Testing Hypotheses about the Sister Group of the Passeriformes Using an Independent 30-Locus Data Set

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Abstract

Although many phylogenetic studies have focused on developing hypotheses about relationships, advances in data collection and computation have increased the feasibility of collecting large independent data sets to rigorously test controversial hypotheses or carefully assess artifacts that may be misleading. One such relationship in need of independent evaluation is the position of Passeriformes (perching birds) in avian phylogeny. This order comprises more than half of all extant birds, and it includes one of the most important avian model systems (the zebra finch). Recent large-scale studies using morphology, mitochondrial, and nuclear sequence data have generated very different hypotheses about the sister group of Passeriformes, and all conflict with an older hypothesis generated using DNA–DNA hybridization. We used novel data from 30 nuclear loci, primarily introns, for 28 taxa to evaluate five major a priori hypotheses regarding the phylogenetic position of Passeriformes. Although previous studies have suggested that nuclear introns are ideal for the resolution of ancient avian relationships, introns have also been criticized because of the potential for alignment ambiguities and the loss of signal due to saturation. To examine these issues, we generated multiple alignments using several alignment programs, varying alignment parameters, and using guide trees that reflected the different a priori hypotheses. Although different alignments and analyses yielded slightly different results, our analyses excluded all but one of the five a priori hypotheses. In many cases, the passerines were sister to the Psittaciformes (parrots), and taxa were members of a larger clade that includes Falconidae (falcons) and Cariamidae (seriemas). However, the position of Coliiformes (mousebirds) was highly unstable in our analyses of 30 loci, and this represented the primary source of incongruence among analyses. Mousebirds were united with passerines or parrots in some analyses, suggesting an additional hypothesis that needs to be considered in future studies. There was no clear evidence that base-compositional convergence, saturation, or long-branch attraction affected our conclusions. These results provide independent evidence excluding four major hypotheses about the position of passerines, allowing the extensive studies on this group to be placed in a more rigorous evolutionary framework.

Key words: Passeriformes, Psittaciformes, landbirds, alignment bias, guide tree.

Introduction

Advances in data collection and bioinformatics have the potential to create a new era for phylogenetics, where large data sets can be collected for hypothesis testing as well as data exploration. This is particularly important when there are multiple conflicting hypotheses regarding relationships, which often occurs when there have been rapid radiations with too little time between divergences for the origin of clear molecular or morphological synapomorphies. For example, several large-scale studies have resulted in conflicting positions for the ctenophores in the animal tree of life (e.g., Dunn et al. 2008; Philippe et al. 2009; Schierwater et al. 2009), indicating that even large data matrices may not support stable consistent relationships. Thus, novel relationships should be rigorously reevaluated even when they are supported by large-scale data sets. Careful analyses of available data matrices can reveal some sources of incongruence (e.g., Philippe et al. 2011), but the collection and analysis of independent data represent an important and complementary strategy.

For the avian tree of life, recent studies have consistently supported three major superordinal clades: Palaeognathae (ostrich, emu, kiwi, and allies), Galloanserae (pheasants, ducks, and allies), and Neoaves (the remaining ~95% of extant avian species) (reviewed by Braun and Kimball 2002; Cracraft et al. 2004). Although these large-scale divisions are clear, the phylogeny of Neoaves remains controversial. Indeed, the extremely short branches at the base of Neoaves combined with the existence of substantial topological incongruence among independent gene trees has been used to suggest that the base of Neoaves might represent an "explosive radiation" or "hard polytomy," which is impossible to resolve with any amount of data (e.g., Poe and Chubb 2004). However, incongruence among data set can be caused by the use of conflicting data and/or poorly fitting models (e.g., Braun and Kimball 2002; Holland et al. 2004; Penny et al. 2008). Additionally, the slowly evolving loci used in some previous studies may have had too little power to resolve the relationship within Neoaves (Chojnowski et al. 2008). A recent large-scale study using nuclear loci, primarily the more rapidly evolving introns,

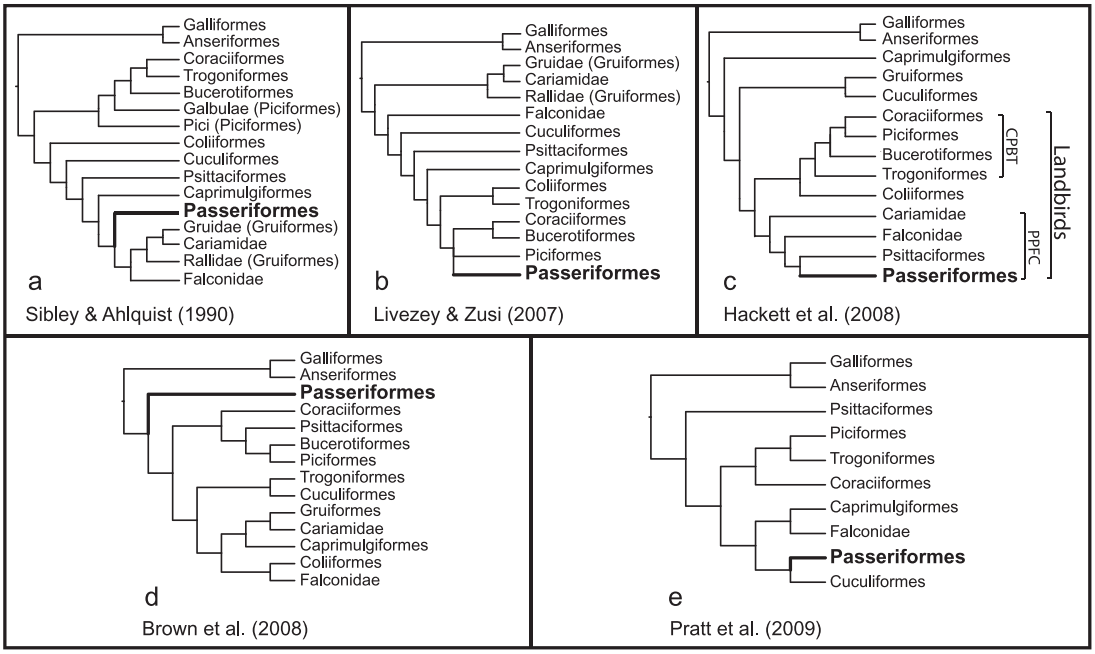

Fig. 1. A priori hypotheses based upon different types of data. (*a*) DNA–DNA hybridization, (*b*) morphological data, (*c*) nuclear DNA sequences, and (*d*) and (*e*), mitochondrial DNA sequences. CPBT, Coraciiformes–Piciformes–Bucerotiformes–Trogoniformes; PPFC, Passeriformes–Psittaciformes–Falconidae–Cariamidae.

has recovered substantial structure among clades within Neoaves (Hackett et al. 2008). However, that study suggested some novel relationships, making it critical both to test these relationships using independent large-scale data sets and to determine whether the relationships could be due to biases or artifacts of phylogenetic inference.

One of the most fascinating questions regarding the phylogeny of Neoaves is the position of Passeriformes (perching birds or passerines), which represent 59% of avian species (Sibley and Monroe 1990) and for this reason are among the best studied of avian orders. Because of the key role that Passeriformes play in many areas of research, ranging from neurobiology, conservation biology, behavior ecology, and evolution among others (e.g., Jarvis and Mello 2000; Sharp et al. 2008), understanding their phylogenetic context is critical to examine information about passerine biology in a larger evolutionary framework. However, the position of passerines within Neoaves has long been unclear. In fact, when Cracraft (2001) divided Neoaves into six major groups, he suggested that Passeriformes alone were one of these groups, emphasizing that there was no convincing evidence to determine the potential sister group of this order.

Several large-scale studies (e.g., Sibley and Ahlquist 1990; Ericson et al. 2006; Livezey and Zusi 2007; Brown et al. 2008; Hackett et al. 2008) have suggested specific hypotheses regarding the relatives of Passeriformes, though there is no consensus among these studies (fig. 1). Using DNA–DNA hybridization, Sibley and Ahlquist (1990) placed Passeriformes deeper within Neoaves as sister to a diverse assemblage of orders (fig. 1*a*). In contrast, using a large-scale morphological data set, Livezey and Zusi (2007) found a polytomy with Passeriformes, Piciformes (woodpeckers and allies), and Coraciiformes (kingfishers and allies) (fig. 1*b*), a result consistent with that given by a single nuclear intron (Fain and Houde 2004). By using nuclear loci, Ericson et al. (2006) supported a clade that includes members of four different groups: Passeriformes, Psittaciformes, Falconidae (falcons), and Cariamidae (seriemas), hereafter called the PPFC clade. Hackett et al. (2008) found the same clade but further resolved it by supporting a Passeriformes–Psittaciformes (hereafter PP) clade (fig. 1*c*; see also figure 3 in Sorenson et al. 2003). Finally, mitochondrial data provided two additional hypotheses. Using partial mitochondrial sequences and extensive taxon sampling, Brown et al. (2008) found deep divergence of Passeriformes making them sister to all other Neoaves (fig. 1*d*). Using whole mitochondria but less extensive sampling of orders within Neoaves, Pratt et al. (2009) found a sister group relationship between Passeriformes and Cuculiformes (fig. 1*e*), a clade that had also been suggested by Mayr et al. (2003) using morphological and nuclear sequence data (see also figure 1 in Sorenson et al. 2003). The relationships found in these different studies define a set of a priori hypotheses that can be tested using independent data.

Taken as a whole, the above hypotheses emphasize the disagreement in the placement of Passeriformes among studies based upon different data sets. This may be driven by two distinct phenomena. First, the disagreement among studies could be related to the rapid radiation at the base of Neoaves. The rapid radiation could have resulted in a hard polytomy (Poe and Chubb 2004), in which case each tree would represent a random resolution. Alternatively, the rapid radiation could have led to a soft polytomy, in which

case the observed incongruence could reflect most or all of the existing data sets having too little power to provide a consistent resolution (Chojnowski et al. 2008). Second, the differences among studies may be due to biases in the data sets that have led to incorrect phylogenetic inferences for most or all of the data sets. To distinguish between these two possibilities, it is important to collect data that are completely independent of the data used to generate the a priori hypotheses (fig. 1) and to examine for potential biases that could affect the results. Thus, we collected data for 28 species including all likely sister groups of Passeriformes from 30 loci (see supplementary tables S1 and S2, Supplementary Material online) that have not been used previously to examine the phylogenetic position of Passeriformes. Although different types of data, such as morphological traits or mitochondrial DNA, have been used in phylogenetic reconstruction (e.g., Livezey and Zusi 2007; Brown et al. 2008), the use of both of these has been considered problematic for the resolution of superordinal relationships in birds (e.g., Braun and Kimball 2002; Mayr 2008). So the data in our study are primarily focused on nuclear introns, which are thought to have greater power to resolve difficult deep phylogenetic problems in vertebrates (Matthee et al. 2007; Chojnowski et al. 2008; Hackett et al. 2008). Moreover, nuclear introns tend to show less complex patterns of molecular evolution relative to coding exons and untranslated regions (e.g., less extreme among-sites rate variation; Hughes and Yeager 1997; Bonilla et al. 2010). However, introns have also been criticized due to both the alignment ambiguities and the loss of signal caused by substitution saturation (e.g., Shapiro and Dumbacher 2001; Morgan-Richards et al. 2008; Pratt et al. 2009). Thus, we examined the impact of these biases, if they exist, by using multiple alignment strategies, testing for saturation, and examining the impact of changes in base composition. This approach provided a rigorous and independent test of the available hypotheses regarding the sister group of passerines.

Materials and Methods

Data Collection

We analyzed data from 28 species including chicken (*Gallus gallus*) and Southern Screamer (*Chauna torquata*) as outgroups (see supplementary table S1, Supplementary Material online). In most cases, more than one species was selected to represent a single order or major clade. Moreover, the 30 loci used in this study were completely independent from the data used in the previous studies that generated the a priori hypotheses (see above). The sequences were primarily noncoding (mostly introns) with little or no flanking exon sequence (see supplementary table S2, Supplementary Material online). The loci were located on 17 chromosomes in the chicken genome, which is expected to limit problems due to linkage given the conservation of avian chromosome structure (Griffin et al. 2007).

Sequences of chicken and zebra finch (*Taeniopygia guttata*) were obtained from the available draft genome sequences (Hillier et al. 2004; Warren et al. 2010). Other sequences were amplified by standard polymerase chain reaction (PCR) using primers developed for this study or were taken from Kimball et al. (2009), Smith (2009) and Smith JV (personal communication), and Cox et al. (2007) (see supplementary table S3, Supplementary Material online for details). PCR products were precipitated using polyethylene glycol 800:NaCl (20%:2.5 M) in preparation for direct sequencing. An ABI Prism 3100-Avant genetic analyzer (PE Applied Biosystems, Foster City, CA) was used to generate sequences using the ABI BigDye Terminator v.3.1 chemistry. Amplicons generated from loci with length polymorphisms did not sequence cleanly, so they were cloned into the pGEM-T (Promega) vector and purified using FastPlasmid Mini-kit (5 Prime GmbH). All samples were sequenced in both directions using amplification primers. Sequencher 4.1 (Gene Codes Corp.) was used to edit sequences and assemble double-stranded contigs. The novel sequences collected in this study have been deposited in Genbank (JN599305–JN599976) and recently published sequences for the *CLTCL1* locus (Braun et al. 2011), which was not included in Hackett et al. (2008), were added to complete the independent 30-locus data matrix.

Sequence Alignment

Accurate sequence alignment is a prerequisite of phylogenetic analyses, and it is clear that alignment can have a major impact on phylogenetic estimation (Ogden and Rosenberg 2006; Smythe et al. 2006). Since the difficulty of multiple sequence alignment represents one of the biggest concerns regarding the use of nuclear introns for phylogenetics (see above), examining the potential for alignment bias using alternative alignment strategies was particularly important for this study.

We conducted analyses using both manual and automated alignments. Most analyses were based on the manual alignment, an approach used in many other studies (e.g., Hackett et al. 2008). We improved the standard manual alignment strategy by conducting "blind alignments." Briefly, sequences were aligned using Mafft v.6.717 (Katoh et al. 2009) and optimized "by eye" in MacClade 4.08 (Maddison DR and Maddison WP 2005), but the order of taxa was randomized for each locus, and taxon names were replaced with random codes prior to the manual optimization. This was done to eliminate any potential bias reflecting prior expectations regarding relationships. These codes were broken, and the alignments for individual locus were combined using a perl program written by E.L.B. We also generated alternative versions of the manual alignment that excluded specific sites. There were a number of large indels that made substantial contributions to alignment length without necessarily providing information useful for reconstructing phylogenetic relationships. To generate data matrices that excluded large indels and regions with potential alignment ambiguity, we used Gblocks 0.91b (Castresana 2000; Talavera and Castresana 2007) and a program ("Gappy"; written by E.L.B.) that excludes gappy

Table 1. Alignment Statistics.

Alignment Methods	Guide Tree[a]	Alignment Parameters[b]	Scores[c]	Alignment Length		Informative Sites	
				Complete[d]	Gblocks[e]	Complete	Gblocks
Manual	—	—	2,429	25,700	10,917	8,614	5,994
	—	Noambiguous	—	24,983	—	8,393	—
	—	Nogappy	—	16,170	—	8,604	—
	—	Noam_nogappy	—	15,776	—	8,384	—
Mafft	—	Default	2,419	25,659	10,926	8,972	6,104
	—	op = 1	2,430	25,576	10,676	8,713	5,929
	—	op = 3	2,410	25,379	10,671	9,126	6,102
	—	ep = 0.01	2,426	25,493	10,844	8,847	6,082
	BR	Default	2,426	25,505	10,752	8,810	6,020
	LZ	Default	2,427	25,476	10,933	8,815	6,119
	HA	Default	2,425	25,504	10,951	8,829	6,116
	SA	Default	2,425	25,468	10,927	8,832	6,119
Prank	BR	Default	2,403	26,747	10,379	8,645	5,639
		12	2,394	26,088	10,074	8,958	5,551
		55	2,405	26,707	10,416	8,586	5,589
	LZ	Default	2,400	28,149	10,412	8,530	5,571
		12	2,382	27,267	10,006	8,928	5,506
		55	2,401	28,044	10,321	8,503	5,534
	HA	Default	2,399	27,800	10,111	8,565	5,414
		12	2,390	27,936	10,094	8,834	5,522
		55	2,400	27,661	10,092	8,523	5,387
	SA	Default	2,406	27,641	10,280	8,535	5,534
		12	2,396	28,248	10,082	8,768	5,513
		55	2,402	27,554	10,322	8,496	5,529
SATe_Mafft	—	Iteration = 50	2,424	24,936	10,828	8,883	6,080
	BR	Iteration = 10	2,414	25,566	10,894	8,979	6,153
	LZ	Iteration = 10	2,418	25,516	10,844	8,875	6,070
	HA	Iteration = 10	2,418	25,129	10,870	8,903	6,136
	SA	Iteration = 10	2,413	25,264	10,848	8,955	6,135
SATe_Prank	—	Iteration = 50	2,407	27,744	10,279	8,398	5,418
	BR	Iteration = −1	2,406	26,939	10,235	8,394	5,474
	LZ	Iteration = −1	2,409	27,135	10,134	8,382	5,377
	HA	Iteration = −1	2,414	26,942	10,330	8,413	5,482
	SA	Iteration = −1	2,408	27,107	10,123	8,461	5,441
Muscle	—	Default	2,432	25,566	10,941	8,647	5,965
T-coffee	—	Default	2,468	26,750	9,704	8,701	5,272
ClustalW2	—	Default	2,281	23,716	11,107	10,323	6,843

[a] Guide trees SA, Sibley and Ahlquist (1990) topology; LZ, Livezey and Zusi (2007) topology; BR, Brown et al. (2008) topology; and HA, Hackett et al. (2008) topology.
[b] Noambiguous, delete ambiguous regions in the manual alignment; Nogappy, delete gappy regions in the manual alignment; Noam_nogappy, delete both ambiguous and gappy sites in the manual alignment. 12: gap rate = 0.01, gap extension = 0.2; 55: gap rate = 0.05, gap extension = 0.5; op, gap open penalties; ep, gap extension penalties.
[c] Sum of the scores for each locus calculated using Core in T-Coffee package based on the complete data matrix. Scores in the upper quartile are underlined.
[d] Complete: Calculation based on complete data matrix.
[e] Calculation based on data sets that generated by Gblocks. Gblocks was not implemented on manual alignments that had already been treated by noise reduction methods (i.e., Noambiguous, Nogappy, and Noam_nogappy).

sites. Gblocks used a criterion that allows the final blocks to have half gap positions (the minimum number of sequences for a conserved position and a flanking position was 15 and 23, respectively, and the maximum and minimum numbers of contiguous nonconserved positions were eight and five, respectively), whereas Gappy was used to exclude sites where nucleotides were present in fewer than three taxa (hereafter "gappy sites"), which was identical to the procedure used by Hackett et al. (2008). Moreover, we manually examined the alignment and identified and excluded regions where the alignment appeared ambiguous (e.g., homopolymer runs). Finally, we generated an alignment with both gappy and ambiguous regions excluded (table 1).

Although manual optimization after automated alignment has the potential to improve alignments because it allows the evaluation of complex features that are difficult to implement in alignment algorithms, it is labor intensive and has the potential to introduce biases based upon prior expectations. Although our blind alignment strategy should limit these biases, it is clearly desirable to examine automated alignment approaches as well. To do this, we also generated alignments using Mafft (Katoh et al. 2009), Prank (Löytynoja and Goldman 2008), SATé (Liu et al. 2009), Muscle (Edgar 2004), T-Coffee (Notredame et al. 2000), and ClustalW2 (Thompson et al. 1994; Larkin et al. 2007). Several different sets of alignment parameters were used in Mafft and Prank to examine their impact upon alignments. Additionally, our independent evidence approach provided an excellent opportunity to explore the sensitivity of multiple sequence alignments to the guide tree since it permits the use of guide trees reflecting the set of a priori plausible hypotheses (e.g., fig. 1) rather than

basing the guide tree upon the data that are being analyzed. Thus, guide trees based upon the first four a priori hypotheses (fig. 1) were used with the programs Mafft, Prank, and SATé. Briefly, the analyses based upon user-specified guide trees used maximum likelihood (ML) estimates of branch lengths for each guide tree, which were obtained for each locus using the manual alignment and PAUP* 4.0b10 (Swofford 2003). Then, each locus was aligned using the corresponding guide tree (with associated branch lengths), a procedure that resulted in four different alignments for each locus, alignment program, and parameter set. Alignments based upon each of the guide trees are identified using the abbreviations SA (Sibley and Ahlquist 1990), LZ (Livezey and Zusi 2007), HA (Hackett et al. 2008), and BR (Brown et al. 2008). The Pratt et al. (2009) topology (fig. 1*e*) was not used as a guide tree due to its limited taxon sampling. Alignments without a user-specified guide tree were also generated using all programs. As described above for the manual alignment, the single-locus alignments for each program and guide tree were combined using the perl program written by E.L.B. Finally, we used Gblocks to exclude sites from these automated alignments to examine the impact of excluding indels and potentially ambiguously aligned regions upon our conclusions.

The length and number of parsimony informative sites in each concatenated alignment are available in table 1. Alignment quality was assessed using the sum of the alignment scores for each locus as calculated using the Core method implemented in T-Coffee. Distances between pairs of alignments were calculated using a column identity metric (using a perl script written by E.L.B). Briefly, every nucleotide in each of the sequences was numbered continuously with no number assigned to gaps or missing data. Then, every column in each multiple sequence alignment was identified based upon the nucleotide numbers in that column. If a column had the same set of nucleotide numbers in both alignments, it was scored as an "identical column." If any sequence had a different nucleotide number in a column, it was identified as a "unique column." This method is sensitive to minor differences between alignments; even if two alignments comprise the same set of site patterns but have columns with different nucleotide numbers, they will have unique columns. The alignment distance we used corresponded to the proportion of unique columns in the alignments, using the minimum value when the proportion of unique columns differed between the two alignments that were compared (supplementary table S4, Supplementary Material online). These alignment distances were used to generate a distance matrix that was clustered by neighbor joining (NJ) to visualize similarities and differences among alignments.

Phylogenetic Analysis

We used the ML criterion and a Bayesian Markov chain Monte Carlo (MCMC) approach to analyze our data. In a set of preliminary analyses based on the manual alignment, we used Modeltest 3.7 (Posada and Crandall 1998) and the second-order variant of the Akaike information criterion (AIC_c) to choose the best-fitting model for ML analyses of the combined matrix and each independent nuclear locus. The best-fitting models for most of the loci were a transversional model+Γ and general time reversible (GTR)+Γ (for complete information, see supplementary table S2, Supplementary Material online). After conducting ML analysis of the combined data set using either the best-fitting model (GTR+Γ+I) in PAUP*4.0b10 (Swofford 2003) or GTR+Γ model in RAxML 7.0.3 (Stamatakis 2006), we found that RAxML and PAUP* exhibited no differences in their ability to identify the optimal tree topology. Since RAxML was computationally efficient and yielded results similar to PAUP*, all other ML analyses were conducted in RAxML using the GTR+Γ model and ten randomized starting trees. Partitioned ML analyses were also conducted in RAxML as described above, defining each locus as a partition. The fit of the GTR+Γ model with and without partitioning was evaluated using the AIC_c with the likelihood scores and numbers of free parameters reported by RAxML.

To further examine the sensitivity of our analyses to the models used for analysis, we used a Dirichlet process mixture of profiles of equilibrium frequencies combined with general exchange rates (the CAT-GTR model) and a Q-matrix mixture model (QMM) models implemented in PhyloBayes 3.3b (Lartillot and Philippe 2004, 2006; Lartillot 2007). Briefly, we ran two MCMC chains using the manual alignment. The runs were stopped after sampling 3,000 trees from each chain. The chains appeared to converge rapidly upon a specific topology, and all discrepancies were lower than 0.1 based upon bpcomp (used to assess convergence in the PhyloBayes package). We evaluated the fit of the CAT-GTR and QMM models by cross-validation (CV) using the HA topology.

We evaluated support in several different ways. First, our primary method of evaluating support was the ML bootstrap (Felsenstein 1985) with 500 replicates or Bayesian posterior probabilities. Second, we conducted "gene-jackknifing" analyses (Hackett et al. 2008) based on manual alignment to test the impact of excluding each individual locus upon the tree topology. After excluding each, we used the remaining data both to search for the ML tree and to examine support using 100 bootstrap replicates. Finally, we measured leaf stability using Phyutility (Smith and Dunn 2008) and the 500 bootstrap result for each combined alignment.

To explore the differences among trees estimated from different alignments and analyses, we calculated RF distances (Robinson and Foulds 1981) among trees in PAUP*. The RF distance approach compares the topology among trees by measuring the number of branches that differ between pairs of trees, so larger numbers reflect greater topological differences. To visualize differences among trees, NJ was used to cluster this matrix of RF distances. To determine whether trees were more similar than expected by chance, RF distances were compared with a distribution of RF distances from a specific tree to 10,000 random trees in which clades that are strongly supported were constrained. Briefly, the random trees were generated as described in Chojnowski et al. (2008), by resolving polytomies in a tree

that includes all branches viewed as uncontroversial (i.e., species within orders or families that are virtually certain to form a clade were constrained to be monophyletic).

We used the same species as Hackett et al. (2008), with the exception of the zebra finch and kea (*Nestor notabilis*), allowing the combination of our data with that study (for the kea, we obtained data for some loci included in Hackett et al. (2008)). Combining our manual alignment with Hackett et al. (2008) resulted in a 49-locus data set (~59 kb of aligned DNA sequence data) that was used for partitioned and unpartitioned ML and ML bootstrap analyses in RAxML, as described above.

Exploration of Potential Phylogenetic Artifacts

We identified loci with potentially biased base composition using two methods. First, we used the χ^2 test of base composition that is implemented in PAUP* to examine the homogeneity of base frequencies across taxa for each locus, limiting our consideration to the base composition of variable sites in the manual alignment (a typical treatment for analyses of base composition; e.g., Phillips and Penny 2003; Harshman et al. 2008). We also calculated relative composition variability (RCV; Phillips and Penny 2003) to describe the base-compositional heterogeneity of each locus. To determine whether loci that exhibit changes in base composition were likely to have had an impact upon our conclusions, we conducted two different ML analyses that excluded either loci that exhibited significant base-compositional heterogeneity based upon the χ^2 test or loci that were in the upper quartile of the RCV values. We also examined whether nucleotide composition bias might drive our conclusions by using NJ to cluster Euclidean distances between vectors of the base composition for variable sites in the manual alignment, as described in Harshman et al. (2008). A C++ program written by E.L.B was used to calculate the RCV values and base-compositional distances (PAUP* was used to conduct the NJ analysis).

Passeriformes exhibit rapid evolutionary rates relative to other birds (e.g., Yuri et al. 2008). To examine the relative evolutionary rates of the taxa included in this study, we calculated the sum of branch lengths from the base of Neoaves. To accommodate phylogenetic uncertainty, branch length estimates were obtained for the four a priori hypotheses for avian phylogeny (SA, LZ, HA, and BR; see above) using the GTR+Γ model in PAUP*. This information was used to select subsets of taxa for analyses that were used to explore the potential for long-branch attraction (LBA).

Although less prone to nucleotide substitutional saturation than mitochondrial sequences (e.g., Armstrong et al. 2001), nuclear introns accumulate substitutions relatively rapidly so saturation represents another potential problem when these relatively rapidly evolving sequences are used to examine ancient divergences (see Philippe et al. 2011). We used the I_{ss} metric proposed by Xia et al. (2003) and implemented in DAMBE (Xia et al. 2003) to examine substitution saturation using the manual alignment. We also conducted ML analyses (with and without partitioning) excluding loci that exhibited saturation based upon this metric to examine the potential impact of saturation upon our conclusions.

Results and Discussion

Independent Evidence Corroborates the Hackett et al. (2008) Topology

RAxML analyses of the independent 30-locus data set that was aligned manually produced a tree remarkably similar to Hackett et al. (2008), for both unpartitioned and partitioned analyses (fig. 2). This is also true for PhyloBayes analyses under both models (fig. 3 and supplementary fig. S1, Supplementary Material online; the CAT-GTR model had a better fit to the data than the QMM model, CV = -5.15 ± 5.25533; Lartillot 2007). Indeed, the Hackett et al. (2008) tree was significantly closer to the trees based upon the novel loci sequenced for this study than to random trees (table 2). In sharp contrast, this was not true for the Sibley and Ahlquist (1990), Livezey and Zusi (2007), and Brown et al. (2008) topologies (table 2). Although the differences in taxon sampling prevented a direct test of the Pratt et al. (2009) hypothesis by comparing the pairwise RF distances, the observed differences between its tree topology (fig. 1*e*) and the phylogeny we obtained (figs. 2 and 3) indicate that it is also quite distant from the trees based on the novel loci.

The novel data strongly corroborated the Hackett et al. (2008) landbird clade, providing 100% bootstrap support/posterior probability for monophyly of this group. This large group excluded Caprimulgiformes, Cuculiformes, and Gruiformes with strong support (figs. 2 and 3), which ruled out three a priori hypotheses (i.e., Sibley and Ahlquist 1990; Brown et al. 2008; and Pratt et al. 2009). The landbirds included the PPFC clade, a second clade comprising Coraciiformes, Piciformes, Bucerotiformes, and Trogoniformes (called CPBT hereafter, this clade is essentially identical to the "woodking" clade of Pratt et al. (2009); see fig. 1), and the Coliiformes. Similarly, the strong support for the CPBT clade (~90% bootstrap support and 100% posterior probability, figs. 2 and 3) excluded the phylogenetic hypothesis of Livezey and Zusi (2007). Although there is no consistent resolution outside the landbirds (figs. 2 and 3), this inconsistency could reflect the limited sampling of these deep-branching taxa. Thus, we will focus within landbirds for the remainder of the paper.

Important areas of congruence between Hackett et al. (2008) topology (fig. 1*c*) and the unpartitioned ML analyses of the manually aligned independent data (fig. 2*a*) included both the PPFC clade (46% bootstrap support) and the sister relationship between Passeriformes and Psittaciformes (the PP clade, 33% bootstrap support). Partitioned ML analyses (which used a model with a better fit to the data based upon the AIC_c) resulted in an optimal tree that united Coliiformes and Psittaciformes (fig. 2*b*), though the partitioned bootstrap consensus tree actually had the PP clade with limited (28%) bootstrap support. An analysis using the CAT-GTR model in PhyloBayes resulted in posterior probabilities of 0.96 and 0.70 for the PPFC and the PP clades, respectively (fig. 3). None of the hypotheses observed in

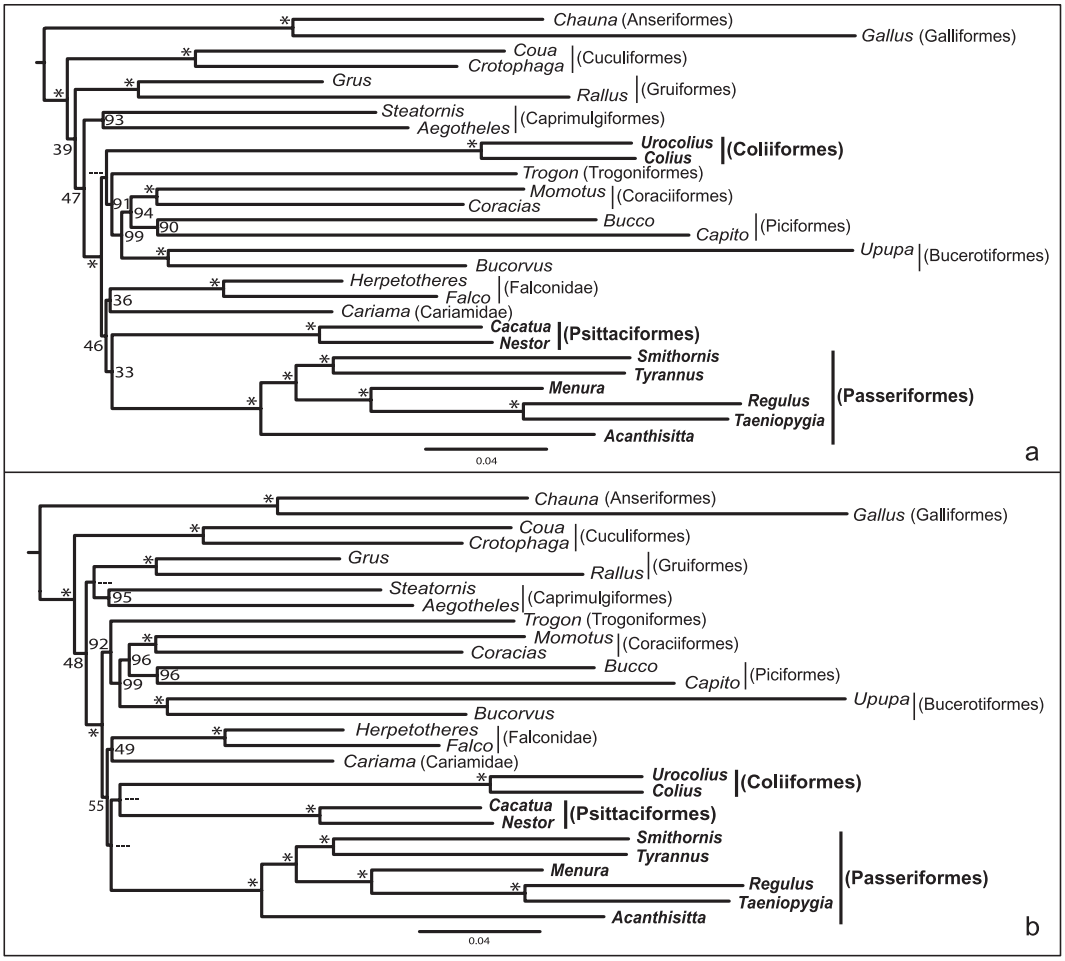

FIG. 2. ML phylogeny based on manual alignment, with bootstrap values. (*a*) unpartitioned ML and (*b*) partitioned ML. *Gallus gallus* and *Chauna torquata* were used as outgroups. * indicates 100% bootstrap support. — indicates clades that were not found in bootstrap consensus trees: Instead, Passeriformes–Psittaciformes obtained 28% bootstrap support, whereas Coliiformes were sister to PPFC with 55% bootstrap support.

the other analyses of these data could be excluded from the 95% credible set of trees; in contrast, all of the a priori hypotheses (fig. 1), with the exception of HA, could be excluded. In short, these analyses presented a clade that includes Passeriformes and Psittaciformes (the PP clade or PP + Coliiformes) and place that group within a larger

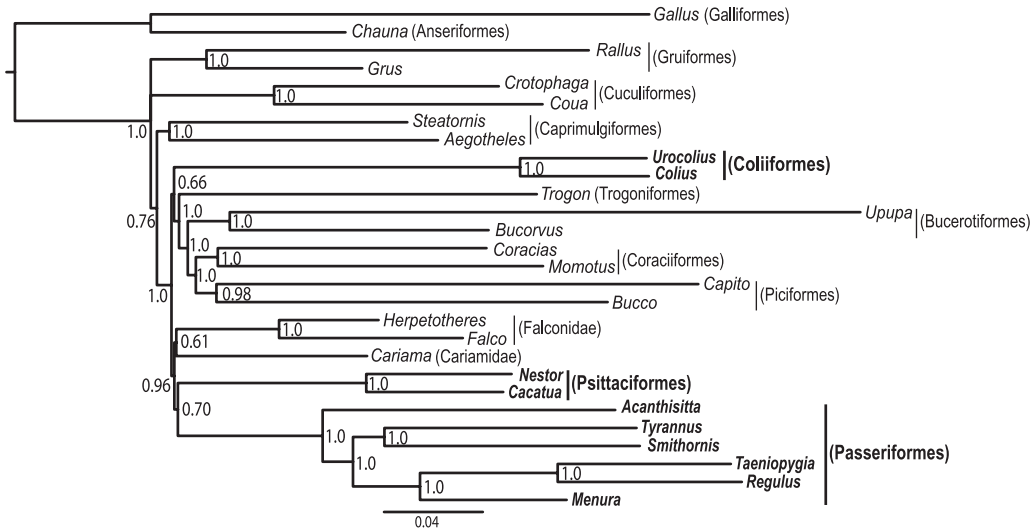

FIG. 3. Consensus tree of the manual alignment using the CAT-GTR model in PhyloBayes. Posterior probabilities (when they are >0.5) are indicated on the tree.

Table 2. RF Distances between the ML Trees for the Manual Alignment and the A Priori Defined Trees.

	Unpartitioned ML		Partitioned ML	
Hypotheses	RF	Probabilities of Matching Random Trees[a]	RF	Probabilities of Matching Random Trees
HA topology	6	<0.0001	12	0.0002
LZ topology	23	0.8509	23	0.8509
SA topology	24	0.0963	24	0.0963
BR topology	20	0.7760	20	0.7760

[a] Probability that the RF distance from trees based upon an a priori hypotheses to the ML tree for the novel loci is less than the RF distance expected for a random tree.

clade that includes Falconidae and Cariamidae (the PPFC or PPFC + Coliiformes clade). Moreover, all of the analyses were able to exclude most of the taxa proposed to be relatives of the Passeriformes (fig. 1).

In order to further explore support for the phylogeny obtained in this study, we conducted gene-jackknifing analyses, finding that 28 of these 30 analyses supported the PPFC and PP clades in either the ML trees, the bootstrap consensus trees, or in both (supplementary table S5, Supplementary Material online). Similar to the situation exhibited by the partitioned ML tree (fig. 2*b*), the two gene-jackknifing analyses that did not find any support for the PPFC or PP clades had the PP clade disrupted by the inclusion of Coliiformes (supplementary table S5 and tree file, Supplementary Material online). Taken as a whole, all analyses supported either the PPFC and the PP clades or an expanded version of these clades that also included Coliiformes, excluding many of the proposed sister groups for Passeriformes (fig. 1).

Alignment Bias—Little Impact upon Landbird Phylogeny

One way to avoid alignment uncertainty is to delete all regions with gaps (Morgan-Richards et al. 2008), but this approach is extremely conservative and can lead to a loss of valuable information. Using Gblocks always resulted in alignments with many fewer informative sites (table 1), although these smaller data matrices still resulted in well-resolved phylogenies (supplementary tree file, Supplementary Material online). Moreover, the majority rule–based consensus tree built with all 34 Gblocks analyses marginally supported the PP clade, placing it sister to the Coliiformes (supplementary fig. S2*b*, Supplementary Material online). The observation that the use of Gblocks did not alter our conclusions suggests that the regions removed by that program have a phylogenetic signal similar to those retained and further suggest that filtering by using Gblocks is not necessary in this case. Additionally, excluding ambiguous regions, gappy sites, or both from the manual alignment retained similar numbers of informative sites (table 1), and the tree topologies recovered were similar to those identified using alignments with no sites excluded (supplementary table S6, Supplementary Material online).

Evaluating the sensitivity of phylogenetic conclusions to alignment uncertainty is likely to represent a better strategy than site exclusion since it does not remove data. Although the alignments generated using different programs (and parameter sets) exhibited variation (table 1, supplementary fig. S3, Supplementary Material online), most had similar alignment scores (table 1), and ML analyses of these alignments resulted in topologies that shared many similarities (e.g., table 3, supplementary table S6 and fig. S4, Supplementary Material online). The only exception was ClustalW2, an alignment program known to perform poorly (e.g., Liu et al. 2009) that had both a low alignment score (table 1) and resulted in tree topologies very different from the others (supplementary fig. S4 and tree file, Supplementary Material online).

Guide trees represent an important parameter for multiple sequence alignment programs, and they have the potential to introduce biases into the topology supported by specific alignments (e.g., Lake 1991; Nelesen et al. 2008). Although the guide trees we used were very different from each other, the alignments based upon different guide trees but using the same program were closer to each other based upon alignment distances than those based upon the same guide tree but using different programs (supplementary fig. S3, Supplementary Material online). This suggests that the guide tree may actually have less impact upon the alignment results than the algorithms used by different multiple sequences alignment programs. The optimal ML trees estimated using the resulting alignments were very similar to each other and to trees obtained from the manual alignment (supplementary fig. S4, Supplementary Material online). Pairwise RF distances among the optimal ML trees ranged from 0 to 12 (not shown), in sharp contrast to the differences among the guide trees used for the sequence alignments (fig. 1*a*–*d*), where the pairwise RF distances ranged from 20 to 24. Moreover, the pairwise RF distances between the optimal ML trees and the original guide trees ranged from 18 to 26 (excluding the HA topology that is very similar to those obtained in all of our analyses), implying that the phylogenetic signal in our independent data set is sufficiently powerful to overcome the influence of diverse guide trees. More support that guide trees had little impact on phylogenetic estimation can also be found in one case where an alignment based on the HA guide tree did not find PP (although it did find PPFC), whereas alignments that were based on other guide trees (e.g., LZ and BR) that yielded high (>80%) bootstrap support for the PP clade (table 3 and supplementary table S6, Supplementary Material online).

The magnitude of the tree differences across alignments was similar to the differences between unpartitioned and partitioned ML analyses of the manual alignment (supplementary fig. S4, Supplementary Material online), emphasizing the relatively limited impact of alignment uncertainty upon our

Table 3. Consensus Results Based on Different Alignments and Methods.

		PP (Bootstrap%)		PPFC (Bootstrap%)		CPBT (Bootstrap%)		Coliiformes-Sister	
Alignment Programs	Guide Trees[a]	All Taxa[b]	−Col[c]	All Taxa	−Col	All Taxa	−Col	Sister Clade	Bootstrap%
Manual	—	33	57	46	85	91	99	PPFC	47
Menura[d]	—	48	63	44	82	91	100	CPBT	52
Menura + *Acanthisitta*[e]	—	27	57	43	84	90	100	CPBT	46
Prank	SA	41	53	51	79	99	100	CPBT	73
	LZ	67	80	No	No	81 (+Col)	99	*Trogon*	81
	BR	75	85	62 (+Col)	62	91	97	Falconidae	81
	HA	99	100	57 (+Col)	85	88	100	PP	52
Mafft	—	23 (+Col)	52	45 (+Col)	69	88	100	Passeriformes	43
	SA	45	73	46 (+Col)	82	90	99	PP	22
	LZ	No	44	64 (+Col)	82	87	100	Psittaciformes	37
	BR	32 (+Col)	50	47 (+Col)	68	89	99	Psittaciformes	37
	HA	56	68	37	65	85	100	CPBT	58
SATe_Prank	—	39	64	45	82	95	100	CPBT	50
	SA	81 (+Col)	90	57 (+Col)	85	98	100	Psittaciformes	71
	LZ	59	62	No	No	85 (+Col)	100	*Trogon*	50
	BR	50	68	49 (+Col)	73	79	99	Falconidae	20
	HA	No	No	44	85	89	100	CPBT	69
SATe_Mafft	—	63 (+Col)	75	53 (+Col)	68	97	99	Passeriformes	43
	SA	65	90	60 (+Col)	81	84	99	PP	46
	LZ	72	85	44	67	86	100	CPBT	66
	BR	81	85	No	53	89	100	CPBT	74
	HA	90	95	48	85	94	100	CPBT	58
Muscle	—	42 (+Col)	63	58 (+Col)	72	91	99	Psittaciformes	32
T-coffee	—	74	88	37	79	83	99	PPFC	49
ClustalW2	—	No	No	No	No	No	No	Psittaciformes	93
% Analyses with clade		68	92	40	88	88	96	36[f]	

NOTE.—Only unpartitioned ML are shown; additional results can be found in supplementary table S6, Supplementary Material online. Genus names are in italics. CPBT, Coraciiformes–Piciformes–Bucerotiformes–Trogoniformes; PP, Passeriformes–Psittaciformes; PPFC, Passeriformes–Psittaciformes–Falconidae–Cariamidae.

[a] Guide trees from SA, Sibley and Ahlquist (1990) topology; LZ, Livezey and Zusi (2007) topology; BR, Brown et al. (2008) topology; and HA, Hackett et al. (2008) topology.

[b] Bold numbers represent the clade supported by bootstrap analyses but not shown in the ML best trees. +Col: Coliiformes are included in the clade and the numbers outside the parenthesis represent supports for the specific clade plus Coliiformes.

[c] −Col: Analyses based on data set that excluded Coliiformes.

[d] Data set has only *Menura* representing Passeriformes.

[e] Data set has *Menura* and *Acanthisitta* representing Passeriformes.

[f] Percentage of analyses where Coliiformes sister to CPBT.

conclusions. The differences among trees obtained using specific guide trees or alignment programs tended to be limited to parts of the tree that were relatively poorly supported, such as the position of the Coliiformes and the relationships among the outgroups to the landbirds. In fact, a majority-rule consensus tree based on all topologies generated by different alignments showed that most clades within landbirds were recovered consistently despite the differences among alignments (supplementary fig. S2, Supplementary Material online). Taken as a whole, the similarities among the trees inferred using the manual and various automated alignments suggest that introns overall have great potential for phylogenetic analyses at this level, extending the results of Chojnowski et al. (2008) who argued that introns are useful based upon their greater sequence variation. Moreover, these analyses suggest that studies like Hackett et al. (2008) that use noncoding data may not exhibit a strong bias due to alignment problems.

Nonhistorical Signals Do Not Appear to Distort Landbird Phylogeny

Both sampling error and systematic error can mislead phylogenetic inference (Phillips et al. 2004; Chojnowski et al. 2008). In the extreme, a data matrix may be so small that it is unlikely to include any genuine synapomorphies (Braun and Kimball 2001). Although increasing data set size reduces sampling error, it will also increase the impact of systematic errors if they are present (e.g., Jeffroy et al. 2006; Philippe et al. 2011). Thus, it is critical to establish that systematic errors are unlikely to have an impact on the conclusions obtained using large-scale data matrices.

Base-compositional heterogeneity is one of the problems known to result in artifactual relationships (e.g., Phillips et al. 2004; Collins et al. 2005). We examined the impact of compositional heterogeneity in two ways. First, we identified and excluded loci that showed potentially problematic deviations from compositional homogeneity. The loci that were removed were the two that showed a significant deviation based upon the χ^2 test and seven in the upper quartile of RCV values (supplementary table S7, Supplementary Material online). Excluding these loci from ML analyses of the manual alignment did not alter support for the PP and PPFC clades (although the Coliiformes were included in some cases) or the CPBT clade (supplementary table S5, Supplementary Material online). Second, we generated NJ trees based on base-compositional distances of

individual loci (as described by Harshman et al. 2008). The PP, PPFC, and CPBT clades were not presented in these trees, suggesting that these clades are unlikely to be united due to base-compositional convergence.

Divergences among major landbird lineages are relatively ancient (mid to late Cretaceous based upon molecular clock analyses; e.g., Ericson et al. 2006; Brown et al. 2008; Chojnowski et al. 2008), and there is substantial rate heterogeneity among lineages (e.g., fig. 2). This creates the potential for LBA (Felsenstein 1978). At first glance, LBA might appear problematic for attempts to establish the position of the passerines in the avian tree of life since passerines have a high rate of molecular evolution (fig. 2; see also Yuri et al. 2008). Indeed, we found that the branches leading to most passerines were long in this study (four taxa were in the upper quartile; supplementary table S8, Supplementary Material online). Although some other landbirds were also associated with long branches (*Upupa*, *Capito*, and both Coliiformes were also in the upper quartile), branch length estimates for parrots, falcons, and *Cariama* were all below the median (supplementary table S8, Supplementary Material online). It has been suggested that LBA can be suppressed by using a site-heterogeneous model such as CAT-GTR (Lartillot et al. 2007), and the presence of the PP and PPFC clades in analyses using CAT-GTR suggest that LBA may not be affecting our conclusions. Additionally, a number of methods to examine LBA have been proposed (reviewed by Bergsten 2005), with one test being the exclusion of taxa with long branches. We were able to use this test because two passerine taxa (*Menura* and *Acanthisitta*) are associated with shorter branches (supplementary table S8, Supplementary Material online). Excluding the long-branch passerines (keeping only *Menura* or *Menura* + *Acanthisitta*) did not alter our conclusions; all analyses supported the PP and PPFC clades (table 3). Although the conditions that lead to LBA can be complex (Hendy and Penny 1989), it is clear that the PP and PPFC clades are robust to changes in taxon sampling designed to reduce branch length heterogeneity.

The observation that non-passerines within the PPFC lineage are not associated with long branches combined with analyses excluding long-branched passerines indicate that LBA is unlikely to have a global impact upon the position of passerines. However, it remains possible that the PP clade reflects LBA since parrots and passerines represent the two longest branches within the PPFC clade. We tested this hypothesis by reducing the taxon sample to only the members of the PPFC clade and 1 of 2 divergent outgroups, *Upupa* or *Gallus*. If there is a high potential for LBA within the PPFC clade, the relevant branch is expected to be attracted to the long outgroup branch, rooting the PPFC clade within the putative PP clade. We did not observe any attraction between the outgroups and the passerines or the parrots (supplementary table S9, Supplementary Material online). Taken as a whole, these analyses indicate that LBA is unlikely to explain the presence of the PPFC clade or the PP clade in our analyses of this independent data matrix.

Substitutional saturation also has the potential to reduce phylogenetic accuracy (Jeffroy et al. 2006; Philippe et al. 2011). Of the 30 loci we examined, eight exhibited some degree of saturation based upon the Xia et al. (2003) I_{ss} metric (supplementary table S7, Supplementary Material online). Excluding those eight loci from analyses of the manual alignment did not have an impact upon our conclusions; both the PP and PPFC clades were supported, and the CPBT clade was monophyletic in analyses that removed these potentially saturated loci (supplementary table S5, Supplementary Material online). Taken as a whole, these analyses suggest that the phylogenetic signal supporting a landbird topology similar to Hackett et al. (2008) is unlikely to reflect systematic error.

Coliiformes—A "Rogue" Taxon

The position of the Coliiformes showed substantial variation among analyses (e.g., fig. 2 and table 3). Coliiformes was sister to the CPBT clade in Ericson et al. (2006) and Hackett et al. (2008), and they were found in this position in around 30% of the analyses reported here (table 3; supplementary table S6, Supplementary Material online). However, whenever the position of Coliiformes was not sister to the CPBT clade, its position was quite variable, allying with Trogoniformes, Psittaciformes, Falconidae, Passeriformes, or forming the sister group with either the PP clade or the PPFC clade in various analyses (table 3; supplementary table S6, Supplementary Material online). Leaf stability tests also supported these observations, as we almost always obtained the lowest values for Coliiformes (see supplementary table S10, Supplementary Material online). Since the Coliiformes are associated with long branches (see above), it seems reasonable to postulate that LBA contributes to their rouge behavior.

The rogue behavior of Coliiformes in our study grouping with a wide variety of taxa (including all potential members in PPFC clade) and disturbing the tree stability may result from the absence of other landbird species, such as those included in Hackett et al. (2008). Indeed, Hackett et al. (2008) reported greater support for the PP and PPFC clades than we observed in this study, despite the inclusion of Coliiformes in their study. However, Hackett et al. (2008) also included other taxa (e.g., Strigiformes) that had not been identified as likely relatives of the Passeriformes and so were not included in this study. This suggests that analyses including other taxa will be necessary to evaluate the position of Coliiformes with confidence.

To examine the influence of Coliiformes on our phylogeny, we also conducted analyses in which Coliiformes were excluded. After Coliiformes were excluded from analyses, we observed more stability in the tree topologies recovered by different analyses (supplementary fig. S4, Supplementary Material online), and the number of analyses that recovered the PP and PPFC clades increased dramatically (table 3, supplementary table S6, Supplementary Material online). Moreover, exclusion of Coliiformes resulted in higher bootstrap support for the PPFC and PP clades in almost all analyses (e.g., table 3). These results demonstrate that Coliiformes did have an influence upon the topologies we obtained; however, these analyses did not allow us to assess whether or not Coliiformes can be excluded as closely related to the Passeriformes.

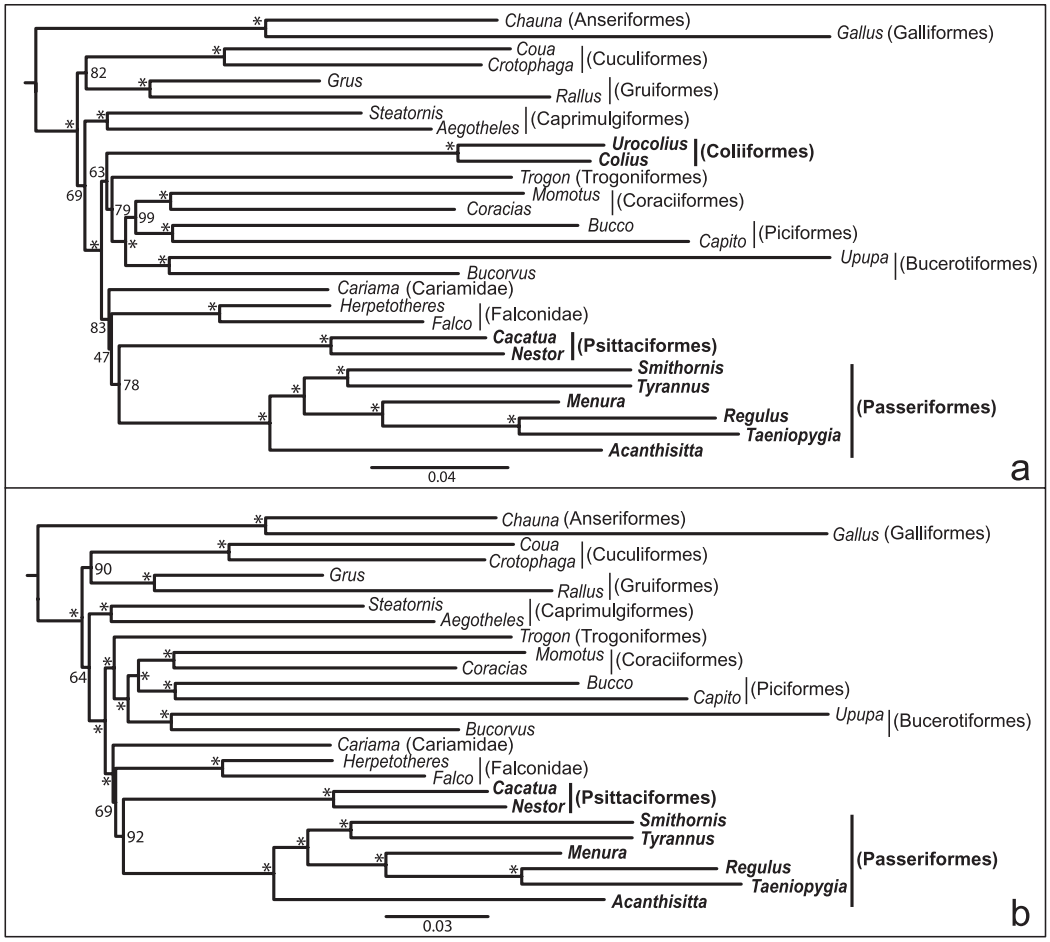

Fig. 4. Unpartitioned ML analyses and bootstrap support based on concatenation of 49 loci. (*a*) With Coliiformes and (*b*) without Coliiformes. * indicates 100% bootstrap support. *Gallus gallus* and *Chauna torquata* were used as outgroups.

Concatenated Analyses—Stronger Support for Landbird Relationships

Although independent analysis provides a rigorous way to test hypotheses, the total evidence approach offers the ability to reduce the impact of variance on tree estimation. Since this study used the same species as Hackett et al. (2008), we constructed a concatenated data matrix that avoided any potential problems related to the use of composite taxa (see Malia et al. 2003, but see Springer et al. 2004 for a contrasting position). Analysis of the concatenated matrix yielded a tree topology identical to that presented in Hackett et al. (2008), although the bootstrap support for the major clades differed (fig. 4*a*). Support for the PPFC and the CPBT clades was higher in the concatenated analyses than in Hackett et al. (2008) (83% vs. 64% and 79% vs.71%, respectively), whereas the PP clade received the same level of bootstrap support in both studies (fig. 4*a*). The failure to obtain higher support with the larger concatenated data set could be caused by the rogue Coliiformes. Indeed, all clades within landbirds were better supported after excluding Coliiformes (fig. 4*b*), with the PP clade obtaining 92% bootstrap support and the support for the PPFC and CPBT clades increasing to 100%. We also examined the LBA effect on the concatenated data set by excluding the long-branch passerines or by excluding all taxa except the PPFC clade and long-branched outgroups (*Upupa* or *Gallus*). We found no evidence of LBA for the PP clade (supplementary tables S6 and S9, Supplementary Material online), suggesting that this larger data set provides strong evidence for the observed landbird relationships.

Changing Our Understanding of Passerine Evolution

The most consistent sister group of Passeriformes was Psittaciformes (60% of all analyses of the complete alignment recovered the PP clade; supplementary fig. S2*c*, Supplementary Material online), although our data cannot exclude the possibility that Coliiformes are sister to Passeriformes (14% of all analyses) or Psittaciformes (15% of all analyses). However, the low leaf stability and inconsistent position of the Coliiformes suggest that a PP + Coliiformes clade is less likely. Given the extensive literature on Passeriformes and that the PP clade appears more likely, it is worth considering the biological implications of a PP clade.

Assuming that Passeriformes are sister to Psittaciformes, as also proposed by Hackett et al. (2008), alters our understanding of avian evolution in several ways. One of the most interesting implications is the origin of song learning, which

occurs in only three orders of birds: Passeriformes, Psittaciformes, and Apodiformes (swifts and hummingbirds, only the hummingbirds exhibit song learning) (Nottebohm 1972). Since songs are critical in mate attraction, territory defense, and species identification, avian song learning has attracted substantial attention (e.g., Hessler and Doupe 1999; Jarvis and Mello 2000; Brenowitz and Beecher 2005). Based on previous phylogenies, it has been suggested that song learning and acquired associated brain structures arose independently in all three orders (e.g., Jarvis et al. 2000). However, our data suggest that song learning may have evolved only twice: once within Apodiformes and once in the ancestor to Passeriformes and Psittaciformes. It is noteworthy that song learning is mainly found in oscine passerines (songbirds) rather than suboscine passerines, which lack forebrain song learning nuclei (Gahr et al. 1993). However, evidence for song learning was recently found in one suboscine (*Procnias tricarunculata*, the three-wattled bellbird; Saranathan et al. 2007), and it remains possible that other suboscines are capable of song learning as well. Thus, it is possible that song learning arose in the common ancestor of the PP clade and was lost in various suboscine lineages.

Another implication of the PP relationship is the biogeographic origin of these orders. The basal divergence within Passeriformes is between Acanthisittidae (New Zealand wrens, represented by *Acanthisitta chloris* in this study) and other passerines, whereas the basal divergence in Psittaciformes is between the Strigopidae (New Zealand parrots, represented by *N. notabilis* in this study) and the other parrots (e.g., Wright et al. 2008). Both Acanthisittidae and Strigopidae are endemic to New Zealand, and the other Passeriformes and Psittaciformes appear to have centers of diversity in parts of Gondwana (e.g., Barker et al. 2002; Wright et al. 2008), suggesting that the biogeographic history of these orders was similar. Indeed, it is reasonable to speculate that the common ancestor may have arisen in New Zealand and dispersed to other parts of the world. Alternatively, the breakup of Gondwana (for details on Gondwana biogeography hypotheses, see Cracraft 2001) could have played an important role in the diversification of these orders, although such a hypothesis is inconsistent with some avian molecular clock studies (e.g., Ericson et al. 2006; Chojnowski et al. 2008).

Landbird Relationships

In addition to the position of passerines, two other clades merit discussion. The first is the relationship between the Falconidae and Cariamidae (the FC of the PPFC clade). Analyses of the novel data weakly supported an FC clade (figs. 2 and 3), in contrast to Hackett et al. (2008) that placed Cariamidae as the deepest branching member of the PPFC clade (fig. 1*c*). Cariamidae has been placed in the Gruiformes in many classifications (e.g., Clements 2007; Livezey and Zusi 2007), but similarities between Cariamidae and raptors (e.g., Falconidae) have been noted (e.g., Jollie 1953; Sibley and Ahlquist 1990). This suggests a Falconidae–Cariamidae clade is plausible, consistent with the biogeographic hypothesis suggested by Ericson (2008). Detailed study of Falconidae and Cariamidae falls outside the scope of the present work, but this group should be examined more rigorously in the future.

The second group of interest is the Coraciiformes–Piciformes clade within the larger CPBT clade. Many classifications unite the Coraciiformes and Bucerotiformes in a single order (the "traditional Coraciiformes"), and a Coraciiformes–Bucerotiformes clade was supported by the morphological analyses of Livezey and Zusi (2007) (fig. 1*b*). Both Ericson et al. (2006) and Hackett et al. (2008) support a close relationship between Coraciiformes sensu stricto (i.e., excluding Bucerotiformes) and Piciformes, rendering traditional Coraciiformes paraphyletic. Analyses of the independent data strongly corroborate the Piciformes–Coraciiformes clade. Overall, these analyses strongly corroborate the hypothesis that traditional Coraciiformes are paraphyletic and provide additional support for the picture of landbird phylogeny that has been emerging from analyses of nuclear sequence data.

Conclusions

These results highlight an important difference between the total evidence and independent evidence approaches. The total evidence analysis resulted in a topology identical to Hackett et al. (2008) but with higher bootstrap support for a number of nodes. In contrast, the independent evidence analyses revealed both areas of agreement with Hackett et al. (2008), highlighting clades that can now be accepted with greater confidence and areas of incongruence that should be further explored. Recognizing areas of incongruence among data sets can facilitate the identification of biases that may result in misleading conclusions, including alignment problems, changes in base composition, saturation, and LBA. Ultimately, the identification of nonhistorical signals that reflect these biases will result in improved estimates of phylogeny.

Using the independent evidence approach, our phylogenetic conclusions excluded all but one a priori hypothesis, demonstrating a close (likely sister) relationship between Passeriformes and Psittaciformes. These further suggest a novel model for the evolution of song learning and a specific biogeographic model for the origin of these clades. Our independent analyses also corroborate additional clades from previous studies, lending further support to the idea that the Neoaves do not represent a hard polytomy and suggesting that further resolution of the avian tree of life may be possible. Furthermore, our data suggest that the potential for alignment ambiguity had limited impact upon our phylogenetic conclusions, though future studies should continue to conduct alignment sensitivity analyses similar to those performed here. Additionally, we found that a single taxon could influence the robustness of the tree topology, emphasizing that careful taxon selection remains very important even with large data matrices.

Supplementary Material

Supplementary tables S1–S10, figures S1–S4, data matrix, and tree file are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Note Added in Proof

A recently published paper by Suh et al. (2011) came to a similar conclusion regarding the parrot-passerine clade using transposable element insertions.

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