist (1990) intimated that the two might be more closely related to one another than to the other two species of finfoots (are to sungrebe), which are endemic to Africa and Asia. Morphological character transformations constrained to this or similar topologies are one-third to one-half as parsimonious as on unconstrained morphology trees. Houde eventually dismissed the previous DNA hybridization results as irreproducible, rejected the monophyly of the limpkin–sungrebe clade, supported the monophyly of finfoots, and reaffirmed their longheld sister relationship to rails (Gadow, 1892; Sibley and Ahlquist, 1972; Olson, 1973; Cracraft, 1982; Houde, 1994).

Several questions are within the scope of the present analysis. (1) Are traditionally recognized Gruidae monophyletic? In particular, should bustards and hemipodes be included in a monophyletic Gruidae or Charadriiformes? Are seriema related to secretary-bird? Are sunbittern or roatelos related to herons? (2) Apart from roatelos, does the first branch in Gruidae separate all ralliform birds from all cramelike birds, as in the subordinal classifications of Cracraft and Sibley and Ahlquist? (3) Are the Psophii of Cracraft monophyletic? If not, then are trumpeters sister to seriemas (as by Cracraft), cranes (as by Sibley and Ahlquist), or rails (as by Olson)? (4) Are finfoots monophyletic, and are they most closely related to the limpkin or to rails? (5) Are sunbittern and kagu sister taxa? (6) Is the fossil Aptornis more closely related to the kagu or to rails?

B. The Gene

12S rDNA is the smaller (about 1 kilobase) of two mitochondrial ribosomal DNAs. rDNA "gene" products are nonprotein-coding rRNAs that complex with proteins to form a ribosome. rRNAs fold onto themselves, like peptides, with evolutionarily conserved secondary structure (Fig. 5.4). The 12S gene can be subdivided into four principal domains, each of which includes self-complementing "stem" and single-stranded "loop" regions. Substitutions within stem regions may be selected against or precipitate compensatory (nonindependent) substitutions to maintain functional stem structure. Single-stranded regions may be involved in temporary base pairing with tRNAs and DNA templates during translation (Watson et al., 1987), and in binding and cross-linking the many proteins that collectively make up the larger ribosomal particles (Noller et al., 1990).

The popular wisdom that loops are evolutionarily more labile than stems is not entirely accurate (Vawter and Brown, 1993; Simon et al., 1994). Some loops contain regions of variable length, but so do some stems. There are motifs within loops that are invariant, from microbes to vertebrates; while some positions within stems are among the most variable of sites in the gene.
FIGURE 5.4 Mitochondrial 12S rDNA, hypothesized structure for domains I–III modified from Sullivan et al. (1995), with stems boxed and numbered according to Van de Peer et al. (1994). The sequence shown is a strict consensus for gruiform birds. Arrows indicate gaps in sequence numbering for alignment with outgroups. Font size of sequence is proportional to site diversity index (Shannon and Weaver, 1949; see Section II.A.6). Insertion/deletion sites are indicated by italics.
The rate of evolution of the 12S rDNA gene is believed to be appropriate to the level of phyletic divergence we aim to address, i.e., within late Cretaceous and Tertiary times (Mindell and Honeycutt, 1990). rDNA has been used for phylogenetic inference at greater and lesser taxonomic ranks (e.g., Kocher et al., 1989; Simon et al., 1994; Cummings et al., 1995).

II. METHODS

A. DNA Sequence Data

1. Sources

Most DNA samples were obtained from ultrafrozen or chemically preserved soft tissues [Gruiformes: Aramus guarauna, Ardeotis (= Choriotis) sp., Helornis fulica, Mesitornis uniclor, Laterallus melanophanus, Rallus longirostris, Turnix sp.; Charadriiformes: Larus heermanni; Ciconiiformes: Phimosus infuscatus] or whole blood [Gruiformes: Anthropoides (= Grus) virgo, Balaeniceps rex, Caraja cristata, Eurypyga helias, Gallinula chloropus, Grus canadensis, Psophia crepitans], but a few were obtained from museum skins and skeletons [Gruiformes: Podica senegalensis, Rhynochetos jubatus, Aptornis (= Apterornis) sp.]. Calcium salts were completely removed from bone by chelation with EDTA. DNA was isolated from tissues by proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation and, when necessary, purification by glass milk or anion-exchange column (Sambrook et al., 1989). The 12S rDNA gene was molecularly cloned by polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Saiki et al., 1988). The gene was amplified intact by priming on conserved flanking tRNAs that readily permit amplification by PCR in novel organisms (e.g., Kocher et al., 1989), except when DNA derived from museum specimens was used (Houde and Braun, 1988; Cooper et al., 1992). Sequencing templates were constructed by a exonuclease digestion of asymmetrically phosphorylated PCR products (Higuchi and Ochman, 1989), which were sometimes gel purified (Kretz et al., 1989). The method of sequencing was direct PCR sequencing using dyeoxy chain termination (Sanger et al., 1977), as modified (Engelke et al., 1988; Sheen and Seed, 1988). Sequencing primers were spaced at internal sites across both strands, specific to chicken sequence, in conserved regions chosen by alignment of sequences of diverged lineages (e.g., Anderson et al., 1981; Clary and Wolstenholme, 1985; Desjardins and Morais, 1990). Sequences were verified from both strands, except in the most 5' region (varies between taxa). Negative controls lacking template DNA were run to check against contaminating DNA in reaction mixtures and pipettors. Negative-control DNA extracts of museum specimens were carried through every step from extraction to sequencing. DNA extraction and PCR setup were performed in a remote, dedicated PCR-free laboratory.
Approximately 870 bases representing domains I–III were obtained for all ingroup taxa except Aptornis, Podica, and Rhynchotes (GenBank accession numbers U76011–76027). The latter are represented by 673, 336, and 388 bases, respectively.

Unpublished complete 12S rDNA sequences of outgroups stone curlew (Charadriiformes: Burhinidae: Burhinus oedicnemus), night heron (Ciconiiformes: Ardeidae: Nyctanassa violacea), and secretary bird (Falconiformes: Sagittariidae: Sagittarius serpentarius) were kindly provided by D. P. Mindell, and the complete sequence of chicken (Galliformes: Phasianidae: Gallus gallus; Desjardins and Morais, 1990), partial sequence of sandpiper, gull, and murre (Charadriiformes: Scolopacidae: Calidris maritima (X76362), Laridae: Larus canus (X76361), and Alcidae: Uria aalge (X76435), respectively; Moum et al., 1994) and stork (Ciconiiformes: Ciconiidae: Ciconia nigra (L33370); Hedges and Sibley, 1994) were obtained from GenBank.

2. Sampling Considerations

We used 17 gruiform species to represent an order that includes 196. Thus, autapomorphies of the species sampled may be mistaken for synapomorphies of families (e.g., Patterson et al., 1993). This problem is diminished because 8 of the 12 gruiform families include 3 or fewer species. Rallidae are the only family with more than 25. Our sampling in no way represents the nominal diversity within Rallidae but should address its interfamilial relations since rallid monophyly is supported by both molecular and morphological studies (Olson, 1973; Sibley and Ahlquist, 1990). We sampled 32% of all genera and 74% of the nonrallid genera in the order. All traditionally recognized gruiform families except the plains-wanderer are represented, making this the most comprehensive investigation of gruiform molecular systematics to date. There is agreement from molecular and morphological phenetic studies that the plains-wanderer is not gruiform (Olson and Steadman, 1981; Sibley and Ahlquist, 1985, 1990).

We sampled one or two individuals (rarely three) per species, with about equal frequency across taxa. Moore (1995) showed that internode lengths in four recently evolved woodpecker species were almost always longer than coalescence time for mitochondrial DNA (mtDNA). Thus, lineage sorting (Avise et al., 1984) should rarely if ever corrupt phylogeny reconstruction for groups with equal or greater internode lengths (i.e., superspecific levels; age of divergence is inconsequential). Gene phylogeny should adhere to organismal phylogeny, and between-species variation should exceed within-species variation. Accordingly, the two most closely related species in this study [Gnus canadensis and Anthropoides (= Gnus vingo] display numerous transition substitutions. We never detected sequence differences in any two samples of one species, except one apparently heteroplasmic individual with a single transition substitution.

Small samples increase the risk that species misidentifications will go unnoticed. We detected one mislabeled specimen (Balearica mislabeled as Carouma) only because
we sequenced other samples of both species. Voucher specimens (a live bird in this case) are no assurance against sample mislabeling.

3. Sequence Alignment

Sequence alignment was initiated with a pairwise similarity measure (MacVector 4.14; Needleman and Wunsch, 1970) and was improved by individual discretion (see below). Sequences were fitted to a map of secondary structure to identify complementary positions (e.g., Kjer, 1995). In so doing, discrepancies between opposite strands resulting from “compressions” (i.e., bases missing on one strand but not the other) were discovered and resolved (Fig. 5.5). The mapping of sequences onto structural models also served to monitor the possible existence of nuclear pseudogenes of mtDNA sequences (Fukuda et al., 1985). The hypothesis of an endosymbiont origin of mitochondria predicts the existence of nuclear copies of mitochondrial genes because the mitochondrial genomes themselves are depauperate in housekeeping genes (Gray et al., 1984). Inasmuch as nuclear pseudogenes are released from selective constraints, loss of conserved binding motifs and stem complementarity would be conspicuously absent in nuclear copies of mitochondrial rDNA. We detected no such instances.

Further improvement of alignment was made according to the principle of interactive phylogenetic weighting (Feng and Doolittle, 1987; Hein, 1990; Konings et al., 1987; Lake, 1991; Mindell, 1991; Thorne and Kishino, 1992). Regions of

![Image](image.png)

**FIGURE 5.5** Sequencing artifact. Autoradiograph of sequencing gel showing a common sequencing artifact in mitochondrial 12S rDNA, domain III, stem 32 (Eurypteryx helias shown). **Left:** Double loading of L strand. **Right:** Double loading of H strand, reverse complemented; arrows indicate G and C bases not evident on opposite strands.
variable length were subjected to successive bouts of phylogeny reconstruction separately from the complete data set using maximum parsimony following alterations of alignment (Section II.A.6). We wanted to determine how "badly" (i.e., counter to available phylogenetic information) alignments could be contrived before traditionally recognized monophyletic families no longer associated with themselves in phylogeny reconstruction (i.e., Gruidae, Rallidae, and Heliornithidae). Some effect of variable alignment was observed, but most often alignment had little or no consequence on phylogeny reconstruction in this study. In this data set synapomorphies of close taxa usually provided sufficient phylogenetic signal to reconstruct sister relationships, whether the synapomorphies are aligned to gaps or to a background of sequence "noise" of questionable homology (i.e., randomized sequence).

When no phylogenetic information was available, we strived to minimize any impact of alignment on phylogenetic inference. When phylogenetic information was available, we made alignments according to a parsimony principle of inverting the fewest number of changes between sequences from well-supported sister taxa. This could just as likely involve the insertion of gaps in nonhomologous positions to maintain sequence alignment as insertion of gaps at homologous positions. Sequence alignments were finalized according to a distance optimality criterion based on majority segregation of purines vs pyrimidines (i.e., minimizing inferred transversions across all taxa without reference to a hypothesis of phylogeny).

4. Transformation Weighting

As transformations saturate, the observed ratio of transversions to transitions deviates significantly from the instantaneous ratio (Brown et al., 1982; Mindell and Honeycutt, 1990; Knight and Mindell, 1993). In other words, the ratio of transversions to transitions appears much closer to 1:1 for deep divergences than for shallow divergences. In spite of this, approximately the same intrinsic difference in rates of transversions to transitions probably occurred throughout the evolutionary history of a group, i.e., all levels of divergence. Thus, weighting schemes for phylogeny reconstruction should attempt to employ the instantaneous ratio rather than one averaged across all levels of divergence, including those saturated. Transversion weighting makes the difference between the recovery or lack of recovery of the traditionally recognized monophyletic clades Gruidae, Rallidae, and Heliornithidae in many of our phylogeny reconstructions. The monophyly of these families is supported in whole or part by a variety of morphological and DNA studies, employing both phenetic and cladistic methodologies (Olson, 1973, 1985; Sibley and Ahlquist, 1990; Krajewski, 1989; Houde, 1994; Krajewski and Fetzner, 1994).

We estimated a ratio of instantaneous rate of transversions to transitions from two most parsimonious phylogenies, one large and not known to be correct (including all 17 gruiform taxa herein), and the other small but believed to be correct (a ladderized tree of Gnis, Anthropoides, Balearica, Aramus, Psophia, and Gallus). Both
trees produced identical results on relative transformation rates. The ratio of observed transversions to transitions was expressed as a function of total substitutions. In several cases where no transversions were observed, they were assigned a value of 1 to preclude the biologically meaningless ratio of infinity. A second-order regression was fitted to the plots and the intercept at one substitution was calculated. The instantaneous ratio is 6:1, even though there was substantial scatter of observed values near the origin (from 2:1 to 19:0).

We also estimated the transformation ratio by fitting a two-parameter model of transversion and transition rates to both the large and small phylogenies (Kimura, 1980). We used TREECALC (Milligan, 1994) to find the transformation ratio with the maximum likelihood (Felsenstein, 1981) for the sequence matrix given a user-specified topology with specified branch lengths. Like the first approach, this estimates the instantaneous rate of transformations rather than an average rate of change over the entire phylogeny. The resulting ratio of 7.3:1 from both large and small phylogenies is in fairly good agreement with the previous estimate. We observed no difference in topology of optimal trees obtained by changing the weighting of transversions from 6 to 7.3 and we used the larger value for the phylogeny reconstructions presented here.

In reality, A/Y transversions appear to outnumber G/Y transversions in our data by about an order of magnitude. In light of the high frequency of transitions, the few G/Y transversions may primarily represent A/Y transversion followed by A/G transition. A weighting scheme of 6:1 for A/Y transversions and 60:1 for G/Y transversions produced similar bootstrap support for the same groups as the 7.3:1 weighting for all transversions, but performed worse in recovering Gruidae monophyly.

Gaps were treated in two ways in parsimony analyses: as missing data and with an intermediate weight of 4 (to satisfy the triangle inequality for weights of all transformation types) with 8:1 transversion-to-transition weights.

5. Position Weighting

The number of substitutions per site was estimated from the most parsimonious tree of 17 ingroup taxa obtained with 7.3-to-1 transversion weighting (Table I). Small

| TABLE I  | Frequencies of Substitutions per Site |
|---|---|---|---|---|---|---|---|---|
| | Total | Variable | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Number of sites | 934 | 395 | 104 | 90 | 76 | 62 | 36 | 20 | 6 | 1 |
| Percentage of sites | 100 | 42.3 | 11.1 | 9.6 | 8.1 | 6.6 | 3.9 | 2.1 | 0.6 | 0.1 |
| Percentage of substitutions | 100 | 100 | 9.4 | 16.2 | 20.5 | 22.3 | 16.2 | 10.8 | 3.8 | 0.7 |
differences in tree topology (i.e., maximum likelihood and neighbor-joining trees) have almost no effect whatsoever on the number of substitutions per site. We nevertheless acknowledge that observed values both overestimate and underestimate actual values.

Four weighting schemes were applied in subsequent bouts of phylogeny reconstruction: equal (weight = 1), reverse (weight = x), inverse (weight = 1/x), and quadratic weights (weight = 1/x^2, where x = number of substitutions per site). Each was used with and without transformation weighting, on complete and partitioned data sets, and in jackknife and bootstrap analyses. Reverse and inverse weighting produced identical trees. Equal weights performed best overall at recovering traditionally recognized families. Quadratic weighting performed worst.

Stems were not weighted differently from loops to account for compensatory substitutions because stems, loops, and bulges evolve at about the same rate (Vawter and Brown, 1993).

Since among-site evolutionary rate variation is known to complicate phylogeny reconstruction (e.g., Milkman and Crawford, 1983; Huelsenbeck and Hillis, 1993), intuition dictates that position weighting would improve phylogenetic estimates. It did not appear to work well in this study.

6. Data Partitioning

Nucleotide data were analyzed in total and in subsets. Variable length and flanking regions that were subject to alternative alignment consisted of sites 82–116, 140–149, 245–259, 320–323, 339–347, 352–358, 409–424, 433–438, 520–528, 707–711, 808, 809, 817–822, and 902–908. Each of the aforementioned position and transformation weighting schemes was used in phylogeny reconstruction with and without these sites removed from the data set. Inclusion of variable-length regions tended to improve tree resolution and bootstrap support of nodes. Many synapomorphies of the traditionally recognized families occur within variable length regions.

We analyzed bases 500–920 (the "12Sa-b" region of Kocher et al., 1989) separately to see whether this region was representative of the entire gene. In short, the answer is no. Although bootstrap values for some clades increased compared with those obtained from the entire data set, the monophyly of cranelike birds was lost.

We partitioned data according to number of changes per site and analyzed each class individually and in groups. This approach addresses among-site evolutionary rate variation and saturation of most-variable sites. Popular wisdom holds that sites that change most are most homoplasious (e.g., Sullivan et al., 1995). Thus, one might rationalize their removal. We were surprised to discover, however, that phylogeny reconstruction using sites that change least yield thousands of equally parsi-monious trees. Least-variable sites appear to be the only ones lacking phylogenetic information.

The inconsequence of saturation in our data is suggested by a regression of site
consistency index, CI, to site diversity index, $H'$ (Shannon and Weaver, 1949). $H'$ is the sum of all nucleotide frequencies at a given position times the natural log of that nucleotide frequency. It is a measure of the amount of variation observed at a position. The maximum value is obtained by equal frequency of each of the four bases at a position; the minimal value is obtained by invariant sites. Site diversity is not a measure of substitution rate. A single substitution can result in either high or low $H'$, depending on where in the phylogeny it occurred. The slope of the regression of $H'$ and CI is $-0.09$ ($P = 0.0006$; variable sites only), indicating that CI does not vary as a function of site diversity in these data. In other words, most-diverse positions are no more or less consistent than least-diverse positions. Substitution rate may be correlated with consistency, but substitution rate and CI cannot be legitimately regressed because their calculations are not independent.

### B. Phylogeny Reconstruction

Trees are constructed and evaluated using parsimony [MP, in MacClade 3.03, (Maddison and Maddison, 1992), and PAUP 3.1.1 (Swofford, 1993); all searches performed with heuristic algorithm and optimized by accelerated transformation], dynamically weighted parsimony (DWP; Williams and Fitch, 1990), maximum likelihood (ML, in PHYLIP 3.5; Felsenstein, 1989), and neighbor-joining [NJ, in MEGA 1.01, (Saitou and Nei, 1987; Kumar et al., 1993)] and PHYLIP 3.5 (Felsenstein, 1981, 1989)]. Figures of trees were created with TreeView (Page, 1996). Empirical base frequencies used in ML and NJ reconstructions are as follows: A, 0.32033; C, 0.28244; G, 0.19667; T, 0.20056.

The recovery of traditionally recognized groups—cranes and limpkin (Gruidae plus Aramidae), rails (Rallidae), and finfoots (Heliornithidae)—within larger phylogeny reconstructions is our standard for the reliability of reconstruction and weighting methods. The monophyly of each of these has traditionally been accepted (e.g., Wetmore, 1960), and is supported at least in part by DNA hybridization and sequences and morphology (Olson, 1973, 1985; Krajewski, 1989; Sibley and Ahlquist, 1990; Krajewski and Fetzner, 1994; Houde, 1994; Houde et al., 1995; and reviews therein). We acknowledge the potential circularity of seeking answers to phylogenetic questions in trees that use the recovery of accepted groups as a standard for evaluating trees.

Dynamically weighted parsimony and NJ using gamma distances are methods that are specifically designed to cope with among-site evolutionary rate variation; yet, these performed worst at recovering expected clades. DWP approximates trees that are otherwise obtained from inverse and quadratic position weighting schemes in MP. We were particularly frustrated by the profound effect that seed trees have on final trees in the WTSUBS program. Had this not been a factor, then we would not have felt constrained by the limits on taxa (i.e., nine) that are allowable in an exhaustive search using the ALLTOPS program. We abandoned the NJ
routine in MEGA after discovering that it could not accept IUPAC symbols. Two-
parameter distances (Kimura, 1980) come closer than gamma distances (Tajima and
Nei, 1984) to producing trees that recover traditionally recognized family groups.

All trees are rooted using chicken (Gallus). Although outgroups are ideally close
sisters to the ingroup and chicken is not close to Gruiformes, certainty of outgroup
status is paramount. Charadriiformes would be the obvious choice for outgroups if
traditional classifications truly embody evolutionary history. However, all of the
other "outgroup" taxa we examined in this study (Charadriiformes, Ciconiiformes,
and Falconiformes) were chosen specifically to test for their potential relations as
unrecognized ingroups. Chicken is the only taxon available that is known with
certainty to be an outgroup.

III. PHYLOGENETIC INFERENCE

A. Results

Certain groupings of taxa were partly or entirely consistent in spite of reconstruc-
tion methods, weighting and data partitioning, and low bootstrap support (Felsen-
stein, 1985). The only clades with MP bootstrap values in excess of 95% in the
complete data set with all weighting and data partitioning regimes were Antho-
poidea—Grus, Rallus—Laterallus—Gallinula, and Eurypygæ—Rhynchochelus. All NJ boot-
strap values for the same clades were lower, suggesting poorer resolution. We be-
lieve the MP tree using 7.3:1 transversion weighting and no position weighting is
the best estimate of phylogeny (Fig. 5.6). We restrict all further discussion to that
tree unless stated otherwise. In it, Heliornis—Podica receives 92% bootstrap support
(if gaps are also assigned weight, then support for finfoots is > 95%). Figure 5.6
summarizes the results of jackknife (Lanyon, 1985) and bootstrap support for each
node in the parsimony analysis under six different weighting schemes on the com-
plete ingroup matrix. Most values are low, especially for deep nodes, and indicative
of high levels of homoplasy. Higher bootstrap values for trees constructed with sub-
sets of ingroup taxa are discussed further in the context of particular issues.

Maximum likelihood and NJ trees differ from the MP tree in movement of
several branches by one node. Maximum likelihood also groups Ardeotis with fin-
foots (Fig. 5.7). Neighbor-joining groups Psophia and Tumix as sisters to cranes, and
groups Balearica with Aramus, rather than with gruines (Fig. 5.8). Neighbor joining
with variable-length regions of sequence omitted and 9:1 transversion weighting
restores gruid monophyly, retains the position of Psophia, and places Tumix as sister
to cranes plus rails. Maximum likelihood and NJ trees are both about 0.5% longer
than the MP tree for character data. All branches on the ML tree are significantly
positive (p < 0.01). All branches on the NJ tree are positive, but many internodes
are extremely short (e.g., Ardeotis—Cariamid).

Cranelike versus raillike birds do not comprise a fundamental dichotomy (i.e.,
FIGURE 5.6  Parsimony jackknife and bootstrap tree of Gruiiformes mitochondrial 12S rDNA, domains I–III, obtained with 7.3:1 transversion weighting using PAUP 3.1.1 (Swofford, 1991). First six numbers shown on each branch are jackknife (Lanyon, 1985) consensus values: (1) 7.3:1 transversion weighting, no position weighting, all characters; (2) 7.3:1 transversion weighting, inverse position weighting, all characters; (3) 7.3:1 transversion weighting, no position weighting, variable length regions excluded; (4) 7.3:1 transversion weighting, inverse position weighting, variable length regions excluded; (5) 8:1 transversion weighting, gaps weighted 4, no position weighting; and (6) 8:1 transversion weighting, gaps weighted 4, inverse position weighting, respectively. Seventh number is the highest bootstrap support obtained under any of the six weighting schemes (100 replicates). Asterisks indicate all weighting schemes that provided bootstrap values of 95% or higher or the single weighting scheme that yielded the highest bootstrap value if less than 95%. Jackknife values preceded by “contra” indicate majority consensus levels that contradict the topology shown. Alternate branching topology and values are shown for Megatobis and Turnix. Variable length regions include sites 82–116, 140–149, 245–259, 320–323, 339–347, 352–358, 409–424, 433–438, 520–528, 707–711, 808, 809, 817–822, and 902–908.
most basal split) in Gruiformes in any of our reconstructions. Nine–taxon trees (*Anthropoides*, *Grus*, *Galinula*, *Laterallus*, *Eurypyga*, *Rhynochetos*, *Gallus*, and any two of *Balearica* and/or *Aramus* and/or *Psophia*; 7.3:1 transversion parsimony; 1000 replicates) yielded bootstrap values >98% in support of a separation of cranes, trumpeter, and rails from sunbittern and kagu (Fig. 5.9). In almost all reconstructions from the complete ingroup data set, seriema, bustard, roatelo, and hemipode are outside of the cranes–rails clade.

Bootstrap support for the trumpeter–rails clade is lacking in the complete data set but rises to 90% in the “12Sa-b” subset with 4:1 transversion weighting. The ML and MP trees agree, but NJ always groups trumpeter with cranes. Parsimony tree lengths are trumpeter–cranes (1215, 30815, unordered and 7.3:1 transversion weights, respectively), trumpeters–rails plus finfoots (1210, 30629), and trumpeters–rails (1214, 30437) (*Aptornis* excluded for simplicity).

Our data support the monophyly of finfoots and their position in the clade of raillike birds, rather than with limpkin among the cranes as suggested by Sibley and Ahlquist (1985, 1990). But trumpeter is important for their placement near rails.
Finfoots group with raillike birds, except (1) when both trumpeter is removed and *Aptornis* is included, with or without transversion weighting (in which case they group with limpkin, sister to cranes), or (2) when gaps are weighted (in which case they group with roatelo, sister to cranes plus limpkin).

Unweighted trees placing *Aptornis* with kagu are 27 unordered steps longer (kagu positioned as in Fig. 5.6) or 102 steps longer (kagu positioned as in Cracraft's Psophii) than a grouping of *Aptornis* with rails. Bootstrap support for a sunbittern-kagu clade exclusive of *Aptornis* is 95–100% in virtually every tree examined. In a 6-taxon tree (*Aptornis, Eurypyga, Gallinula, Gallus, Laterallus, Rhynochetas; 7.3:1 transversion parsimony; 1000 replicates*) of only bases 200–920 for which the *Aptornis* sequence is complete, bootstrap support is 99% for rails, 99% for rails plus *Aptornis*, and 98% for sunbittern-kagu (Fig. 5.10). The decay index for the *Aptor-
FIGURE 5.9 Consensus 9-taxon parsimony bootstrap tree of Anthropoides, Grus, Gallinula, Laterallus, Eurypyga, Rhynochetos, Gallus, and any 2 of Balearia and/or Aramus and/or Psophia (1000 replicates, heuristic parsimony with 7.3:1 transversion weighting of mitochondrial 12S rDNA, domains I–III, using PAUP 3.1.1; Swofford, 1991). Bootstrap values in excess of ≥90% in support of a cranes–rails clade, exclusive of sunbittern–kagu, are labeled. The Psophia of Cracraft are polyphyletic. Cranes and rails do not form a fundamental dichotomy in Gruiformes, as is widely presumed (compare to Figs. 5.2 and 5.3).

mis–rails node is 6% in this tree (expressed as a percentage to account for 7.3:1 transversion weighting). Although optimal transversion parsimony trees indicate a sister relationship between Aptornis and finfoots in the full data set, bootstrap support for this grouping is always ≤82%.

Bustard, seriema, roaetelo, and hemipode tend to group in consistent positions, but none of the positions are well supported and some weighting regimes produce

FIGURE 5.10 Parsimony bootstrap tree showing the relationship of Aptornis to rails, not kagu (1000 replicates heuristic parsimony with 7.3:1 transversion weighting, sites 200–920 of mitochondrial 12S rDNA, domains I–III, for which Aptornis sequence is complete, using PAUP 3.1.1; Swofford, 1991; compare to Fig. 5.2). Bootstrap values are labeled.
different results. Most consistent is a tendency for seriema and bustard to form a clade that is sister to the cranes plus rallid-like birds. In a 9-taxon tree (Anthropoides, Grus, Laterallus, Gallinula, Ardeotis, Cariama, Eurypyga, Rhynochetos, Gallus; 7.3:1 transversion parsimony; 1000 replicates) bootstrap support for the bustard-seriema clade is 88% and its sister relation to cranes—rails is 92% (Fig. 5.11).

Seriema, bustard, roatelo, all Charadriiformes, heron, and ibis form a clade when all outgroups are included in transversion parsimony. Relaxation of weighting removes half of the Charadriiformes and both Ciconiiformes from this clade. None of these nodes receive strong bootstrap support.

Hemipode is sister to all Gruiformes except sunbittern—kagu. In an 8-taxon tree (Anthropoides, Grus, Laterallus, Gallinula, Tornix, Eurypyga, Rhynochetos, Gallus; 7.3:1 transversion parsimony; 1000 replicates), bootstrap support for a sister relationship of hemipode to cranes plus rails is 95% (Fig. 5.12).

The shortest MP tree without transversion weighting includes roatelo as a member of the rallidlike clade, sister to trumpeter. This, however, is only 2–10 steps shorter than 4 dissimilar trees, grouping roatelo with seriema and/or bustard or hemipode. Roatelo has a strong tendency to cluster with Charadriiformes (Lime, Ursia, Calidris) and ibis (Phimosus), and always groups with gull-murre when they are included, regardless of weighting regime. The highest bootstrap values we obtained in support of roatelo—Charadriiformes is 87% (all taxa; 8:1 transversion parsimony, gaps weighted 4, inverse position weighting; 100 replicates).

**B. Discussion**

Several groups are consistently supported by our analyses, in spite of what may first appear to be conflicting results from different methods of reconstruction and low
FIGURE 5.12 Parsimony bootstrap tree showing the relationship of hemipoles among Gruiformes (1000 replicates heuristic parsimony with 7.3:1 transversion weighting of mitochondrial 12S rDNA, domains I–III, using PAUP 3.1.1; Swofford, 1991; compare to Fig. 5.3). Bootstrap values are labeled.

bootstrap support. Many of the nodes with low bootstrap support in the complete data set receive high support with fewer taxa. While the complete phylogeny (Fig. 5.6) is not robust, it agrees with well-supported trimmed trees (Figs. 5.9–12). Questions posed in Section 1.A are addressed in order here.

1. Are traditionally recognized Gruiformes monophyletic? Taken at face value, our data suggest that Gruiformes are not monophyletic. Kagu and sunbittern always assume a position more basal than putative outgroups, except chicken. Bustards, seriema, and roatelo form a clade with Charadriiformes, so Gruiformes may be paraphyletic. This accords with the description by Olson (1985) of the osteology of bustards as resembling glareoloid Charadriiformes and the inability of Sibley et al. (1993) to consistently separate Gruiformes from Charadriiformes. We did not address the monophyly of Charadriiformes in our study.

Hemipoles are not distant outgroups to Gruiformes as proposed by Sibley and Ahlquist (1990), but stone curlew (Burhinus) appears to be closely related to them in the absence of transformation weighting.

Seriema never groups with secretary bird, as suggested by Verheyen (1957). Instead, they appear to be ecologically, behaviorally, and morphologically convergent.

Neither sunbittern nor roatelo group with herons, as suggested by Olson (1979, 1985). However, roatelo are the most problematic taxon studied. Their position is unstable and poorly supported in our reconstructions. This is unfortunate since morphology has thus far provided little guidance on their relationships and we provide their first molecular data here. Preliminary observations without formal character analysis reveal no compelling morphological synapo-
morphies to unite roatelos with either Charadriiformes or Gruiformes (P. Houde, personal observation). Their alliance with Charadriiformes among Gruiformes in our gene phylogeny may be spurious.

Roatelos have probably survived a long period of isolation in Madagascar. Their pairwise genetic distances to ingroups are larger than for any other pair of taxa, even exceeding all other chicken—Gruiformes distances. Thus, roatelos either represent an ancient lineage, unrelated to Gruiformes, or an unusually rapid rate of evolution has erased any evidence of that relationship in their 12S rDNA. If the latter, then this is quite the opposite of the evolutionary slow-down of some other insular Malagasy endemics (Bonner et al., 1981).

2. Apart from roatelos, does the first branch in Gruiformes separate all rail-like birds from all crane-like birds, as in the subordinal classifications of Cracraft (1982) and Sibley and Ahlquist (1990)? No, all methods of reconstruction strongly support a clade that includes cranes and rail-like birds to the exclusion of seriemas, hemipodes, kagu, and sunbittern, and probably roatelos and bustards. The cranes and rail-like birds appear to be a monophyletic group.

3. Are the Psophii of Cracraft monophyletic? Clearly not, as trumpeters and Aptornis are among the cranes—rails clade, seriemas appear to be sisters to bustards, and kagu and sunbittern are far removed from all others. Reconstructions showing monophyly of the Psophii of Cracraft are 60 unordered steps longer than optimal trees. While Aptornis is closely related to rails, the same is only weakly supported for trumpeters. Our result explains the difficulty people have had in distinguishing the position of trumpeters relative to rails and cranes. Trumpeters truly are intermediate between the other two. Olson's (1973) analogy of trumpeters to primitive rails agrees well with our result (paraphyly of trumpeters is not implied).

4. Are finfoots monophyletic, and are they more closely related to the limpkin or to rails? Finfoots are monophyletic and group among rail-like birds. This is corroborated by our own DNA hybridization experiments and cladistic analysis of morphology (Houde, 1994; Houde et al., 1995). Although only weakly upheld by the data set at hand, this consensus of our findings should dispel any lingering acceptance of the limpkin—sungrebe clade proposed by Sibley and Ahlquist (1985, 1990). The fact that they are as close as they are is a surprise, indeed, and underscores the previously unrecognized phyletic proximity of crane-like and rail-like birds.

5. Are sunbittern and kagu sister taxa? Yes, this relationship is supported strongly by all analyses. Kagu-like fossils from the early Eocene of Wyoming and middle Eocene of Germany (Hesse, 1988, 1992) place peculiar Amazonian and New Caledonian distributions of these monotypic families into perspective. The distributions of fossils and neotaxa suggest a fairly cosmopolitan pantropical—temperate distribution of “eurypygoids” in the warm forests of the early Tertiary. The modern kagu and sunbittern appear to be relicts of this radiation surviving in the most isolated forest refugia.
6. Is the fossil Aptornis more closely related to the kagu or to rails? There is no question that Aptornis is much closer to rails than to kagu. The original description by Parker (1866) of the skull of Aptornis in comparison to trumpeters was remarkably insightful (considering trumpeters to be like primitive rails, as by Olson, 1973). The phylogenetic informativeness of postcranial morphological characters is all but obliterated by gigantism and the shift of locomotory dependence from the wings to the legs in this flightless bird.

Many of our results conflict markedly with the DNA hybridization studies of Sibley and Ahlquist (1985, 1990). The 12S rDNA sequences support a cranes–rails clade and a sunbittern–kagu clade, and a sister relationship of trumpeters and finfoots to rails. Sibley and Ahlquist proposed that all other Gruiformes except hemipodes are closer to cranes than rails are to cranes, that kagu is closer to everything except rails and hemipodes than it is to sunbittern, and that trumpeter is sister to cranes (Fig. 5.3). We also disagree with their placement of hemipodes far from Gruiformes. They presented a dendrogram that supports the sister relationship of bustards and seriencas that we found, but they did not speculate on its veracity (Sibley and Ahlquist, 1990: Fig. 335).

IV. MOLECULAR EVOLUTION

A. Sequence Divergence

Pairwise Kimura distances (Table II) provide a rough guide to relative amounts of sequence divergence in the 12S rDNA of Gruiformes, although they cannot be considered uniformly proportional to divergence times. Temporal calibrations of sequence divergence apply neither across taxa nor across genes, and perhaps not through time itself (Ayala, 1986; Britten, 1986; Sheldon, 1987; Mindell et al., 1996). Saturation of sequence divergence by multiple hits on individual sites is a convincing mechanism for compression of genetic distance relative to time (Mindell and Honeycutt, 1990). Opposite effects could hypothetically result from other as yet poorly characterized factors, including pervasive environmental mutagens, aspects of the natural history and population structure of a species, phyletic radiation, lateral gene transfer by viruses, or genome transfer by hybridization.

All sites are not equally available to substitution, and the choices of which are used and how they are weighted significantly alter divergence estimates (Pesole et al., 1992). For this reason, we present the highest (variable sites only; 7.3:1 transversion weighting) and lowest (all sites, no weighting) pairwise distances (Table II), rather than intermediate distances (i.e., all sites with weighting and variable sites without weighting). Although the number of sites unavailable to vary may be overestimated by considering only those that are observed to vary, distances based on all sites definitely underestimate invariant sites. Moreover, the divergence of chicken from Gruiformes is ancient (e.g., probably Cretaceous), so most sites available for variation should show it.
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*Above diagonal: 7:3:1 transversion weighting, variable sites only; below diagonal: unweighted, all sites included.

*Missing data.
Statistically significant differences in evolutionary rates among Gruiformes are identified by ranking unweighted two-parameter distances within monophyletic groups, summed across all outgroups ("multiple comparisons for ranked data in randomized block" of Zar, 1974; "SNK" of Houde, 1987). Although not significant, among the cranes genetic distances involving Gnuus appear low while those of crowned crane appear high, in accordance with the observations of Krajewski (1989). Limpkin exhibits significantly lower distances than crowned crane (\(\alpha = 0.05; 14\) outgroups). Accordingly, in a 6-taxon tree rooted to chicken, trumpeter undergoes 57 substitutions, limpkin only 32, crowned crane 55, and the two gruine cranes have 53 and 54. The vastly different branch lengths within the cranes-limpkin clade may complicate the recovery of the expected topology of limpkin sister to cranes vs sister to crowned crane or outgroups (Nei, 1991; Huelsenbeck and Hillis, 1993).

Rail and sungrebe distances appear high, although not so much as trumpeter. Among rails, Gallinula distances are significantly lower than Laterallus (\(\alpha = 0.001\)) and Rallus (\(\alpha = 0.05; 15\) outgroups). No other robust relative rate tests are justifiable because of missing data in the only other clades for which independent evidence of monophyly is available. However, roatele has the highest distance to chicken of all Gruiformes.

Neighbor-joining places trumpeter with cranes but MP and ML include it with the rails, so we tested its distances using both phylogenies. When included in the cranes clade, trumpeter distances are observed to be significantly higher than all the others (\(\alpha = 0.001; 13\) outgroups). Trumpeter distances are not different than any rails when included in that clade (\(\alpha = 0.05; 10\) outgroups).

B. Evolutionary Dynamics of 12S rDNA

One cannot help but be struck by the conservation of both sequence and secondary structure between rDNAs of such disparate groups as bacteria and vertebrates (Van de Peer et al., 1994). Yet, on this broad evolutionary scale one also appreciates the elongation, shortening, and complete loss of some stems. The small-scale events that lead to such large-scale patterns require comparison of the genes in both relatively closely and distantly related organisms (Kjer, 1995; Hickson et al., 1996). Here, we describe some of the small-scale variation in the 12S rDNA of Gruiformes. The variation we note has negative implications for the general practice of matching sequences to structural maps of rDNA constructed from unrelated organisms. Our stem nomenclature follows Van de Peer et al. (1994).

1. Stem Migration

We noted movements of complementary bases upstream and downstream within stems. This "stem migration" seems to result more from substitutions that affect
complementarity than from insertions and deletions. It may result in extended or reduced base complementarity, but usually involves the migration of one or both sides of the stem region. These discrepancies between taxa are reflected in the irregularly boxed stems 8 and 26 of Fig. 5.4.

Stem 8 is the most variable (Figs. 5.4 and 5.13). Its distal segment is flanked on both sides by single-stranded loops of variable length. Similarities in sequence and stem position are apparent among closely related taxa, but identification of homologous sites is difficult across all Gruiformes. The stem consists of five pairs of complementary bases in all taxa except the hemipode (one mismatch) and trumpeter (which seems to have six). The upstream side of the stem in rails has a pattern of YYCCT, seriema and bustard have CCTTA, limkin and crowned crane have CCTAR, and gruine cranes have CCTAT. The pattern in trumpeter (GCTTAC) is the same as in gruines (i.e., CCTAY), except an additional purine is added to the 5' end. A 5' purine is otherwise a uniquely shared character of the stems of sunbittern and kagu (RCCCTT).

Alignment of the upstream CCT of stem 8 in all taxa invokes considerable stem migration. Phylogenetic weighting produces an alternate alignment that is both more parsimonious and invokes less stem migration. On the downstream side of stem 8 most taxa have a homologous sequence of AGG (i.e., aligned), complementing the upstream CCT (minor differences in hemipodes and roatelo). The stem of sunbittern and seriema (kagu data missing) migrates 1 base downstream, while that of rails migrates 2 bases downstream relative to the AGG sequence and the stem of other Gruiformes.

In roatelo, stem 24 migrates upstream 1 base on the downstream side of the stem by transition substitutions. In crowned crane, roatelo, and sunbittern, stem 26 appears to migrate upstream 1 base on the upstream side of the stem, and upstream 2 bases on the downstream side of the stem. It is difficult to infer the location of stem 26, however, because it consists of only two pairs of bases. This is reflected in the ambiguity of Fig. 5.4, in which the stem is shown to overlap stem 24. Hypothetically, such overlap could accurately reflect temporally alternate or tertiary structural interactions as in stem 22, but there is no independent evidence for such phenomena here.

All the birds examined here differ from mammals in stems 24, 27, and 47. The entirety of the avian stems 24 and 27 migrate one position proximal compared with mammals (i.e., upstream on upstream side, downstream on downstream side). Stem 47 is inferred to have elongated distally relative to mammals (i.e., downstream on upstream side, upstream on downstream side).

2. Compensatory Substitutions

We made anecdotal notes on frequency of compensatory substitutions while counting putative synapomorphies for clades of interest. Unlike insertions and deletions within stems (Section IV,B,3), most substitutions within stems precipitate compen-
FIGURE 5.13 Partial sequence alignment of Gruiiformes mitochondrial 12S rRNA. The consensus sequence is for Gruiiformes only, but gaps in consensus sequence are shown to permit alignment with outgroups. Stems are underlined and labeled. Upper-case lettering, canonical pair in stem; upper-case italics, noncanonical pair in stem; lower-case, nonpairing base. Positions 77–120 illustrate migration of stem 8 and an uncompensated insertion within the stem at position 119 in Psophia. Positions 721–750 illustrate replication slippage in the distal loop of stem 42 as a mechanism for sequence length variation. Positions 824–840 illustrate shortening of stem 47 in Rhynchotus by an uncompensated deletion at position 830.
satory substitutions on the complementary side. Of those that we tracked, no fewer than 64% of compensations that occur do so within the period of one internode in the phylogeny reconstruction. Some that are located in the inferred distal extension of stem 47 remain uncomplemented over several internode periods, but such delays are not unique to this stem. Noncanonical pairing or nonpairing may be favored in these instances, or they may reflect our inability to detect all substitutions in the depths of the tree.

We may have observed an early event in the process of compensatory substitution. This involves an apparent example of heteroplasm in Rallus. Both C and T bands occur at site 372 in an otherwise clear and unambiguous sequence autoradiograph. This site is in a complementary position to site 402 in stem 24, where Rallus is unique in possessing a transversion substitution from A to T. The C represents a transition substitution from the T state at site 372. Neither C nor T complement with site 402. We speculate that the heteroplasm represents temporary relaxation of selection for sequence conservation related to the process of compensation. Stated differently, all noncomplementary bases may be tolerated equally, transitions would likely be the first form of variants to appear, and complementary bases would be selectively favored when they arise, eventually stabilizing the sequence.

3. Insertions and Deletions

We noted uncompensated insertions and deletions in stems that create or eliminate bulges (Figs. 5.4 and 5.13). Crowned crane and limpkin share a deletion of a 1-base bulge on downstream side of stem 3 (position 315). Trumpeter possesses a 1-base uncomplemented addition in stem 8 (position 119). Kagu exhibits a unique 1-base uncompensated deletion on the first base of the upstream side of stem 47 (position 830). All three rails exhibit a synapomorph 1-base uncompensated deletion on the downstream side of stem 48 (position 902).

Replication slippage may accelerate length variation in polynucleotides, and dinucleotide repeats in a variety of systems (Tautz et al., 1986; Hancock and Dover, 1990; Weston-Hafer and Berg, 1991; Degouën et al., 1991; Wolfson et al., 1991). The terminal loop of stem 42 varies from 5 to 12 bases in Gruiformes (Figs. 5.4 and 5.13). Cranes synapomorphically possess the most bases here. The loop begins with a variable number of pyrimidines (up to eight; almost all Cs) that immediately follow a C in the stem. The stem of hemipode ends with a T, and its loop begins with poly(T) instead of poly(C). The next segment of the loop includes up to three purines (mostly As), followed by a variable number of bases of which most are pyrimidines, especially Cs. This appears to be an example of length variation by slippage. Slippage seems to occur more readily than transitions, which in turn exceed transversions. Accordingly, crowned crane is the only member of the cranelike birds to exhibit a substitution among the 3- to 9-base poly(C) of the first segment, and it is a transition. Replication slippage may also be involved in the length variation at positions 83-107, 140-145, 659-660, and 817-820.
After a long history of substitutions has overwritten the earmarks of replication slippage, it may be impossible to distinguish chance similarities from homology. In variable-length regions, it may be prudent not to attempt to force sequences into alignment in the interest of phylogenetic inference.

4. Among-Site Rate Heterogeneity

Site variability, or substitution rate per site, differs profoundly between higher taxonomic groups. This is somewhat surprising in light of the sequence conservation between bacteria and vertebrates at some positions. Sullivan et al. (1995) identified 29 most-variable sites in their study of 12S rDNA in sigmodontine rodents, a group that is considerably less diverged among themselves than are Gruiformes. While the maximum number of changes per site they observed was three, we infer sites to have changed as many as eight times in our phylogeny of Gruiformes. Of 21 most-variable sigmodontine sites of which we could determine the homology in Gruiformes, 8 are invariant in Gruiformes. 3 changed only once, 3 twice, 3 three times, 3 four times, and 1 changed six times. This is clearly a different distribution of among-site rate variability between the two groups. Thus, the evolutionary dynamics of 12S rDNA may differ substantially between birds and mammals, and possibly contribute to the higher levels or resolution in some nonavian 12S rDNA reconstructions of even deeper divergences than those in Gruiformes (e.g., Cummings et al., 1995).

Phylogenetic informativeness is often thought of as inversely related to the rate at which changes occur, and weighting schemes are designed accordingly. But they need not be. The lengths of the monotonous polynucleotides in loop 42 may have evolved rapidly but they are obviously highly correlated with phylogeny (Section IV,B,3). For example, in the first segment rails have one C residue, finfoots have two, trumpeters and limpkin have three, and cranes have five to eight. Further similarities are exhibited by sunbittern—kagu—hemipode and seriema—bustard and finfoot—Aptornis—roatelo.

C. Character Evolution of 12S rDNA

Many regard nucleotide substitutions as stochastic or effectively neutral within the recognized constraints of positional variation in evolutionary rates and differences in rates between evolutionary lineages. By limiting consideration of frequency of nucleotide substitution merely to positional effects of evolutionary rate, one may overlook the ways in which nucleotide substitutions may be functionally correlated or adaptively specialized (Margoliash et al., 1976; Hancock and Dover, 1990; Irwin et al., 1991; Gillespie, 1991; Ma et al., 1993).

Positions of synapomorphously and homoplasiously shared derived characters of individual clades may be identified on a structural map of the gene, and tested for
fit to models of expected distributions of nucleotide substitutions. Derived characters that are concentrated in functional or structural regions of a gene and that differ significantly from expected distributions of nucleotide substitutions might represent adaptive specializations.

1. Expected Probability of Substitution

The expected probability of substitution for any site is estimated by dividing the number of times that site changed by the sum of all nucleotide substitutions at all sites in a most-parsimonious phylogeny of many taxa. Expected substitution frequencies for a functional or structural region is simply the sum of expected substitution probabilities of all sites included in a region that is otherwise defined by independent criteria (e.g., protein-binding domain or secondary structure).

We partitioned the 12S rDNA molecule into large regions on the basis of protein binding, and small regions corresponding to stem/loop structure. The large regions include sites 339–540 in domain II, which broadly encompass the binding domain of ribosomal proteins S6+18, and sites 610–670 and 770–930 in domain III, which collectively include the binding domains of proteins S7 and S19 (Noller et al., 1990; Ehresmann et al., 1990). The small regions include positions 339–452 and 796–861 within the larger sets.

We summed all nucleotide substitutions (from 0 to 8 per site; Section II,A,5; Table I) in each these regions in the most-parsimonious phylogeny and divided those sums by the total number of nucleotide substitutions in the phylogeny ($n = 1110$) to calculate the percent of substitutions detected in each region, our estimate of expected substitution probabilities for each region. Across all Gruiformes, only 21.5% of all sites but 34.3% of all substitutions occur in large region domain II, 27.8% of sites but 26.5% of substitutions in large region domain III, 12.1% of sites but 23.0% of substitutions in small region domain II, and 7% of sites but 10.0% of substitutions in small region domain III. The expected probability of substitution is greater in domain II than in domain III.

2. Distribution of Synapomorphies

Casual observation suggested to us that substitution frequencies were different in some clades than the whole of Gruiformes. We used chi-square (Zar, 1974) to test whether the relative frequencies of substitutions (i.e., synapomorphies) on individual branches differed significantly from the whole phylogeny in each of these regions (Section IV,C,1; substitutions defining the clade were subtracted from the whole to ensure independence of sets being compared). We performed this test on both the branches uniting trumpeter with ralllike birds and uniting trumpeter with cranelike birds, since our data support the former sister relationship only weakly. Parsimony bootstrap values support inclusion of trumpeters in the cranes–rails group, but does not significantly favor a grouping with either cranes or rails (Fig. 5.9).
Raillike birds ("rails" below) consisted of a star phylogeny for *Aptornis*, *Gallinula*, *Heliornis*, *Laterallus*, *Podica*, *Rallus*. Cranelike birds ("cranes" below) consisted of a star phylogeny for *Anthropoides*, *Balearica*, *Aramus*, and these were rooted by a star clade of *Ardeotis*, *Cariana*, *Mesitornis*, *Turnix*, sisters sunbittern–kagu and finally chicken. Twenty-five putative synapomorphies unite trumpeter with "cranes" and 18 are shared by trumpeter and "rails" (Fig. 5.14). We did not consider a phylogeny with trumpeters as sister to both "cranes" and "rails" because this parsimony reconstruction was 2.2% longer than the others.

Significant differences from expected frequencies of substitutions were apparent in the large region of domain II in the branch uniting trumpeters—"rails," and in all of the small regions for both trumpeters—"rails" and trumpeters—"cranes" ($p < 0.025$). Eliminating compensatory substitutions from consideration does not change the results. Thus the evolutionary rate in some functional and structural regions differs significantly between some gruiform clades, as both phylogenies gave significant results.

3. Distribution of Synapomorphy versus Homoplasy

Patterns of molecular synapomorphy and homoplasy can be elucidated by comparing mutually exclusive sets of putative synapomorphies indicated by alternate hypotheses of phylogeny for a single taxon (C) relative to its candidate sisters (A and
B; Fig. 5.14). The putative synapomorphies of C to each of clades A and B are mutually exclusive as long as A and B are sisters to one another (exclusive of C).

We tested for differences in frequencies of putative synapomorphies in functional and structural regions between the mutually exclusive hypothetical clades, trumpeters—"rails" and trumpeters—"cranes" (Fig. 5.14). This asks whether trumpeters—"cranes" and trumpeters—"rails" tree topologies affect the positions of putative synapomorphies differently. Using a chi-square contingency table with Yates correction (Yates, 1934; Zar, 1974), we found that the two phylogenies exhibit significant differences from one another in the frequencies of synapomorphies in the regions examined except for large region, domain II (large region, domain III, $p = 0.001$; small region, domain II, $p = 0.025$; small region, domain III, $p = 0.05$).

What value could there be in making a comparison with at least one tree that must be erroneous? Minimally, we have shown that synapomorphies and homoplasies are distributed differently; but we believe we have done more. Because (1) sets of synapomorphies defining two mutually exclusive hypothetical clades must in reality include at least one set of homoplasies, (2) both sets represent derived states (i.e., not plesiomorphies, as determined from outgroups), (3) distributions of those sets differ significantly between alternative phylogenies, and (4) those differences correspond to functional and structural regions of the gene, both genic divergence and convergence are observed to localize in functional and structural regions. This demonstrates variation in among-site evolutionary rates between sister taxa that could possibly represent (different) adaptive specializations at the molecular level.

Convergence may be implicated when homoplasiously shared characters coincide with discrete structural or functional parts of an organism or a gene, as they are in this example. We emphasize a distinction between suites of convergent characters and homoplasious noise. The former may be under the influence of a unifying selective agent, possibly affecting secondary structure and molecular interactions. We cannot distinguish whether our observation results from selection or is merely a byproduct of other factors.

There may be explanations for the different groupings of shared characters in the trumpeters—"rails" and trumpeters—"cranes" trees that do not invoke adaptive specialization. Because the rate of evolution in domain II exceeds that of domain III overall (Section IV,C,1), then the higher than expected number of shared characters in domain III of trumpeters—"rails" might represent phylogenetic signal while the higher than expected number of shared characters in domain II of trumpeters—"cranes" might be an attraction of long branches (Felsenstein, 1978). This explanation may not account for the less than expected numbers of shared characters in domain II of trumpeters—"rails" and domain III of trumpeters—"cranes."

We used Mann–Whitney U (Zar, 1974) to test whether synapomorphies of trumpeters—"cranes" (domain II) differ from synapomorphies of trumpeters—"rails" (domain III) in evolutionary rate (i.e., number of substitutions per site; Table I; both large and small regions were tested). This differs from the observation that domain II has a higher expected substitution probability overall than domain III.
because it examines the evolutionary rate on a site-by-site basis. We also tested whether all synapomorphies of trumpeters—"cranes" differed from all synapomorphies of trumpeters—"rails" in evolutionary rate. No test detected significant differences in substitution rates between sites defining the trumpeters—"cranes" and trumpeters—"rails" clades ($p > 0.2$). This suggests that the difference in distributions of synapomorphies and homoplasies we observed cannot be attributed simply to rate differences among sites.

V. IMPLICATIONS OF 12S EVOLUTION FOR PHYLOGENETIC INFERENCE

The lack of a clear resolution of many aspects of gruiform phylogeny from 12S rDNA is disappointing. 12S rDNA strongly supports relationships of some recently diverged taxa, but not of more distant taxa. Even some traditionally accepted family groups do not receive robust support from our data. What is the cause for the lack of phylogenetic resolution in these data?

12S rDNA is not entirely saturated by homoplasious substitutions at the levels of gruiform divergence because it performs well at resolving much older divergences. Yet it exhibits sufficient noise to hinder resolution of a phylogeny that may be characterized by relatively short internodal branches (Fig. 5.8). Low jackknife values are symptomatic of such noise (Fig. 5.6). Our phylogenetic hypotheses must be interpreted cautiously. A gene phylogeny may not always accurately reflect organismal phylogeny (Avise et al., 1984; Wu, 1991; but see Moore, 1995). Discrepancies in phylogenetic reconstructions derived from different genes demonstrate that all cannot accurately reflect organismal phylogeny (Felsenstein, 1988; Bremer, 1988; Pamilo and Nei, 1988; Wheeler and Honeycutt, 1988; Hendy and Penny, 1989; Doyle, 1992; Sanderson and Doyle, 1992).

We chose 12S rDNA partly because it includes both evolutionary labile and conserved regions, and therefore should have a broad window of resolution for addressing recent and ancient divergences. However, among-site variation in evolutionary rate, among other factors, proves to impede rather than enhance the successful recovery of gene phylogenies. Phylogeny reconstruction is sensitive to substitution bias (Brown et al., 1982; Knight and Mindell, 1993), differences in evolutionary rates at the level of the organism (Britten, 1986; Sheldon, 1987), gene (Ayala, 1986) or nucleotide position (Milkman and Crawford, 1983), homoplasious evolution (Wilkinson, 1991), composition bias (Collins et al., 1994), and differences in branch lengths, tree symmetry, and number of taxa (Nei, 1991; Huelsenbeck and Hillis, 1993). The factors that influence the reliability of tree-building methods is well understood for only simple conditions (Hendy and Penny, 1989; Rohlf et al., 1990; Nei, 1991; Navidi and Beckett-Lemus, 1992; Huelsenbeck and Hillis, 1993; Kim et al., 1993; Zharkikh and Li, 1993).

Whatever lack of resolution is symptomatic of 12S rDNA data, it probably does
not accrue from positional rate variation as traditionally perceived because neither position weighting nor data partitioning improves tree resolution. Instead, it may result from differences in evolutionary rates between taxa and differences in positional rates between taxa. The conserved sequences and secondary structures of small subunit rDNA shared by prokaryotes and vertebrates belie dramatic differences in evolutionary lability of homologous regions between different lineages. This is evident at a large scale by the observation that a quarter to a third of the most variable sites in rodents are invariant in Gruiiformes. It is evident at the small scale by significant differences in regional substitution rates between sister clades. High rates of substitution, thus, are not confined to particular sites across taxa; they are found in different locations in different lineages.

VI. SUMMARY

We performed phylogenetic reconstructions using 12S rDNA sequences from representatives of all the currently recognized families of Gruiiformes. We found rails closer to cranes than many other Gruiiformes widely believed to be close to cranes. We suggest that trumpeters are closer to rails than to cranes, but suggest that they are intermediate between the two. Among a clade of rail relatives are the sungebre and finfoots and the fossil Aptornis. Kagu and sunbittern are each the only close relative of the other, and are the most distant of all Gruiiformes examined. We make several observations and inferences on the evolutionary dynamics and character evolution of the 12S rDNA molecule, including (1) variation in secondary structure resulting from stem migration and uncompensated insertions and deletions within stems, (2) replication slippage as a mechanism of sequence length variation in loops, (3) differences in per-site substitution rates between birds and mammals, (4) the process of compensatory substitution in stems, and (5) differences in distributions of synapomorphies and homoplasies that are spatially correlated with functional and structural regions of 12S rDNA. A robust but simplified estimate of the instantaneous ratio of rates between transversions to transitions is calculated for the 12S rDNA of Gruiiformes.

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