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Molecular phylogeny of songbirds (Passeriformes) inferred from mitochondrial 16S ribosomal RNA gene sequences

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Abstract

Phylogenetic relationships among the families of passerine birds have been the subject of many debates. These relationships have been investigated by using a number of different character sets, including morphology, proteins, DNA–DNA hybridization, and mitochondrial DNA gene sequences. Our objective was to examine the phylogenetic relationships of a set of passerine songbirds (Oscines) and to test the taxonomic relationships proposed by Sibley and Ahlquist (1990). We sequenced 1403 aligned bases encompassing the mitochondrial transfer-RNA-Valine and 16S ribosomal RNA genes in 27 species from 14 families (including a Suboscine outgroup). Our results differ in significant ways from the superfamily designations of Sibley and Ahlquist by questioning the monophyly of the Sylvioidea and by placing the Regulidae in the Corvoidea.
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1. Introduction

The order Passeriformes is a large monophyletic assemblage of birds whose interrelationships are poorly understood. The group is defined by just a few morphological synapomorphies including the features of the palate, spermatozoa, forelimb and hind limb muscles, and feet (Raikow, 1982). However, beyond these morphological traits, passerines also differ from other birds in certain continuous traits. They have a metabolic rate that tends to be higher than other birds of comparable size, and they have relatively large brains and superior learning abilities, especially with respect to vocalizations (Sheldon and Gill, 1996). It is argued that because of a combination of their morphological, neurological, behavioral, and ecological adaptations, the passerines radiated unlike any other avian group (Fitzpatrick, 1988). It appears that the passerines were so successful, and radiated so rapidly during the late Tertiary, that the lines of demarcation among families and higher groups are now poorly defined (Feduccia, 1995).

Relationships among the families of passerine birds have been the subject of many debates over the years (Sibley and Ahlquist, 1990). With the development of new molecular systematic techniques, the debate over relationships within the passerines has become even more heated. These relationships have been investigated using a number of phylogenetic tools including morphology (Beecher, 1953), tissue proteins (Stallcup, 1961), DNA–DNA hybridization (Sheldon and Gill, 1996; Sibley and Ahlquist, 1990), nuclear gene sequences (Barker et al., 2002), and mitochondrial gene sequences (Chikuni et al., 1996; Edwards et al., 1991; Seutin and Birminham, 1997). However, despite the attention to this phylogenetic problem, many familial relationships within the Passeriformes remain unresolved.

Lack of resolution in previous studies has been attributed to two causes, short internodes separating most of the major groups, and methodological problems within individual studies. Previous estimates of relationships within the Passeriformes indicate that the passerine tree is characterized by short internodes separating most major groups. Relative brief times between branching events leave little opportunity for diverging clades to acquire synapomorphies (Lanyon, 1988),

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which can result in indistinct groups. As a result, early researchers complained that classifying passerines was unusually difficult because so many of the groups seem to grade into one another (Sheldon and Gill, 1996). Both morphological and molecular studies have been frustrated by this large number of seemingly intermediate forms within the passerines.

Morphological analyses have been especially difficult because of the great similarity among the passerine families and a high level of convergent evolution exhibited by these birds. Except for the larks (Alaudidae) and the swallows (Hirundinidae), there are apparently no other families that can be defined unequivocally by anatomical characters (Mayr, 1956). In addition, passerines have repeatedly and independently evolved into morphologically similar ecotypes in different parts of the world, leading to much convergence among characters (Sibley and Ahlquist, 1990). Beecher (1953) attempted to produce a phylogeny of the songbirds (Oscines) based mostly on the jaw musculature and other characters of the head; however, due to the possibility that the real significance of jaw musculature is functional rather than phyletic it has been critcized (Mayr, 1956).

Molecular phylogenetic results have been fraught with a lack of resolution, as well, due to the lack of phylogenetic signal in the genes chosen for the studies. The mitochondrial cytochrome oxidase I gene investigated by Seutin and Birminham (1997) was found to possess high levels of homoplasy in comparisons above family level. The few studies performed using the mitochondrial cytochrome *b* gene have had similar results (Chikuni et al., 1996; Edwards et al., 1991). Moore and DeFillippis (1997) suggest that the phylogenetic information from the cytochrome *b* gene is only reliable in passerine birds at divergences of up to about 9 million years ago. Using the fossil record (Feduccia, 1995) and the DNA–DNA hybridization calibration of Sibley and Ahlquist (1990, $\Delta T_{50}H$ 1.0 = 2.3 million years of divergence) it appears that even the most recently evolved families are probably 12–16 million years old. Consequently, it is unlikely that cytochrome *b* will be useful for inferring relationships among passerine families, although for a somewhat counter view see Klicka et al. (2000).

DNA–DNA hybridization studies have been the most taxonomically intensive of the molecular studies performed on these birds (Figs. 1 and 2). The phylogeny produced by Sibley and Ahlquist (1990) is both the best known and the most criticized (Fig. 1). The results of their study have been criticized on two main points: (1) failure to account for variable rates of evolution among the birds, and (2) the lack of testing for branch robustness or confidence on their trees (Sheldon and Bledsoe, 1993). These methodological problems have cast doubt upon the classifications proposed by Sibley and Ahlquist, especially since the phylogeny produced by a subsequent DNA–DNA hybridization study conflicted with their results. For example, Sheldon and Gill (1996) found that Sibley and Ahlquist's division of the passerines into three clades was not supported, since two of those clades were found to be polyphyletic (Fig. 2).

Despite the controversy concerning the methodology used by Sibley and Ahlquist (1990), their extensive study of the phylogeny of the birds of the world is both the most complete and most frequently cited in avian

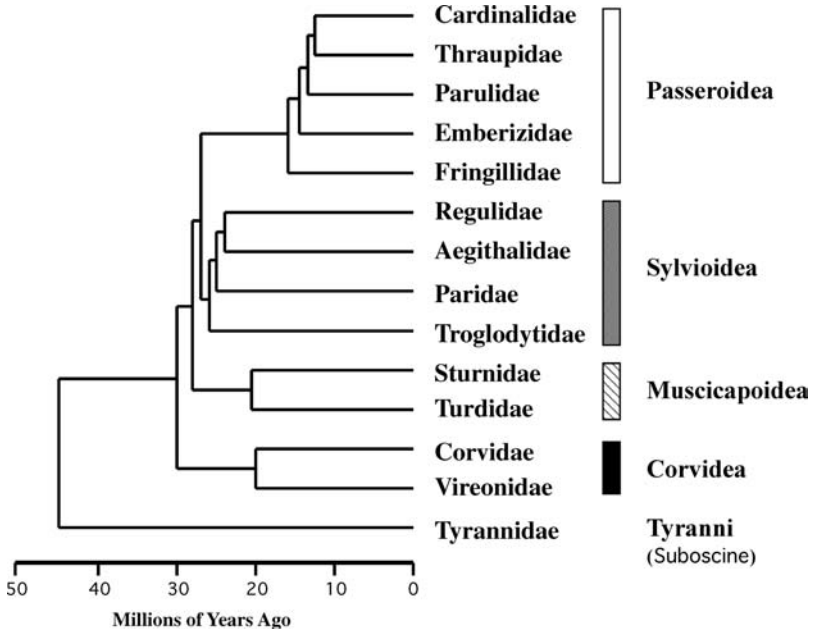

Fig. 1. Phylogeny of the songbird (Oscine) families examined in the present study as proposed by Sibley and Ahlquist (1990, Figs. 369, 370, 384, and 385) based on DNA–DNA hybridization data. Superfamily designations are shown to the right of the phylogeny. Times of divergence are based on their passerine calibration of $\Delta T_{50}H$ 1.0 = 2.3 million years of divergence.

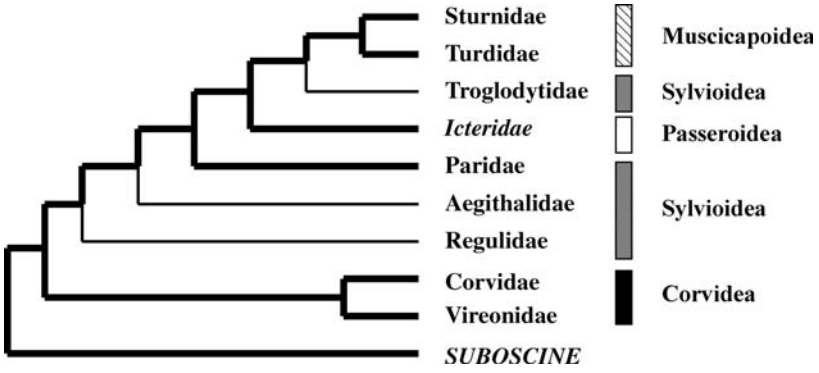

Fig. 2. Phylogeny of the songbird (Oscine) families examined in the present study as proposed by Sheldon and Gill (1996) based on DNA–DNA hybridization data. The thick lines indicate strong phylogenetic support, whereas the thin lines suggest weaker support. Superfamily designations according to Sibley and Ahlquist (1990) are shown to the right of the phylogeny. The Icteridae are italicized because they were not used in the present study, but are considered as the representative taxon for the Passeroidea by Sheldon and Gill (1996).

studies. Also, several of the higher level divisions proposed by Sibley and Ahlquist have been confirmed by other molecular studies. For example, the separation of the Passeriformes into two distinct clades, the Oscines and the Suboscines, is supported by mitochondrial DNA (mtDNA) sequences (Edwards et al., 1991), and morphological and behavioral studies (Wyles et al., 1983). The division of the Oscines into the parvorders Passerida and Corvida has also been independently confirmed by another DNA–DNA hybridization study (Sheldon and Gill, 1996), although not by nuclear (Barker et al., 2002) or mtDNA sequences (Edwards et al., 1991). However, the other divisions put forward by Sibley and Ahlquist (1990) are less substantiated.

The purpose of this study is to define the phylogenetic relationships among a set of songbird (Oscine) families, and to test the accuracy of the superfamily designations of Sibley and Ahlquist (1990). We chose mitochondrial ribosomal RNA (rRNA) genes as the basis of this analysis because they include both evolutionary labile and conserved regions (Hillis and Dixon, 1991; Mindell and Honeycutt, 1990). A number of studies have used rRNA genes to examine the phylogenetic relationships among birds. However, the previous studies have focused on examining higher relationships, such as the relationships among orders (Hedges et al., 1995; Mindell et al., 1997; van Tuinen et al., 1998, 2000), or have used a different ribosomal gene, the 12S rRNA (Houde et al., 1997). Therefore, it appears that these mitochondrial genes should have a broad window of resolution for addressing recent and ancient divergences among the Passeriformes.

2. Materials and methods

2.1. Collection of specimens

The species names and current classification of the specimens used in this study are listed in Table 1, which includes 27 species from 24 genera in 14 families. The exact collection data for each specimen can be obtained from the authors. Specimens were collected and placed on wet ice in the field and then transferred to a −80 °C freezer.

2.2. DNA isolation

Total genomic DNA was isolated from approximately 3 mm^3 of tissue, which was removed from the pectoral muscle of the bird. This tissue was ground using a tube-fitting pestle in a 1.5 ml tube containing 450 μl of grinding buffer (0.1 M EDTA, 100 mM Tris, pH 8.0, 1% SDS, 0.2 M NaCl). The homogenate was incubated for 1 h at 45 °C in a water bath. Following heat incubation, 1/10 volume saturated KCl was added and the solution was incubated on ice for 1 h. Protein particles were pelleted and the clear supernatant was removed, then 1/25 volume 5 M NaCl and 2 volumes of 95% cold ethanol were mixed with the supernatant and chilled for 15 min. The DNA was pelleted, dried, and then resuspended in 200 μl of PCR water.

2.3. PCR amplification

Double-stranded PCRs were run in a 50 μl volume with a surface layer of mineral oil. Included in the 50 μl volume were 2 μl of purified total DNA template, 5 μl of each primer (10 pM/μl), 5 μl of dNTP (10 μM), 10 μl of 5× Buffer C (Invitrogen), and 0.4 μl of 10× Taq polymerase (Promega). The primers used for double-stranded amplification were 12Sa and 16Sbr (Palumbi et al., 1991) which amplify a segment approximately 2080 base pairs (bp) in length (Table 2, Fig. 3). All reactions were subjected to 30 cycles of denaturing (94 °C, 45 s), annealing (50 °C, 1 min), and extension (72 °C, 2 min). Amplified PCR products were cleaned prior to sequencing using a PEG precipitation protocol (Kusukawa et al., 1990).

Table 1
Taxa examined in this study, with names and family affiliations according to the American Ornithologists' Union Checklist (1998)

Family	Taxon	Common name	GenBank Accession Nos.
Tyrannidae	*Empidonax oberholseri*	Dusky Flycatcher	AF202806, AF202833
Vireonidae	*Vireo gilvus*	Warbling Vireo	AF202804, AF202831
	Vireo huttoni	Hutton's Vireo	AF202805, AF202832
Corvidae	*Cyanocitta stelleri*	Stellar's Jay	AF202802, AF202829
	Aphelocoma californica	Western Scrub-Jay	AF202803, AF202830
Paridae	*Poecile rufescens*	Chestnut-backed Chickadee	AF202795, AF202822
	Baeolophus inornatus	Oak Titmouse	AF202796, AF202823
Aegithalidae	*Psaltriparus minimus*	Bushtit	AF202798, AF202825
Troglodytidae	*Troglodytes aedon*	House Wren	AF202797, AF202824
Regulidae	*Regulus calendula*	Ruby-crowned Kinglet	AF202801, AF202828
Turdidae	*Catharus guttatus*	Hermit Thrush	AF202800, AF202827
Sturnidae	*Sturnus vulgaris*	European Starling	AF202799, AF202826
Parulidae	*Vermivora celata*	Orange-crowned Warbler	AF202788, AF202815
	Dendroica coronata	Yellow-rumped Warbler	AF202786, AF202813
	Oporornis tolmieie	MacGillivray's Warbler	AF202789, AF202816
	Wilsonia pusilla	Wilson's Warbler	AF202787, AF202814
Thraupidae	*Piranga ludoviciana*	Western Tanager	AF202791, AF202818
Emberizidae	*Pipilo maculatus*	Spotted Towhee	AF202784, AF202811
	Spizella passerina	Chipping Sparrow	AF202785, AF202812
	Passerella iliaca	Fox Sparrow	AF202783, AF202810
	Zonotrichia leucophrys	White-crowned Sparrow	AF202781, AF202808
	Zonotrichia atricapilla	Golden-crowned Sparrow	AF202780, AF202807
	Junco hyemalis	Dark-eyed Junco	AF202782, AF202809
Cardinalidae	*Passerina amoena*	Lazuli Bunting	AF202790, AF202817
Fringillidae	*Carpodacus purpureus*	Purple Finch	AF202792, AF202819
	Carpodacus mexicanus	House Finch	AF202793, AF202820
	Coccothraustes vespertinus	Evening Grosbeak	AF202794, AF202821

Table 2
Oligonucleotides used for amplification and sequencing

Primer	Sequence	Location
12Sa	5′-AAACTGGGATTAGATACCCCACTAT	1729–1753
16S500	5′-GTCGTAACAAGGTAAGTGTACCG	2231–2253
16Sc	5′-TACCTTTTGCATCATGGTCTAGC	2546–2568
16Sars	5′-GTATTGAAGGTGATGCCTGCC	3234–3254
16S840	5′-GTTCTTGCTAAATCATGATGC	2574–2554
16Sa	5′-ATGTTTTTGGTAAACAGTCG	3214–3195
16Sbrs	5′-GTCCTGATCCAACATCGAGG	3727–3708
16Sbr	5′-CCGGTCTGAACTCAGATCACGT	3804–3783

Note. The location values correspond to the complete chicken mitochondrial genome (Desjardins and Morais, 1990).

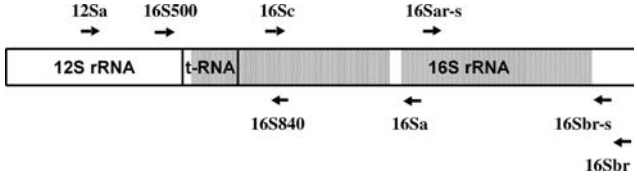

Fig. 3. Primers used to amplify and sequence the 1403 aligned base pairs of DNA. The universal primers 12Sa and 16Sbr were used for the initial amplification. Passerine specific sequencing primers were designed for internal sequencing (see Table 2 for primer sequences).

2.4. DNA sequencing

All sequencing was done via dye terminator cycle sequencing on an ABI 377 Automated Sequencer following the protocol specified by the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Revision B, August 1995, Perkin–Elmer). Primers used for sequencing are presented in Table 2 and shown in Fig. 3.

2.5. Sequence alignment

All final sequences used were obtained by reconciling sequences from both the forward and reverse sequencing runs. The initial alignment of sequences was produced using the Sequencher 3.0 analysis program. Conserved regions were first identified and aligned, and the gaps were assigned so that the fewest number of changes occurred. However, a secondary structure approach was used to construct the final alignment (Hickson et al., 1996; Kjer, 1995). An approximation of the secondary structure for 16s rRNA

of Passeriformes was created by superimposing the sequences over the proposed Gymnotiformes rRNA secondary structure (Alves-Gomes et al., 1995). By comparing the superimposed sequences, it was possible to define segments corresponding to loops and stems, establish base pairing, and improve the alignment. The alignment of the variable region was further corrected using this same method with the secondary structure proposed by Parker and Kornfield (1996) for cyprinodonitid killifishes. Alignment of areas of the sequence outside of the model was executed as conservatively as possible.

2.6. Preliminary sequence analysis

Sequences were evaluated for overall base composition bias and among taxa base composition. The base composition bias statistic was calculated according to Irwin et al. (1991) and ranges in value from zero to one; zero indicating no bias and one showing complete base composition bias. An extreme overabundance of one nucleotide state can increase the tendency for those sites to become saturated (Irwin et al., 1991). In addition, a strongly skewed mutation bias can violate the assumption in parsimony analysis that there is an equal probability of change at all sites (Perna and Kocher, 1995).

2.7. Phylogenetic analysis

A variety of model based methods, in addition to maximum parsimony, were employed to infer phylogenetic relationships. Parsimony has been shown to be inconsistent under certain situations when dealing with molecular sequence data (Huelsenbeck, 1995; Hasegawa and Fujiwara, 1993; Kuhner and Felsenstein, 1994), so maximum likelihood approaches were also used. All parsimony and maximum likelihood analyses were performed using the computer program PAUP* 4.0 (Swofford, 2001).

Maximum parsimony searches were conducted using heuristic search methods with tree bisection-reconnection (TBR) branch swapping, collapse of zero-length branches, and equal weighting of all characters. Two parsimony analyses were conducted: one including all sites in the alignment, and the other excluding regions which contained gapped sites that might be subject to alignment error (indels). Confidence in the resulting tree topologies was assessed by performing a bootstrap test (Felsenstein, 1985) using 300 replicates.

In addition to searching for trees under the maximum parsimony criterion, we also searched for trees using maximum likelihood. In order to determine which model best fit the data, a series of nested [i.e., the null hypothesis (H_0) is a special case of the alternative hypothesis (H_1)] hypotheses were performed on various nucleotide substitution models. An initial neighbor-joining (NJ) tree based on the Jukes–Cantor distance (JC) was generated, and then a likelihood ratio test (LRT) was performed (Goldman, 1993) to test the models. We calculated the test statistic as $2(\ln L_0 - \ln L_1) = -2\ln \Lambda$, where L_0 and L_1 are the likelihood values under the null and alternative hypotheses, respectively. We calculated the associated probability using a χ^2-distribution with the degrees of freedom equal to the difference in number of free parameters between the two models. The models tested included the simplest substitution model, the Jukes–Cantor model (JC, Jukes and Cantor, 1969), which assumes that all nucleotide substitutions are equally probable and that the nucleotides occur in equal frequencies. The more complicated Hasegawa, Kishino, and Yano model (HKY85, Hasegawa et al., 1985) allows the transition and transversion rate to differ and incorporates observed average nucleotide frequencies. Finally, the most parameter rich model tested was the general time-reversible model (GTR, Lanave et al., 1984; Rodriguez et al., 1990; Tavare, 1986), which incorporates observed average base frequencies and allows for rate variation among six substitution types. In addition to the nucleotide models other parameters were investigated. These included the extent of among site rate variation (α value of the Γ-distribution estimated with eight rate categories) along with the number of invariable sites (I). After the best-fit model was found, we performed a heuristic search using the same branch swapping techniques as described when using maximum parsimony. The search was started using the initial parameter estimates from the NJ tree, but once a better tree was found we reestimated the parameters and searched again. This process was continued until it converged on the same maximum likelihood tree. Bootstrap tests were performed once again, but this time using 100 replicates.

Maximum likelihood was also used for additional phylogenetic tests. To test the null hypothesis of a molecular clock for our dataset, we used a procedure proposed by Felsenstein (1993). This test uses a LRT to determine if there is a significant difference between the likelihood scores obtained from an analysis where the branch lengths are unconstrained as compared to an analysis that constrains the branch lengths so that all the tips are contemporaneous. Once again, the likelihood test statistic is assumed to be approximately equal to a X^2-distribution with $n-2$ degrees of freedom, where n equals the number of taxa sampled (Felsenstein, 1981). In addition, competing tree topologies based on previous phylogenetic hypotheses were compared using the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) to test for significant difference in tree lengths. This test was performed using RELL with 1000 bootstrap replicates and the results evaluated as a one-tailed test.

3. Results

The passerine sequence obtained for our study consisted of 1403 bp of aligned sites that spans the chicken mtDNA positions 2286–3184 and 3230–3722 (Desjardins and Morais, 1990). In the chicken, the complete sequence of the t-RNA-Valine gene is 72 bp in length, and the 16S rRNA gene is 1620 bp in length. The passerine sequence analyzed in the present study consisted of a partial sequence of 61 bp for the t-RNA-Val gene, and a partial sequence of 1342 bp for the 16S rRNA gene (see Table 1 for GenBank Accession Nos.). Of these 1403 bp there were 467 variable sites (370 excluding outgroup) and 303 parsimony informative sites (284 excluding outgroup). The base composition bias statistic calculated was relatively low (bias = 0.146). A χ^2 test for homogeneity of base frequency among taxa was non-significant when including all characters in the analysis ($P = 0.999$).

The parsimony analysis resulted in three most parsimonious trees (length = 1482). The strict consensus tree with bootstrap values is presented in Fig. 4. To examine the effect of the alignment on the analysis, gapped positions (indels) were removed, which created a data set of 1313 positions. The parsimony analysis with indels removed resulted in four most parsimonious trees (length = 1259). The consensus of these trees is virtually the same as for the complete dataset (tree not shown). The indel removed consensus still maintains the superfamily Passeroidea, but does include *Passerella iliaca* and *Spizella passerina* in the Embirizidae, as well as not including *Coccothraustes vespertinus* in the Fringillidae. At higher levels of resolution the Paridae is not sister to the Passeroidea, but left unresolved with the Sylvioidea. The final difference is that *Troglogytes aedon* and *Psaltriparus minumus* are not united together, but are left unresolved with the other Sylvioidea. The only difference present in the indel removed consensus tree relative to the complete dataset tree consists of a lack of resolution for certain taxa, but not an entirely different tree, suggesting that the gapped positions (indels) are not contributing a disproportionate effect on the topology. The main effect produced by removing the gapped positions was to slightly lower the bootstrap support at some of the nodes, which is expected since fewer characters are present in the reduced dataset. Since the inclusion of the indel characters does not appear to have any adverse effects on recovering the phylogeny, all subsequent analyses were conducted using all the characters.

The results of the maximum likelihood model selection are presented in Table 3. The maximum likelihood model determined using the LRT suggested that the best model for these data was the GTR + I + Γ. The like-

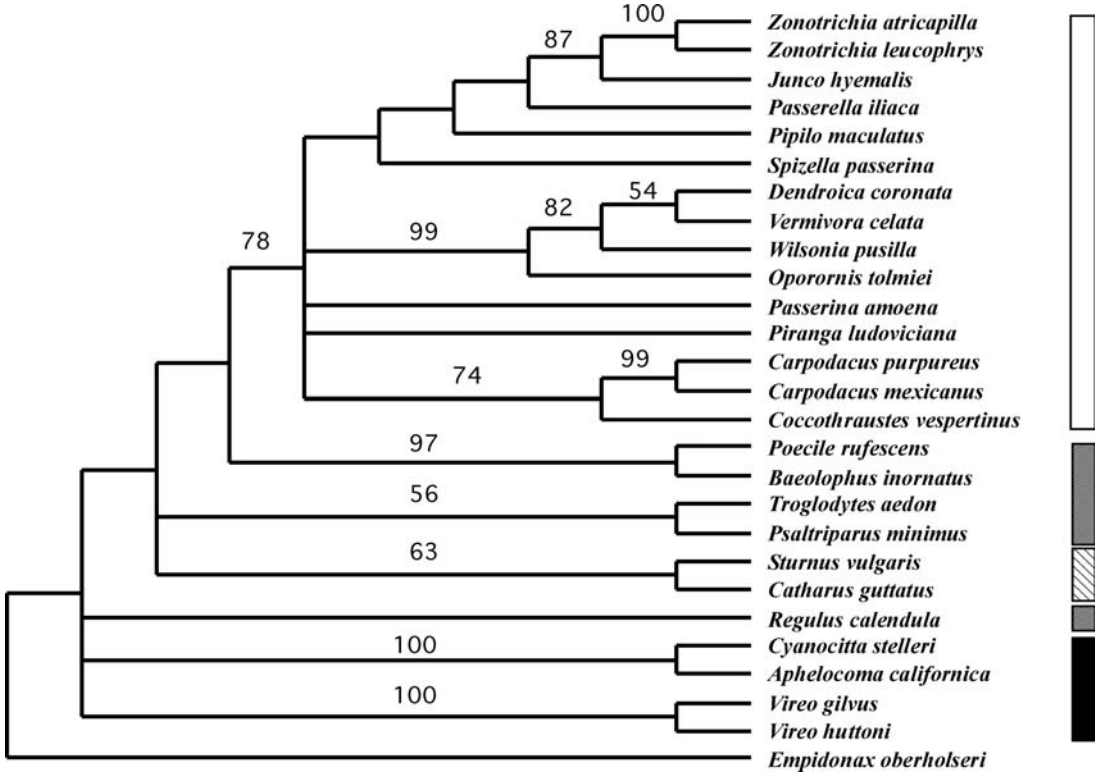

Fig. 4. Strict consensus of the three most parsimonious trees (length = 1482). Numbers at the nodes represent bootstrap frequencies of 300 bootstrap replicates. Superfamily designations according to Sibley and Ahlquist (1990) are shown to the right of the phylogeny (see Fig. 1).

Table 3
Maximum likelihood analysis of hierarchical substitution models for the 16S rRNA sequence data, based on a NJ tree of JC distances

H_0 vs. H_1	$-\ln L_0$	$-\ln L_1$	$-2\ln\Lambda$	df	P
JC vs. F81	10086.402	9996.756	179.3	3	<0.0001[a]
F81 vs. HKY85	9996.756	9683.682	626.1	1	<0.0001[a]
HKY85 vs. GTR	9683.682	9523.068	321.2	4	<0.0001[a]
GTR vs. GTR + Γ	9523.068	8425.453	2195.2	1	<0.0001[a]
GTR + Γ vs. GTR + I + Γ	8425.453	8418.395	14.1	1	<0.0002[a]

Note. Likelihoods were evaluated with the likelihood ratio test (LRT) as described in Section 2. The abbreviations for the models and parameters are: JC, Jukes and Cantor (1969); F81, Felsenstein (1981); HKY, Hasegawa et al. (1985); GTR, general time-reversible model (Lanave et al., 1984; Rodriguez et al., 1990; Tavare, 1986); Γ, shape parameter of the gamma distribution estimated with eight rate categories; I, proportion of invariable sites.

[a] Hypothesis rejected.

lihood search using this model resulted in one maximum likelihood tree with a $-\ln L = 8404.25091$ (Fig. 5), which is considered the best estimate of the phylogeny. The parameter values as estimated from this tree were: $A \Longleftrightarrow C$: 3.12165, $A \Longleftrightarrow G$: 5.92054, $A \Longleftrightarrow T$: 2.367, $C \Longleftrightarrow G$: 0.45845, $C \Longleftrightarrow T$: 18.1457, $G \Longleftrightarrow T$: 1.0 for the GTR model, estimated base composition was $A = 0.35957$, $C = 0.25139$, $G = 0.17678$, $T = 0.21226$, and $\alpha = 0.3059$ for the Γ-distribution, and $I = 0.34727$ for the proportion of invariable sites. Maximum likelihood was also used to test for a molecular clock. The molecular clock tree produced with the same parameter estimates above gave a likelihood score of $-\ln L = 8430.99075$, which indicates that the molecular clock should be rejected ($\chi^2 = 53.5$, df = 25, $P = 0.0008$).

Results of the Shimodaira–Hasegawa tests based on the GTR + I + Γ model with the parameters specified above are presented in Table 4. These results reveal that the 16S rRNA tree statistically conflicts with the proposed DNA–DNA hybridization phylogeny of Sibley and Ahlquist (1990). However, when examining subsets of the Sibley and Ahlquist (1990) hypotheses, such as forcing the superfamily Sylvioidea to be monophyletic, or rearranging the familial relationships in the superfamily Passeroidea, then the two different phylogenetic

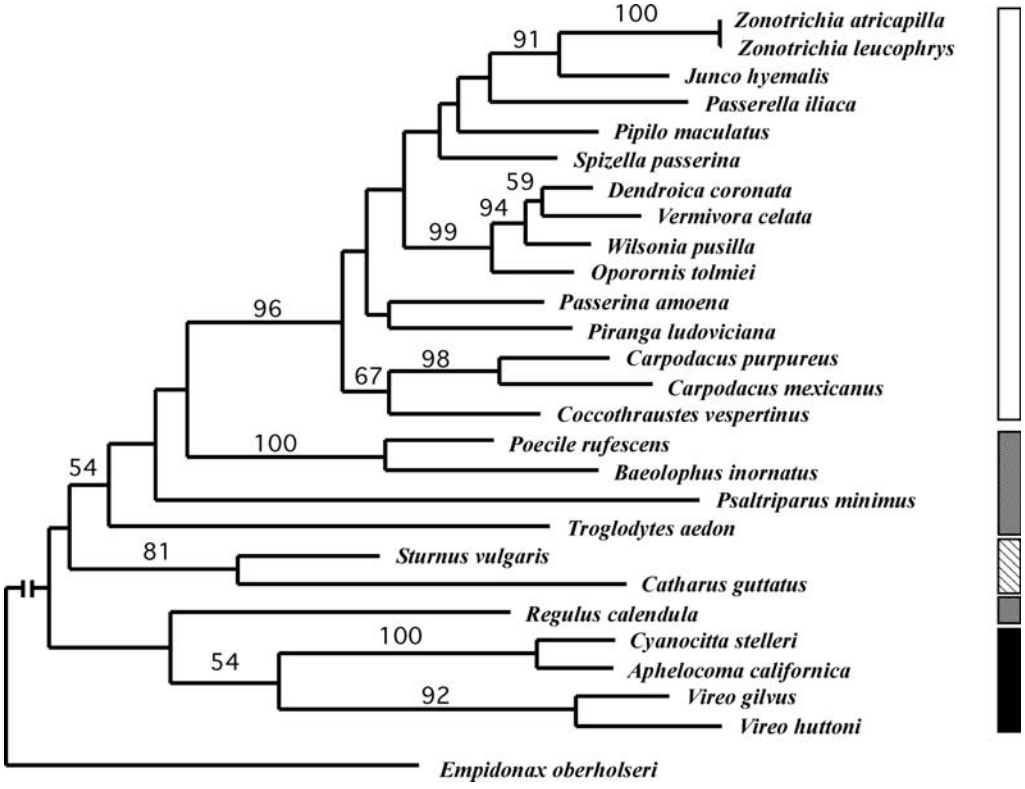

Fig. 5. Phylogeny of the songbirds (Oscines) inferred from the maximum likelihood tree ($-\ln L = 8404.25091$) based on the GTR + I + Γ model (see text for details and parameter estimates). Superfamily designations according to Sibley and Ahlquist (1990) are shown to the right of the phylogeny (see Fig. 1).

Table 4
Shimodaira–Hasegawa tests comparing the 16S rRNA maximum likelihood tree to previously suggested songbird phylogenies

Tree	$-\ln L$	Difference $-\ln L$	P^a
Present study	8404.25091	(best)	
Sibley and Ahlquist (1990)	8437.29069	33.03978	0.015*
Sylvioidea monophyly	8426.39047	22.13956	0.068
Corvida monophyly	8404.56966	0.31875	0.933
Passeroidea relationships	8410.94248	6.69157	0.508
Sheldon and Gill (1996)	8415.86583	11.61491	0.308
Klicka et al. (2000)	8406.71091	2.45999	0.722
Barker et al. (2002)	8431.64201	27.39110	0.020*

[a] Probability of getting a more extreme *t*-value under the null hypothesis of no difference between the two trees (one-tailed test). Asterisked values in the table indicate significant difference at $P < 0.05$.

hypotheses are statistically indistinguishable. The same is true of the 16S rRNA comparison to the Sheldon and Gill (1996) passerine phylogeny, which is also based on DNA–DNA hybridization, and the Klicka et al. (2000) familial relationships among the superfamily Passeroidea, which are inferred from cytochrome *b* and NADH mtDNA sequences. In both cases they are statistically indistinguishable, although the proposed phylogenies are different. However, the recent phylogeny proposed by Barker et al. (2002) is statistically different from the phylogeny proposed here by using the Shimodaira–Hasegawa test.

4. Discussion

Overall, the present study, which is based on mitochondrial 16S rRNA mtDNA gene sequence, does not support the relationships put forth by Sibley and Ahlquist (1990) as shown by the Shimodaira–Hasegawa test (Table 4). However, at various levels in the phylogeny general agreement among studies can be found. Of the six families investigated in our study that contained more than one taxon, all six were maintained as monophyletic groups in accordance with the AOU checklist (1998). At the next level, some of the superfamily designations set forth by Sibley and Ahlquist (1990) are supported by our study. The Corvoidea, Muscicapoidea, and Passeroidea each form cohesive groups, but with differing amounts of support. The grouping of Vireonidae and Corvidae into the Corvoida is inferred, but lacks bootstrap support. The grouping of the families Turdidae and Sturnidae into the superfamily Muscicpoidea is confirmed (Barker et al., 2002; Sheldon and Gill, 1996; Sibley and Ahlquist, 1990) and well supported. In addition, the superfamily Passeroidea is well supported by our dataset, as has been previously suggested by a number of studies (Barker et al., 2002; Bledsoe, 1988; Klicka et al., 2000; Sibley and Ahlquist, 1990).

The most glaring difference between the phylogenetic relationships recovered in our study and the study described by Sibley and Ahlquist (1990) is in the placement of the Kinglets (*Regulus*) in the superfamily Sylvioidea. In our study, the Regulidae is considered part of a monophyletic group along with the Vireonidae and Corvidae. In the phylogeny of Sheldon and Gill (1996) another arrangement is suggested; *Regulus* appears as the sister taxon to all other passerids. Finally, the nuclear gene tree of Barker et al. (2002) infers another relationship, which places *Regulus* as a sister taxon to the Muscicapoidea. Traditionally, Kinglets have been considered typical sylvioids, close to leaf-warblers (Mayr and Amadon, 1951), but Sibley and Ahlquist (1990) separated the Kinglets into their own family, though retained them as sylvioids. Sheldon and Gill (1996) found that in the raw data Sibley and Ahlquist provided for the Regulidae (1990: Figs. 278 and 285) the measured distances were substantially longer than those illustrated in their Fig. 350, or as shown in the tapestry phylogeny (1990: Fig. 380). Thus, the raw data produced by Sibley and Ahlquist (1990) seem to be consistent with the findings of our study and of Sheldon and Gill (1996) and Barker et al. (2002), that is they indicate an unexpectedly large divergence between the Regulidae and other traditional sylvioids. However, the Shimodaira–Hasegawa tests are unable to statistically differentiate among these hypotheses based on the 16S rRNA dataset (Table 4).

The relationships within the superfamilies as described by Sibley and Ahlquist (1990) vary from one study to another. Due to the sampling used in our study, there are only two superfamilies in which the familial relationships can be compared. Within the Passeroidea, the familial relationships described by DNA–DNA hybridization in the study of Bledsoe (1988) are congruent with those found in our study. However, they differ from the findings of Sibley and Ahlquist (1990), and again from those of Klicka et al. (2000). However, the Shimodaira–Hasegawa tests are unable to statistically differentiate among these different hypotheses, so these relationships as inferred by the 16S rRNA gene sequence data appear to be only weakly supported.

The relationships among the Sylvioidea, on the other hand, are quite different among all the studies. As already mentioned, Sibley and Ahlquist (1990) consider

the Sylvioidea as a monophyletic group and show Aegithalidae as being more closely related to the Regulidae with the Paridae and the Troglodytidae basal to this group. Other DNA–DNA hybridization work (Sheldon and Gill, 1996) reported the Troglodytidae as a monophyletic group with the Muscicapoidea (Sturnidae and Turdidae), and not with Paridae, Aegithalidae, and Regulidae. The study by Barker et al. (2002) suggests a similar arrangement. Our study suggests that none of these families combine to make a monophyletic group, however, once again there is no statistical difference among studies based on the Shimodaira–Hasegawa test (Table 4). This is not surprising given the low support at the internal nodes connecting the various sylvioid families and the families of the Muscicapidae and it appears that these internodes are very short. Short internodes at these positions could help explain some of the discrepancy between our study and those of Sibley and Ahlquist (1990) and Sheldon and Gill (1996). The difference between our study and Sibley and Ahlquist (1990) could be explained in two different ways. It is possible that the Sibley and Ahlquist (1990) phylogeny is more robust because of the inclusion of more taxa, which could possibly fortify short internodes (Swofford et al., 1996). Alternatively, the Sibley and Ahlquist (1990) estimate of phylogeny may contain mistakes because of the problems in their experimental design and data analysis (Sheldon and Bledsoe, 1993). Many of the short internodes presented by Sibley and Ahlquist (1990) might be expected to collapse when assessed by a bootstrap analysis (Sheldon and Gill, 1996).

The separation of the Oscines into the monophyletic parvorders Corvida (Corvidae and Vireonidae) and Passerida (other Oscine families of our study), as suggested by Sibley and Ahlquist (1990), was not recovered by the 16S rRNA sequences. This is due to the paraphyletic position of the Passerida. However, the superfamily Corvidea monophyly was reaffirmed by our analysis, but with no support. The corvine affinity of *Vireo* was first suggested by Sibley and Ahlquist (1982), and later confirmed by protein studies (Johnson et al., 1988), and independent DNA–DNA hybridization (Sheldon and Gill, 1996). Prior to these studies *Vireo* was commonly considered to be close to the New World nine-primaried oscines (Mayr and Amadon, 1951).

In contrast to the other studies mentioned, the findings of our study are extremely different from the phylogeny produced using mitochondrial cytochrome *b* sequence for a selected number of Passeriformes in Japan (Chikuni et al., 1996). The most obvious difference between these two phylogenies is that the cytochrome *b* phylogeny proposes that the Emberizidae are a basal clade, sister to the Corvidae. This is in direct conflict with both the DNA–DNA hybridization results (Bledsoe, 1988; Sheldon and Gill, 1996; Sibley and Ahlquist, 1990), nuclear gene sequences (Barker et al., 2000), and the results of our study. There are two possible explanations for this discrepancy. First, the study performed by Chikuni et al. (1996) used extremely divergent outgroups, including Columbiformes, Strigiformes, Piciformes, and Anseriformes. The choice of such divergent outgroups could have affected the outcome of their phylogeny. Second, and probably most importantly, they chose to use cytochrome *b* sequence data to infer the phylogeny. As they stated, the relationships among the families of the order Passeriformes were unclear, since they found that their dataset was saturated with substitutions in comparisons above family level.

Even though the phylogeny produced in our study is relatively robust to various types of analyses, it lacks bootstrap support at many of the deeper branches. The best supported branches in our phylogeny are the ones connecting the most closely related taxa (species within a genus) and the moderately divergent taxa (genera in families). Presumably, there are not enough sites in our dataset to resolve the relationships among different passerine families with confidence. One hypothesis that could account for at least part of the weak resolution in the deeper branches of the phylogeny, is the possibility of a narrow time window in which differentiation may have occurred within the Passeriformes. Both DNA–DNA hybridization and mtDNA analyses have shown that the branch separating songbirds (Oscines) from other perching birds (Suboscines) is more strongly supported than most branches within songbirds (Edwards et al., 1991; Sibley et al., 1988). These results appear to show that the lineage leading to the songbirds is long and that the radiation of the songbird families occurred within a short time relative to the divergence of songbirds from other perching birds. For example, a star-like phylogeny appears to be the case for the members of the family Emberizidae. Based on the resolution of other genera within a family, it would be expected that these moderately recent nodes within the Emberizidae would have high support. Instead, what we find is that the internodes in these clades are extremely short and have very little support. This same pattern was also found in other mtDNA studies of this group. Seutin and Birminham (1997) found that most internal branches in the emberizine clade were very short and not statistically positive in length. Bootstrap analyses of their cytochrome oxidase I dataset provided only limited support for these branches. Consequently, even the addition of appropriate DNA sequence data may not support these deeper branches.

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