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Monophyly and relationships of wrens (Aves: Troglodytidae): a congruence analysis of heterogeneous mitochondrial and nuclear DNA sequence data

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Abstract

The wrens (Aves: Troglodytidae) are a group of primarily New World insectivorous birds, the monophyly of which has long been recognized, but whose intergeneric relationships are essentially unknown. In order to test the monophyly of the group, and to attempt to resolve relationships among genera within it, sequences from the mitochondrial cytochrome *b* gene and the fourth intron of the nuclear β-fibrinogen gene were obtained from nearly all genera of wrens, from their relatives as suggested by traditional taxonomy and DNA–DNA hybridization analyses, and from additional passerines. Maximum likelihood analysis of the two data sets yielded maximal congruence between independently derived estimates of relationship, outperforming a variety of weighted parsimony methods. Hierarchical likelihood ratio tests indicated that the two gene regions differed significantly in every estimated parameter of sequence evolution, and combined analysis of the two data sets was accomplished using a heterogeneous-model Bayesian approach. Independent and simultaneous analyses of both data sets supported monophyly of the wrens (excluding one recently added member, the monotypic genus *Donacobius*) and a sister-group relationship between wrens and the gnatcatchers (*Polioptila*). Additionally, strong support was found for paraphyly of the genus *Thryothorus*, and for a sister-group relationship between the genera *Cistothorus* and *Troglodytes*. Analyses of these data failed to resolve basal relationships within wrens, possibly due to ambiguity in rooting with a distant, species-poor outgroup. Analysis of the combined data for wrens alone yielded results which were largely congruent with relationships inferred using the complete data set, with the benefit of stronger support for relationships within the group. However, alternative rootings of this ingroup tree were weakly supported by nucleotide substitution data. Insertion–deletion events suggest that the genus *Salpinctes* may be sister to all other wrens.

1. Introduction

The family Troglodytidae (*sensu* Mayr and Greenway, 1960) comprises some 75 species of generally small, active, highly vocal insectivorous birds. The family is limited in distribution to the New World (except *Troglodytes troglodytes*), with a center of diversity in Central America (Brewer, 2001). Members of this family have been the subject of a wide variety of ecological and behavioral studies, including studies of mating systems (Johnson et al., 1993, 1994; Rabenold et al., 1990; Leonard, 1990), male multiple-nest-building behavior (Evans, 1997; Evans and Burn, 1996; Leonard and Picman, 1987), vocal dialects and repertoires (Catchpole and Rowell, 1993; Kroodsma, 1977; Kroodsma and Canady, 1985; Kroodsma et al., 1999; Morton et al., 1986; Verner, 1975), vocal duetting (Brenowitz and Arnold, 1986; Farabaugh, 1982; Levin, 1996a,b), and cooperative breeding (Rabenold, 1990; Skutch, 1935). Since it has been the target of such intensive studies of a wide variety of significant characteristics, the family presents excellent opportunities for integrative, comparative analyses of ecological and behavioral evolution. However, complete understanding of variation within the family must incorporate information on historical associations among species and genera (e.g., Harvey and Pagel, 1991). To date, no comprehensive hypothesis of phylogenetic relationships among wrens exists, and the

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relationship of the family to other passerines remains a matter of contention.

Hypotheses of the relationships of the Troglodytidae were until recently limited to circumscriptions of the family's contents, and associations of the family with others in linear taxonomic series. The contents of the family have been stable over the last century, after several genera were eliminated from the family and recognized as members of the babbler subfamily Timaliinae and the family Sylviidae (sensu Mayr and Cottrell, 1986; Mayr and Paynter, 1964), though the enigmatic genus *Donacobius* was recently assigned to the family (A.O.U., 1983; Clench et al., 1982). The taxonomic placement of the family relative to other passerine families has varied, but several groups have traditionally been associated with the wrens, including the dippers (Cinclidae; e.g., Sharpe, 1881), mimic-thrushes (subfamily Miminae, family Muscicapidae; *sensu* Mayr and Greenway, 1960; e.g., A.O.U., 1886), creepers (Certhiidae; e.g., Beecher, 1953; Ridgway, 1904), and nuthatches (e.g., Beecher, 1953). Most recent traditional classifications place the Troglodytidae near the Cinclidae and Mimidae in linear series (Mayr and Amadon, 1951; Mayr and Greenway, 1960; Wetmore, 1960).

Recently, genetic data have been applied to the problem of wren relationships. Sibley and Ahlquist (1990) proposed a hypothesis of relationships for the Class Aves, based upon analysis of DNA–DNA hybridization distance data, including explicitly phylogenetic hypotheses of relationship among wrens and their putative allies. Within wrens, Sibley and Ahlquist obtained very little resolution, with most branches shorter than estimated experimental error in hybridization distance measurements. Regarding wren relationships to other groups, Sibley and Ahlquist proposed a novel hypothesis: that wrens (including *Donacobius*, Sibley and Ahlquist, 1984) are the sister-group to a clade containing the gnatcatchers and gnatwrens (genera *Polioptila*, *Microbates*, and *Ramphocaenus*) and the verdin (genus *Auriparus*). The former genera have been placed together as subfamily Polioptilinae of the Muscicapidae (sensu lato) by Mayr (1946), and Mayr and Paynter (1964), while the latter genus is generally considered the a member of the penduline tit family Remizidae (e.g., A.O.U., 1998). Sibley and Ahlquist found the Certhiidae (the creepers, *Certhia* and *Salpornis*) to be the sister-group to the wren/gnatcatcher/verdin clade, and the Sittidae (the nuthatches and wallcreeper, *Sitta* and *Tichodroma*) as the sister to all of these. These genetic results supported previous notions of a link between wrens and treecreepers and nuthatches, while rejecting any affinity with the babblers (which they placed within a newly defined Sylviidae), mimic-thrushes (placed as sister-group to the traditional Sturnidae within another superfamily), and dippers (also placed in another superfamily). A subsequent DNA–DNA hybridization study by Sheldon and Gill (1996) supported Sibley and Ahlquist's arrangement of the gnatcatchers and gnatwrens, treecreepers, and nuthatches (represented by *Polioptila*, *Certhia*, and *Sitta* respectively) as the closest relatives to the wrens. However, their data agreed with traditional taxonomy in placing *Auriparus* with the penduline-tits. Also, their data supported placement of the mimic-thrushes as the sister-group to the starlings, well separated from the wrens. Many of these hypothesized relationships among wrens, creepers, nuthatches, and mimic-thrushes were recently corroborated using nuclear DNA sequences (Barker et al., 2002).

Hypotheses of relationship among genera of wrens are extremely few, although some connections among genera have been suggested by past taxonomic practice. Sumichrast's wren (*Hylorchilus sumichrasti*) was originally assigned to the genus *Catherpes*, and later removed to its current genus based upon more extensive material (reviewed in Atkinson et al., 1993). Sibley and Monroe (1990) suggested returning the genus to *Catherpes* (e.g., Sibley and Monroe, 1990), based upon vocal similarities, but subsequent discovery of the vocally distinctive Nava's wren (*Hylorchilus navai*) suggested retention of the genus *Hylorchilus* (Atkinson et al., 1993). The cañon wren *Catherpes mexicanus* has also been linked with the rock wren (*Salpinctes obsoletus*), and at least one widely used taxonomy (Mayr and Greenway, 1960) subsumes both species within *Salpinctes*. Recently Rice et al. (1999) found, based on mitochondrial DNA sequence data, that the timberline wren (*Thryorchilus browni*) was nested within the genus *Troglodytes*, suggesting paraphyly of the latter genus. Other than these, there are no hypotheses of relationship among wren genera except the various linear series of taxa.

The work reported here was performed in order to test previous notions of phylogenetic relationships of wrens (Aves: Troglodytidae). Specifically, this work attempts to test the monophyly of the family, to determine its sister-group relationships, and to determine relationships among genera within the family. As suggested by some authors (Hillis, 1987; Barrett et al., 1991; Flook et al., 1999; but see Bull et al., 1993; Reed and Sperling, 1999), data from two character systems which differ in their rates of divergence (the mitochondrial cytochrome *b* gene and the fourth intron of the nuclear β-fibrinogen gene) were collected in the hope that they would provide complementary information at different hierarchical levels. Though this strategy has been advocated in the literature, considerable disagreements have arisen over methodological approaches to analyzing the data obtained (Brower et al., 1996; Bull et al., 1993; Chippindale and Wiens, 1994; De Quieroz et al., 1995; DeSalle and Brower, 1997; Huelsenbeck et al., 1996; Krajewski et al., 1999). In particular, combining data sets with fundamentally different evolutionary dynamics can be

problematic (Bull et al., 1993; Caterino et al., 2001; Wilgenbusch and de Quieroz, 2000). The sequence data reported here were analyzed both independently and in combination, using parsimony, maximum likelihood, and Bayesian approaches, specifically addressing the issue of among-partition variation in evolutionary dynamics.

2. Materials and methods

2.1. Data collection and alignment

2.1.1. Taxon sampling strategy

Taxon sampling in this study was designed in order to: (1) test the sister group relationships and monophyly of wrens, (2) test the placement of *Donacobius* relative to the traditional families Mimidae and Troglodytidae and (3) determine intergeneric relationships among wrens. Choice of taxa was guided primarily by the phylogenetic hypotheses of Sibley and Ahlquist (1990) (see also Harshman, 1994), Sheldon and Gill (1996), and Barker et al. (2002). The taxa sampled, following the sequence of Sibley and Monroe (1990), are listed in Table 1. A single suboscine (*Pipra pipra*) was chosen as an outgroup for estimation of interfamilial relationships. All of the superfamilial groupings of passeridan passerines were sampled (Muscicapoidea, Sylvioidea, and Passeroidea), as well as two members of the putative sistergroup to the Passerida, the parvorder Corvida (Sibley and Ahlquist, 1990). Within the Muscicapoidea, sampling included a thrush (Muscicapidae: Turdinae), a mimic-thrush (Sturnidae: Miminae), and a starling (Sturnidae: Sturninae). Within the Sylvioidea, sampling included all of the putative wren outgroups, including the nuthatches (Sittidae), treecreepers (Certhiidae: Certhiinae), and gnatcatchers and gnatwrens (Certhiidae: Polioptilinae). Within the wrens, all genera were sampled except the monotypic genera *Ferminia*, *Thryorchilus*, and *Uropsila* (13 genera, not including *Donacobius*). It should be noted, however, that *Thryorchilus* has recently been allied to the genus *Troglodytes* in a molecular study by Rice et al. (1999). Two representatives were included for the genera *Campylorhynchus* and *Cistothorus*, and five species were sampled from the genus *Thryothorus*, which contains a large percentage of wren diversity (27/75 species; Sibley and Monroe, 1990). All other genera were represented by an individual from one species each (10 genera, two of which are in any case monotypic). Two members of the Passeroidea were included in the analysis in order to complete sampling of the superfamilies within the parvorder Passerida. Under the assumption that relationships among the sylvioid taxa sampled here are similar to those indicated by DNA hybridization, this sampling strategy allows rigorous testing of the membership of *Donacobius* in both the traditional Troglodytidae (Mayr and Greenway, 1960), and Mimidae.

2.1.2. Amplification and sequencing of cytochrome b and β-fibrinogen intron 4

Total genomic DNA was extracted from ~100 mg of each tissue sample by standard proteolytic digestion, phenol/chloroform extraction, and concentration by centrifugal dialysis (Centricon 100, Amicon); alternatively, some samples were extracted using commercially available protocols and reagents (PureGene, Gentra Systems; QiaAmp, Qiagen). Preparation of cytochrome *b* sequencing template was accomplished by primary PCR amplification of a majority of the gene using the primer pair B1a/B8, and subsequent reamplification of three segments (B1a/B2a, B3/B6, and L15507/B8; see Table 2) from a gel plug (5 μL of initial PCR run on a 1% low melting point agarose [FMC] gel, excised and melted in 200 μL ddH_2O) of this initial amplification. Primary amplifications were performed in 25 μL total reaction volumes [25–50 ng template DNA, 1× amplification buffer (1.5 mM final $MgCl_2$, Boehringer–Mannheim), 0.5 U *Taq* polymerase (Boehringer–Mannheim), 0.2 μM in each primer, 80 μM in each dNTP], with thermal cycling parameters for initial amplifications as follows: an initial 3 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 30 s at 51 °C, and 75 s at 72 °C, followed by a final 3-min extension at 72 °C. Primary amplifications of the fourth intron of the β-fibrinogen gene were accomplished as for cytochrome *b*, except using the FIB3/FIB4 primer pair (Table 2), and an extension time of 30 s. Reamplifications were performed in 50 μL volumes (using 2 μL of melted gel plug as template), with conditions as for primary amplifications, excepting an increase of 2° in annealing temperature, and an extension time of 30 s. Reamplification products were purified by one of two methods: (1) centrifugal dialysis using a Microcon 100 (Amicon), twice adding 500 μL ddH_20 to the 50 μL reaction volume and spinning at 500*g* for 15 min; or (2) dsDNA extraction using the GeneClean protocol (Bio101). Dialyzed PCR products were quantified and diluted to 25 ng/μL final concentration, while genecleaned products were resuspended in 25 μL ddH_2O.

Several intron 4 PCR products (Table 1) were cloned in order to detect heterozygotes and length variants. Samples selected for cloning were those for which clean double-stranded sequence could not be obtained directly from PCR product. Cloning was accomplished by ligation of PCR product (using T4 DNA ligase [Promega]) into *Eco*RV-digested (Pharmacia Biotech) pBluescript II (Stratagene), modified to contain thymine overhangs by treatment with dTTP and Taq polymerase. Ligated plasmids were used to transform *Escherichia coli* strain DH5α cells (Gibco), and selected for inserts by growth on LB plates with IPTG and X-Gal (5′–3′). Positive colonies were grown overnight in LB, and the presence

Table 1
List of taxa included in this study, following the taxonomy of Sibley and Monroe (1990), including lengths of sequences obtained from β-fibrinogen intron 4, and sample data

Species and taxonomy	I4	Source[a]
Suborder Tyranni		
Pipra pipra	609	LSUMNS B7079
Suborder Passeri: Parvorder Corvida		
Meliphaga gracilis	603	*fide* A.C. Driskell
Dasyornis broadbenti	609	*fide* A.C. Driskell
Suborder Passeri: Parvorder Passerida		
Superfamily Muscicapoidea		
Sialia sialis	589[b]	FMNH 350787
Sturnus vulgaris	585[b]	FMNH 389606
Dumetella carolinensis	585	FMNH 350635
Superfamily Sylvioidea		
Sittidae		
Sitta pygmaea	593	FMNH 343324
Certhiidae: Certhiinae		
Certhia familiaris	575	FMNH 351158
Certhiidae: Troglodytinae		
Donacobius atricapillus	570	FMNH 1772 [SML88-246, MPEG]
Campylorhynchus brunneicapillus	590	FMNH 342076
C. megalopterus	590	MZAH 8711
Odontorchilus cinereus	593	FMNH 1789 [DW3682, MPEG]
Salpinctes obsoletus	585	MVZ 170087
Catherpes mexicanus	580	FMNH 5470 [BEHB033, MZAH]
Hylorchilus sumichrasti	581	MZAH [OMVP1132, MZAH]
Cinnycerthia peruana	588	ZMUC O2450 [NK13, MDS]
Cistothorus platensis	592	FMNH 350634
C. palustris	592	FMNH 333341
Thryomanes bewickii	591	MZAH 9734
Thryothorus coraya	585[b]	FMNH 339666
T. maculipectus	583	MZAH 7828
T. ludovicianus	588	AMNH 20929
T. leucotis	592	MUSP 73431
T. guarayanus	589	FMNH 334541
Troglodytes aedon	592	FMNH 343273
Henicorhina leucosticta	594	FMNH 343285
Microcerculus marginatus	597[b]	LSUMNS B11839
Cyphorhinus arada	595	FMNH 1775 [ATP86-142, MPEG]
Certhiidae: Poliptilinae		
Polioptila caerulea	585[b]	FMNH 343322
Cisticolidae		
Prinia bairdii	444	FMNH 355824
Zosteropidae		
Zosterops senegalensis	601	FMNH 346671
Superfamily Passeroidea		
Acanthidops bairdii	580	LSUMNS B16267
Zeledonia coronata	580	LSUMNS B16178

[a] Source indicates the specimen voucher for each sample. If voucher data were unavailable, then the tissue number is listed, the institution where the voucher is located is indicated, and collector's number provided in brackets. ANSP, Philadelphia Academy of Natural Sciences; FMNH, Field Museum of Natural History; LSUMNS, Louisiana State University Museum of Natural Science; MDS, Museo de Salango, Ecuador; MPEG, Museu Paraense Emilio Goeldi; MUSP, Museu de la Universidad de Saõ Paolo; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley; MZAH, Museo de Zoología "Alfonso L. Herrera," Universidad Nacional Autónoma de México; and ZMUC, Zoological Museum, University of Copenhagen.
[b] Cloned samples.

of appropriately sized inserts confirmed by electrophoresing 40 μL phenol/chloroform-extracted culture on an agarose gel, visualized with ethidium bromide. Cultures of three positive colonies per PCR product were purified for sequencing using the PERFECTprep plasmid DNA purification protocol and reagents (5′–3′).

Table 2
List of primers used in amplification and sequencing, numbered according to location in the *Gallus* genome (Desjardins and Morais, 1990) for mitochondrial primers, and the human β-fibrinogen gene (Chung et al., 1991) for intron primers

Primer	Sequence (5′–3′)	Location[a]
B1a	ccatccaacatctcagcatgatgaaa	L14990[b]
B3	atctgcatctacctacacatcgg	L15191[c]
L15506	ctcaccttcctacacgaaacagg	L15506[b]
B2a	cccctcagaatgatatttgtcctca	H15298[b]
B6	gcgtaggcgaataggaagtatca	H15709
B8	ggagtcttcagtctctggtttacaagac	H16065[b]
FIB3	ctgtaatatcccggtggtttcagg	S4706
FIB4	atttcagatgtttcacctccctttc	AS5291

[a] For mitochondrial primers, L indicates binding to heavy strand, H to light strand; for intron primers, S indicates binding to the sense strand, and AS to the anti-sense strand.
[b] From Helm-Bychowski and Cracraft (1993).
[c] From Lanyon and Hall (1994).

Purified PCR products were cycle-sequenced using 50–100 ng template (or 2–4 μL purified product for GeneCleaned samples), amplification primers, and either the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq DNA Polymerase, FS; Perkin–Elmer) or the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (with AmpliTaq DNA Polymerase, FS; Perkin–Elmer), according the manufacturer's directions, except using 10 μL reaction volumes. Purified plasmid preparations were sequenced using dRhodamine sequencing chemistry and the primer pair SK/KS (SK 5′-CGCTCTAG AACTAGTGGATC-3′, KS 5′-TCGAGGTCGACGGT ATC-3′). Sequencing reactions were either purified using CentriSep columns (samples sequenced with FS; Princeton Separations, Princeton, NJ), or ethanol precipitated according to the manufacturer's directions (samples sequenced with dRhodamine). Reactions were then electrophoresed on an ABI 377 automated sequencer (Applied Biosciences, Perkin–Elmer). All nucleotide positions were sequenced from both strands.

Sequences obtained using the cytochrome *b*-targeted primers were between 1069 and 1093 bases in length (depending on the size of the intergenic spacer and tRNA-Thr fragment in individual taxa), corresponding to positions 14,991–16,064 of the *Gallus* mitochondrial genome (Desjardins and Morais, 1990). Sequences obtained from direct sequencing of PCR product or cloning using β-fibrinogen intron 4 primers varied from 468 to 633 bases in length, corresponding to positions 4707–5290 of the human gene (counting from the first base of the glutamine codon which codes for the first amino acid of the mature protein, equivalent to bases 5266–5849 of GenBank Accession No. M64983; Chung et al., 1991). Intron–exon boundaries were examined, exon sequences confirmed against available avian sequence (Weissbach et al., 1991; unpublished data), and conserved 5′ and 3′ intron motifs (Perlman et al., 1990) identified. Contig alignments were created using Sequencher (Genecodes, Ann Arbor, MI). Multiple alignment of cytochrome *b* sequences was accomplished by eye in Sequencher. Appropriate coding of cytochrome *b* sequences was verified by translation into amino acids using the avian mitochondrial code (Desjardins and Morais, 1990; implemented in MacClade v4.0, Maddison and Maddison, 2000). Multiple alignment of intron sequences was accomplished by hand modification of an initial alignment obtained using Clustal W (Thompson et al., 1994).

2.2. Phylogenetic analysis

2.2.1. Preliminary examination of data

All phylogenetic analyses were conducted using PAUP* (v4.0b10; Swofford, 1998). The sequence data were examined in a variety of ways prior to phylogenetic analysis. Since heterogeneity in base composition is known to affect phylogenetic inference (Galtier and Gouy, 1998; Lockhart et al., 1994; Yang and Roberts, 1995), variation among sequences in base pair composition was examined. Base composition homogeneity was tested using a χ^2 analysis of base frequencies across taxa, examining each coding position separately for the cytochrome *b* data. This test does not correct for phylogenetic relationships among taxa, but should be conservative, since phylogenetic autocorrelation will reduce the probability of detecting the effect of shifts in base composition. For base frequencies in partitions which showed significant heterogeneity, χ^2 goodness-of-fit analyses were also conducted for base frequencies of each sequence relative to the overall mean values, correcting for multiple comparisons.

2.2.2. Separate phylogenetic analyses: parsimony

A variety of parsimony analyses were performed in order to assess the impact of weighting on apparent conflict between the data sets. Analyses of cytochrome *b* data performed included: (1) equally weighted parsimony, (2) elimination of third position data, (3) elimination of third position transitions only, (4) Rodrigo's (1992) modification of Wheeler's (1990a) combinatorial weights approach, and (5) analysis of amino acid translations. Intron 4 sequences were analyzed under equally weighted parsimony alone, treating alignment gaps in two different ways. Regardless of gap treatment, indels were treated as missing data within the matrix, though gapped regions were always included (excepting one, see Section 3). The matrix was analyzed either in this form alone, or with potentially shared indel events coded separately as potentially informative characters. Indel events were coded where the gapped region exhibited: (1) uniformity of indel length, and (2) conservation of flanking sequence. Taxa which shared a stretch of indels in the final alignment were coded as potentially

sharing an insertion/deletion event. During parsimony analyses, all searches were heuristic, using the tree-bisection and reconnection (TBR) branch-swapping algorithm, with 50 random addition sequences of taxa (or more, depending on the discovery of multiple islands of equally parsimonious trees). Support for inferred relationships was evaluated using the bootstrap (Felsenstein, 1985).

2.2.3. Separate phylogenetic analyses: maximum likelihood

Analyses under the maximum likelihood criterion were preceded by an evaluation of alternative models of sequence evolution using a fixed tree generated by the neighbor-joining method (Saitou and Nei, 1987) with Jukes–Cantor distances (Jukes and Cantor, 1969). The initial topology chosen had little effect on the parameter values estimated (results not presented). Models evaluated included the F81 (Felsenstein, 1981), HKY85 (Hasegawa et al., 1985), and general time-reversible (Yang, 1994), allowing for invariant sites, Γ-distributed rate heterogeneity, or a combination of both for each model (Gu et al., 1995). Additionally, each model was evaluated both with and without the assumption of a molecular clock, in order to determine the significance of rate heterogeneity among lineages (Felsenstein, 1981). The significance of increases in likelihood caused by addition of parameters (e.g., allowing for among-site variation in rates) was tested using the likelihood ratio statistic $(-2\ln\Lambda = 2[\ln\lambda_2 - \ln\lambda_1]$, where λ_1 is the likelihood of the restricted model), under the asymptotic assumption of a χ^2 distribution (Huelsenbeck and Crandall, 1997; Huelsenbeck and Rannala, 1997). The model selected was the least parameter rich which could not be significantly improved by additional parameters. Subsequent to model evaluation and selection, a heuristic search with ten random addition-sequence replicates and TBR branch-swapping was performed, fixing the model parameters inferred for the starting tree. In order to identify all equally likely resolved topologies, branches of effectively zero length were not collapsed (option lcollapse = no). In order to test the robustness of the search to parameter value choice, the model parameters were reestimated on the initial optimal trees, and tree searches were repeated with the adjusted parameter estimates. Nodal support was evaluated using the bootstrap, with model parameters fixed across replicates, and initial trees for swapping obtained by neighbor-joining.

2.2.4. Separate phylogenetic analyses: Bayesian

Analysis of these data sets using Bayesian methods, as implemented in MrBayes (version 3.0; Altekar et al., in press; Huelsenbeck and Ronquist, 2001) was also performed. For each data set, the optimal model parameterization as determined by maximum likelihood methods was assumed, with uniform interval priors, except for base frequencies, which were assigned a Dirichlet prior (Huelsenbeck and Ronquist, 2001). Analyses were conducted using Metropolis coupling with four incrementally heated Markov chains (MC^3; default heating parameter), each chain assigned to an independent processor using the distributed-memory parallelization implemented in MrBayes v3.0 (Altekar et al., in press; compiled on a 16 processor Sun Fire 6800, with 64-bit, 900 MHz UltraSparc III architecture, Sun Microsystems, Santa Clara, CA). Chains were run for 2×10^6 generations, and sampled every 100. The log-likelihood of each chain was evaluated as a function of generation number, in order to determine the minimum number of generations to discard from the beginning of the chain as "burn-in." Posterior estimates of model parameters and taxon bipartitions were derived from the complete sample of chains minus those discarded as burn-in. At least two runs from random starting points were performed for each data set, and the equilibrium log-likelihood, parameter values, and bipartition frequencies of each run were compared to evaluate the stability of posterior estimates (Huelsenbeck et al., 2002; Huelsenbeck and Ronquist, 2001).

2.2.5. Evaluation of congruence

Congruence of phylogenetic estimates from the individual data sets was evaluated both qualitatively and quantitatively. First, the results of separate analysis of each data set were qualitatively compared in terms of topological congruence using the symmetric-difference metric (Robinson and Foulds, 1981). In this way, the analytical conditions and assumptions most favorable to the null hypothesis of homogeneity of phylogenetic signal were identified. The significance of any incongruence which could not be eliminated by varying analytical assumptions was evaluated by bootstrap proportions estimated for the most congruent analyses (Felsenstein, 1985), and by Bayesian posterior probabilities. Significant incongruence was inferred where conflicting nodes were recovered in separate analyses, with support exceeding criterion as estimated by either of these measures. Criterion for bootstrap was set at 75%, in excess of the often-cited 70% level (Hillis and Bull, 1993), and for the Bayesian posterior probabilities at 0.95, as these latter are argued to have a straightforward statistical interpretation (Huelsenbeck and Ronquist, 2001; Larget and Simon, 1999; but see Suzuki et al., 2002).

2.2.6. Combined analysis

Combined analyses were pursued under the analytical conditions which most favored congruence in separate analyses of the two data sets. Prior to combined analysis using model-based approaches, process-parameter homogeneity between the two data sets was evaluated by

maximum-likelihood model-fitting with PAML v3.12, (Yang, 1997). A hierarchy of models enforcing parameter homogeneity and relaxing this constraint were fit, using the "Mgene" option of the "baseml" program. The following parameters were either constrained to homogeneity or allowed to vary between partitions: gene-specific substitution rates (c), base composition (π_i), relative rates of nucleotide substitution classes (r_{ij}), and pattern of among-site rate heterogeneity (discrete approximation to the Γ-distribution, four rate categories, parameter α). PAML does not currently allow estimation of a proportion of invariant sites (p_{iv}), so this parameter was not evaluated. The significance of parameter heterogeneity was evaluated by the χ^2 approximation to the likelihood ratio, as for the model evaluations previously described. The likelihood of each heterogeneous model was compared to that of the more restrictive homogeneous model, for each parameter tested. Given that two data sets differ in evolutionary dynamics, the assumption employed in maximum likelihood analysis that site patterns are identically distributed is inappropriate. Unfortunately, simultaneous maximum likelihood analysis of data sets using multiple models of substitution (heterogeneous-model likelihood), though theoretically feasible (e.g., Yang, 1996), has not been implemented with an efficient search algorithm to date. The closest approximation to such a search which has been implemented is a Bayesian analysis via Markov chain Monte Carlo, allowing significantly heterogeneous parameters to vary independently among partitions (MrBayes v. 3.0; Altekar et al., in press). Bayesian analysis of the combined data was performed as for the individual data sets, freeing significantly different parameters (as evaluated by likelihood) to vary between the two partitions, using the "unlink" and "prset" options.

3. Results

3.1. Sequence characteristics

Sequences of the cytochrome *b* gene were obtained for all taxa (GenBank Accession Nos. AY352520–AY352549; the sequence of *Acanthidops* was obtained from GenBank [AF489878], and the sequences of *Meliphaga* and *Dasyornis* were provided by A.C. Driskell [AY353241, AY353242]), corresponding to positions 14,991–16,035 (1045 bases of 1143) of the *Gallus* mitochondrial genome (Desjardins and Morais, 1990). Base composition of these sequences was fairly typical for mitochondrial DNA of birds in general (Edwards et al., 1991; Hackett, 1996; Kocher et al., 1989; Kornegay et al., 1993; Nunn and Cracraft, 1996), showing highest skew at third positions, with successively less skew at second and first positions respectively (Table 3). Homogeneity across taxa was not rejected for cytochrome *b* first and second positions (Table 3). However, the χ^2 test for third positions indicated significant heterogeneity across all taxa, though heterogeneity was not significant within the Troglodytidae alone ($\chi^2 = 57.11$, df = 54, $p = 0.36$). Sequence-specific goodness-of-fit tests for third position base frequencies indicated that only sequences of *Pipra*, *Dasyornis*, and *Dumetella* deviated significantly from the overall mean values ($\chi^2 = 101.5$, 21.0, 18.6, respectively, df = 3, $p < \alpha = 0.002$ with correction for multiple comparisons). Patterns of variation within cytochrome *b* also corresponded to those previously noted for the gene in other birds. Of codon third positions, 97.4% were variable, with 91.8% of variable positions potentially informative under the parsimony criterion. For first and second positions, 31.3 and 12.6% were variable, respectively (with 75.2 and 56.8% of variable sites potentially informative). Uncorrected pairwise distances for cytochrome *b* ranged from 6 to 20% divergence within the ingroup, and up to 25% in comparisons to *Pipra*.

Sequence of the fourth intron of the β-fibrinogen gene was obtained for all taxa (GenBank Accession Nos. AY352550–AY352580; sequences of *Meliphaga* and *Dasyornis* were provided by A.C. Driskell [AY353243 and AY353244]). Clonal diversity for those taxa for which multiple clones were sequenced was minimal (four positions varied between clones, out of 2446 positions sequenced for multiple clones: 0.16%), though one case of apparent heterozygosity for a single base pair insertion–deletion event was detected (position 160 of the *Sturnus* sequence). The region of this indel event was eliminated from phylogenetic analysis (see below). Base composition of the intron was relatively constant across taxa, and skewed toward adenine and thymine residues (Table 3), suggesting placement in an AT-rich isochore

Table 3
Average nucleotide composition of cytochrome *b* and β-fibrinogen intron 4

Partition	A	C	G	T	χ^2
Cytochrome *b*-1st position	0.235	0.299	0.243	0.223	14.66
Cytochrome *b*-2nd position	0.195	0.262	0.130	0.413	2.60
Cytochrome *b*-3rd position	0.377	0.474	0.044	0.105	273.62*
β-Fibrinogen Intron 4	0.312	0.169	0.177	0.342	14.07

Base proportions are shown for each coding position of cytochrome *b*. The χ^2 values refer to the two-way test of independence implemented in PAUP* (Swofford, 1998), with df = 96 ($p < 0.01$ indicated by an asterisk).

(Aota and Ikemura, 1986; Ikemura and Aota, 1988). Sequences varied in length between 444 bp (*Prinia*) and 609 bp (*Pipra* and *Dasyornis*), with a median of 590 bp. Alignment of these sequences was fairly straightforward, yielding few regions of significant ambiguity (only one region, including bases 153–165, was excluded from analysis). An exception was the sequence obtained for *Prinia*, which was significantly shorter than those of other taxa sampled, and which additionally included a stretch of bases with questionable homology to other sequences in the alignment (bases 250–460 of the alignment). This region of the sequence was treated as missing data for *Prinia*. The final alignment indicated the presence of 17 regions with potentially informative insertion–deletion events. Of these 17 regions, 13 exhibited characteristics (see Section 2) which justified coding and analysis as separate characters (see Appendix A). Levels of variation in this alignment of the intron were relatively high in percentage of positions variable (59.6%), but most variability was due to singleton substitutions, as only 39.6% of all variable sites were potentially informative under the parsimony criterion. Uncorrected sequence divergence among taxa for intron 4 ranged from 0.4 to 13.5% in the ingroup, and up to 17.9% in comparisons to *Pipra*.

3.2. Evaluation of congruence between cytochrome b and intron 4

Topological comparisons between the trees derived from separate phylogenetic analyses of the complete cytochrome *b* and β-fibrinogen intron 4 data sets are summarized in Fig. 1. The minimum value possible for the Robinson–Foulds symmetric difference metric is zero (where no bipartitions differ between trees), while the maximum is twice the number of bipartitions in a fully-resolved unrooted tree ($2n-6$, which is in this case 60; when all bipartitions in the two trees conflict). In general, three types of analysis of the cytochrome *b* data yielded topologies most similar to those found in analyses of the intron data: parsimony analysis with exclusion of third position transitions, combinatorial weights, and maximum likelihood (Fig. 1). These treatments of the cytochrome *b* data yielded topologies which had mean differences from intron 4 topologies on average 8.2 difference units better than comparisons of trees from equally weighted parsimony, parsimony excluding third position data, or parsimony analysis of inferred amino acid sequences. Addition of gap information to parsimony analysis of the intron 4 data did not appreciably affect congruence with cytochrome *b* trees. However, maximum likelihood analysis of the intron 4 data did slightly increase congruence, and the maximum congruence between independent phylogenetic estimates from the two data sets was obtained when maximum likelihood was used as the analysis criterion for both. For this reason, only the results of likelihood-based analyses (maximum likelihood and Bayesian) are reported here in detail, and the quantitative evaluation of congruence is based on these.

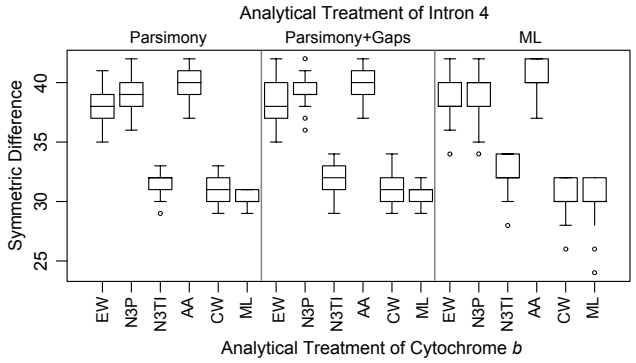

Fig. 1. Comparison of topologies from separate analyses of cytochrome *b* and intron 4 data. Labels on the abscissa indicate treatment of the cytochrome *b* data (EW, equally weighted parsimony, N3P, parsimony with elimination of third positions, N3TI, parsimony with elimination of third position transitions, AA, parsimony analysis of amino acid translations, CW, parsimony with combinatorial weights, and ML, maximum likelihood), and the three sections of the plot area labelled above indicate treatments of the intron 4 data (parsimony both without and with informative gaps coded as separate characters, ML, maximum likelihood). The boxplots summarize pairwise symmetric differences between all unique equally parsimonious or likely trees obtained for the two data sets with each combination of analytical approaches.

Likelihood model evaluations of the two data sets indicated substantially different evolutionary dynamics for the two gene regions. Analysis of the cytochrome *b* data yielded the GTR + I + Γ model as the best fit (versus GTR + I, $-2\ln\Lambda = 659$, df = 1; GTR + Γ, $-2\ln\Lambda = 89$, df = 1; HKY85 + I + Γ, $-2\ln\Lambda = 842$, df = 4; all $p \ll 0.01$). Additionally, the molecular clock could be rejected for these data, though by a much narrower margin ($-2\ln\Lambda = 50$, df = 31, $p = 0.02$). The best-fit model for the intron 4 data was GTR + Γ (versus HKY + Γ, $-2\ln\Lambda = 29$, df = 4, $p \ll 0.01$; the optimization algorithm of PAUP* would not assign a non-zero p_{iv} when fitting the GTR + I + Γ model). The GTR + I model yielded a slightly lower likelihood than GTR + Γ ($\Delta\ln[\lambda] = 6.2$): this is probably not a significant difference though this cannot be readily evaluated, as the two models are not nested. For these data, the molecular clock could not be rejected ($-2\ln\Lambda = 41$, df = 31, $p = 0.11$).

A heuristic search for the cytochrome *b* data under the GTR + I + Γ model (with initial estimates fixed) identified three equally likely trees, the strict consensus of which lacked resolution at one node (Table 4, Fig. 2A). Bootstrap analysis indicated that most recovered nodes had very little support, as only 16 of the 29 retained taxon bipartitions were recovered in more than 50% of bootstrap replicates, and only 11 in more than 75% (the criterion value chosen for this study, Fig. 2A).

Table 4
Summary of model parameters and tree scores for maximum likelihood and Bayesian analyses of cytochrome *b* and β-fibrinogen intron 4 for all taxa (r_{1J} refer to parameters of the GTR model of substitution, α is the parameter of the Γ-distribution for rate heterogeneity, and p_{iv} is the estimated proportion of invariant sites)

	Maximum likelihood		Bayesian-separate[a]		Bayesian-combined[b]	
	Cytochrome *b*	Intron 4	Cytochrome *b*	Intron 4	Cytochrome *b*	Intron 4
# Trees (# Nodes[c])	3 (29)	405 (25)	NA	NA	NA	NA
$-\ln(L)$	11877.5	3944.1	11879.3 (10.4)	3987.5 (7.1)	15904.9 (10.1)	←joint estimate
Length	4.078	1.021	25.8 (3.6)	1.16 (0.06)	14.6 · 1.59 (0.01)	14.6 · 0.07 (0.01)
r_{AC}	2.697	1.634	0.106 (0.05)	2.053 (0.49)	0.081 (0.04)	2.122 (0.49)
r_{AG}	8.983	4.819	10.731 (3.89)	5.950 (1.17)	8.263 (2.55)	6.004 (1.19)
r_{AT}	1.413	0.689	0.725 (0.31)	0.868 (0.21)	0.613 (0.23)	0.857 (0.20)
r_{CG}	0.372	1.918	0.267 (0.16)	2.219 (0.56)	0.212 (0.12)	2.189 (0.55)
r_{CT}	9.117	4.021	9.424 (3.42)	4.891 (1.01)	7.601 (2.23)	4.880 (0.96)
α	1.004	3.174	0.300 (0.03)	3.885 (1.83)	0.308 (0.03)	9.456 (8.64)
p_{iv}	0.481	NA	0.381 (0.02)	NA	0.384 (0.03)	0.071 (0.05)

[a] Means across retained Markov chain samples ($n_{cytb} = 19,400$ generations, $n_{I4} = 19,700$), standard deviations in parentheses.
[b] As for a, $n_{combined} = 19,900$ generations.
[c] Number of nodes retained in strict consensus.

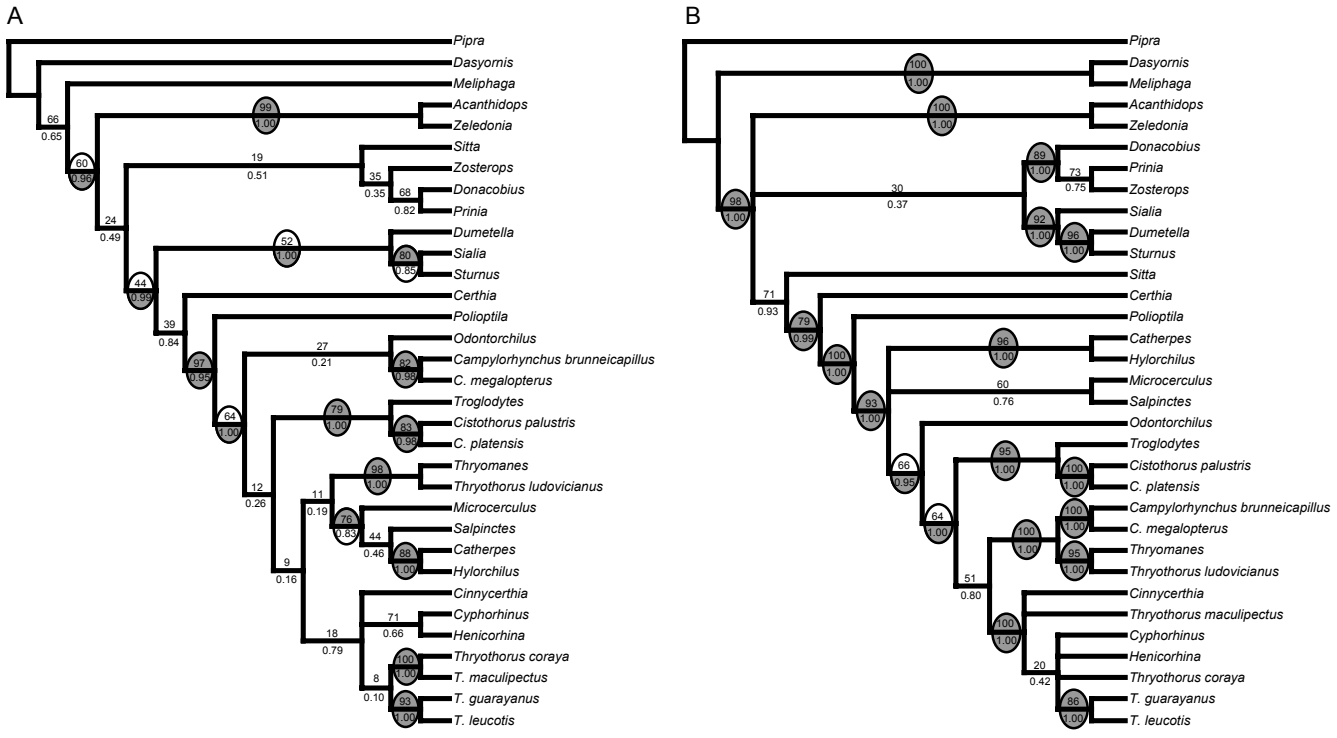

Fig. 2. Strict consensus of maximum likelihood trees obtained from separate analyses of cytochrome *b* (A) and β-fibrinogen intron 4 (B). Numbers of trees, parameter estimates, and likelihoods are reported in Table 4. Numbers above each branch are the percentage of bootstrap replicates ($n = 100$) in which that branch was recovered, and numbers below each branch are estimated Bayesian posterior probabilities (19, 400, and 19,700 sampled trees for each data set, respectively). Branches with either bootstrap percentages $\geqslant 75$ or estimated Bayesian posteriors $\geqslant 0.95$ are highlighted with ellipses, and the values exceeding these criteria shaded.

The majority rule consensus of trees sampled in the Bayesian analysis of the cytochrome *b* data was similar to the maximum likelihood trees recovered, and the estimated posterior nodal probabilities yielded similar conclusions. Searches enforcing the molecular clock for the intron 4 data were not feasible as many divergences were closely spaced in this data set, and search times under the clock constraint increased to unrealistic lengths. Heuristic searches using the preferred model (without a clock, and with initial parameters fixed) yielded 405 equally likely trees, the consensus of which yielded irresolution at four nodes (Table 4, Fig. 2B). The

strict consensus was surprisingly well-resolved (Fig. 2B, 25/30 possible nodes retained), given the large number of trees obtained. Levels of bootstrap support for nodes on the strict consensus was fairly high, with 23/25 retained nodes found in $\geqslant$50% of bootstrap replicates, and 17/25 found in $\geqslant$75% (Fig. 2B). As for the cytochrome *b* data, the consensus of trees obtained from Bayesian analysis of the intron data was similar to the consensus of maximum likelihood trees, and the posterior probabilities obtained yielded essentially identical conclusions.

These analyses yielded only one case where significant conflict was indicated. Both data sets recovered a group containing the genera *Sialia*, *Dumetella*, and *Sturnus*. However, within this group, the cytochrome *b* data consistently grouped *Sialia* and *Sturnus* (80% of bootstrap replicates), while the intron data yielded strong evidence for grouping of *Dumetella* and *Sturnus* (96%). Bayesian analyses of the same data failed to recover significantly conflicting hypotheses of relationship, as the estimated posterior probability of the branch uniting *Sialia* and *Sturnus* was only 0.85 for the cytochrome *b* data (Fig. 2A). Conversely, both data sets supported critical relationships, including monophyly of the traditional Troglodytidae (i.e., exclusive of *Donacobius*; 64 and 93% of bootstrap replicates for cytochrome *b* and intron 4 respectively, estimated posterior probabilities of 1.00 for both data sets), and a sister-group relationship between the Troglodytidae and *Polioptila* (97 and 100%, 0.95 and 1.00). The presence of only a single point of significant conflict, the ambiguity of its significance, and its irrelevance to the primary phylogenetic hypotheses being tested (see Section 2), suggested that combined analysis of the two partitions was warranted.

3.3. Testing homogeneity of substitution process

Given that maximum likelihood analysis of the two data sets maximized congruence between them, analysis of the combined data under maximum likelihood was preferred. Prior to this analysis, the validity of the assumption of evolutionary process homogeneity was evaluated. Under the maximum likelihood criterion, the significance of among-gene heterogeneity in best-fit models of nucleotide substitution was estimated (Tables 5 and 6). Likelihood ratio tests indicated that the two data sets differed significantly in their best-fitting substitution models and parameter values (Table 6), rejecting homogeneity between the two data sets in every parameter examined. Because of the high values of these statistics, their significance is not affected by correction for multiple comparisons (28 comparisons suggests an $\alpha = 0.002$, and all values reported here have $p < 0.001$). The extremely high significance values for these tests prompted evaluation of the validity of the use of the χ^2 approximation to the null distribution for $-2\ln\Lambda$. For the simplest case of each model parameter being tested for homogeneity (highlighted in bold in Table 6), the null distribution of the test statistic was evaluated by simulating the data under the simplified model (100 simulated data sets per model comparison generated with Seq-Gen v1.1 [Rambaut and Grassly, 1997], using parameter values estimated by the baseml program of PAML v3.12), and calculating the likelihood of the simulated data sets under the null (homogeneous; the condition of the generating model) and alternative (heterogeneous) hypotheses. In all cases except that of the $2 + 1\Gamma$ versus 1 comparison, the Monte Carlo-generated null distribution matched the χ^2 approximation extremely well, as evaluated qualitatively by quantile–quantile plots

Table 5
Likelihoods of heterogeneous model evaluations using PAML (π_i are the base frequency parameters, $\boldsymbol{R}$ is the substitution rate matrix, α is the parameter of the Γ-distribution of rates)

Model #	π_i	$\boldsymbol{R}$	α	Branch lengths	Molecular clock	$-\ln(\lambda)$	G[a]	Mgene[a]	Malpha[a]
1	Equal	Equal	Equal	Equal	No	16261.6	No	0	0
$2+1\Gamma$	Equal	Equal	Equal	Proportional	No	16214.9	Yes	0	0
$2+2\Gamma$	Equal	Equal	Unequal	Proportional	No	16128.8	Yes	0	1
$3+1\Gamma$	Unequal	Equal	Equal	Proportional	No	16151.2	Yes	2	0
$3+2\Gamma$	Unequal	Equal	Unequal	Proportional	No	16053.7	Yes	2	1
$4+1\Gamma$	Equal	Unequal	Equal	Proportional	No	16174.8	Yes	3	0
$4+2\Gamma$	Equal	Unequal	Unequal	Proportional	No	16087.5	Yes	3	1
$5+1\Gamma$	Unequal	Unequal	Equal	Proportional	No	16098.1	Yes	4	0
$5+2\Gamma$	Unequal	Unequal	Unequal	Proportional	No	16018.3	Yes	4	1
6-NC	Unequal	Unequal	Unequal	Non-proportional	No	15934.4	Yes	1	1
6-C	Unequal	Unequal	Unequal	Non-proportional	Yes	15982.7	Yes	1	1

Under the headings π_i, $\boldsymbol{R}$, and α, an entry of "equal" indicates that the corresponding parameter was assumed uniform across partitions, and an entry of "unequal" indicates that the parameter was estimated separately for each partition. Under the heading of branch lengths, "equal" indicates that a single rate was assumed for the two partitions, "proportional" that a constant describing relative branch lengths for the two partitions (c) was estimated, and "non-proportional" that all branch lengths were optimized independently for the two partitions.

[a] These headings indicate settings used in analyses with PAML. Inclusion of the G statement with the input data matrix allows recognition of multiple process partitions. The Mgene parameter indicates the non-rate-heterogeneity parameters to be allowed to vary between partitions, and Malpha indicates when patterns of among-site rate variation are allowed to vary.

Table 6
Likelihood ratio tests of model parameter heterogeneity between cytochrome *b* and β-fibrinogen intron 4 (parameters and model numbers as in Table 5, $-2\ln\Lambda$ = likelihood ratio statistic, df = degrees of freedom for χ^2 approximation)

Hypothesis tested	General model	Restricted model	$-2\ln\Lambda$	df
Homogeneous rate distribution (α)	**2 + 2Γ**	**2 + 1Γ**	172.1	1
	3 + 2Γ	3 + 1Γ	195.1	1
	4 + 2Γ	4 + 1Γ	174.6	1
	5 + 2Γ	5 + 1Γ	159.5	1
Homogeneous base composition (π_i)	**3 + 1Γ**	**2 + 1Γ**	127.3	3
	3 + 2Γ	2 + 2Γ	150.2	3
	5 + 1Γ	4 + 1Γ	153.3	3
	5 + 2Γ	4 + 2Γ	138.3	3
Homogeneous rate matrix (***R***)	**4 + 1Γ**	**2 + 1Γ**	80.2	5
	4 + 2Γ	2 + 2Γ	82.7	5
	5 + 1Γ	3 + 1Γ	106.2	5
	5 + 2Γ	3 + 2Γ	70.7	5
Equal branch lengths	**2 + 1Γ**	**1**	93.5	1
Proportional branch lengths	**6-NC**	**5 + 2Γ**	167.9	36
Molecular clock	6-NC	6-C	96.6	62

All values are significant at $\alpha \leqslant 0.05$, correcting for multiple comparisons. The χ^2 approximation to the null distribution of $-2\ln\Lambda$ for model comparisons highlighted in bold was validated via Monte Carlo simulation (see Section 3).

(results not shown). In the exceptional case, the Monte Carlo-generated null distribution did not appear to follow a χ^2 distribution with 1 degree of freedom. Regardless of this discrepancy, the high value of the statistic obtained exceeded 100% of the empirically derived null values, indicating heterogeneity in average substitution rate between the two partitions.

3.4. Combined analysis

Subsequent to model evaluation, the cytochrome *b* and intron 4 data were analyzed simultaneously using Bayesian methods, allowing the partitions to vary independently in their substitution parameter estimates. As optimal models varied between the partitions, the more general of the two parameterizations was used (GTR + I + Γ, without a molecular clock), and all parameters were allowed to vary between the data sets. Although the parameter analysis above indicated that independent branch lengths fit the data significantly better than proportional branch lengths, only the latter is implemented in MrBayes v3.0, and this option was used. The majority rule consensus of 19,800 trees sampled from the Markov chains at stationarity is presented in Fig. 3, and the parameters obtained in these analyses are summarized in Table 4. The consensus tree obtained was extremely similar to those obtained in maximum likelihood and Bayesian analysis of the intron 4 data alone. The traditional Troglodytidae was recovered. The genus *Donacobius* was excluded from the family, and strongly supported as part of a group including *Zosterops* and *Prinia*. *Polioptila* and *Certhia* were strongly supported as successive sister-groups to the wrens, with weak support for *Sitta* as sister to all the former. Within wrens, all genera except *Thryothorus* were supported as monophyletic, and four strongly supported intergeneric groups were recovered. The first of these comprised four of five *Thryothorus*, *Henicorhina*, *Cinnycerthia*, and *Cyphorhinus*. The second comprised the genus *Campylorhynchus*, along with its sister-group of *Thryomanes* and *Thryothorus ludovicianus*. The third comprised the genera *Cistothorus* and *Troglodytes*. Finally, the genera *Catherpes* and *Hylorchilus* were joined with high posterior probability. Additional nodes within the wrens received only weak support.

3.5. Additional analyses of the Troglodytidae and Polioptila

Most of the lack of resolution in relationships among the taxa sampled here could be isolated to three portions of the tree. First, basal relationships among distantly related passeridan groups (e.g., wrens and allies, muscicapoids, sylvioids, and passeroids) were ambiguously resolved. This is perhaps unsurprising given the lack of dense taxon sampling at this level of comparison. Among closely related taxa, the clade containing four *Thryothorus*, *Cinnycerthia*, *Cyphorhinus*, and *Henicorhina* was unresolved, probably due to insufficient variation to resolve closely-spaced divergences. The final major region of irresolution was among basal lineages within the Troglodytidae (especially *Salpinctes*, *Microcerculus*, *Hylorchilus*/*Catherpes*, and *Odontorchilus*). This lack of resolution could be due to a lack of sufficient variation to resolve these basal relationships. Alternatively, the distant, species-poor sister-group to the wrens (*Polioptila*) could be problematic (Wheeler, 1990b; Smith, 1994). For this reason, phylogenetic

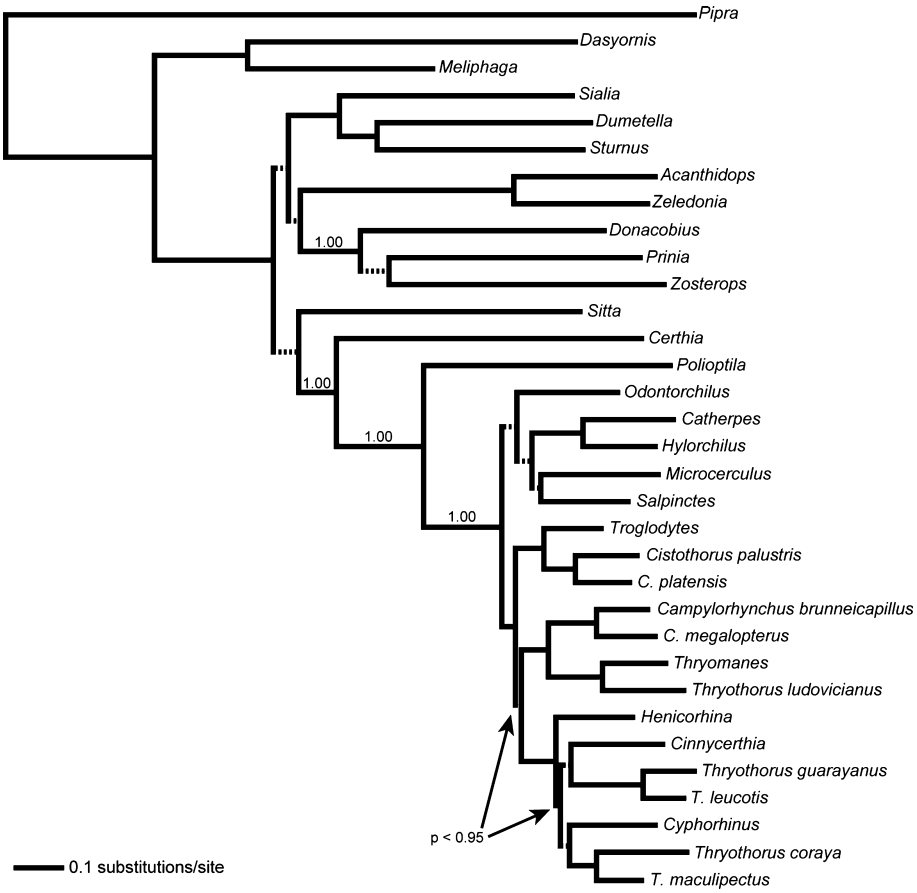

Fig. 3. Results of heterogeneous-model Bayesian analysis of cytochrome *b* and β-fibrinogen intron 4 for the complete data set. Shown is the 50% majority rule consensus of 19,900 trees sampled from a single run of MC^3, with additional compatible nodes present in fewer than 50% of samples retained. Branch lengths are proportional to the expected number of substitutions per site, estimated as the mean value of the branch in all samples of the Markov chain where the branch appeared. All branches had estimated posterior probabilities $\geqslant 0.95$, excepting those highlighted by dashing: selected values relevant to hypotheses being tested are shown.

analyses were repeated using pruned data sets containing wrens plus their sister-group (as indicated by the complete data set and both genes) and wrens alone.

The combined Bayesian analysis was repeated for the two data sets, including the traditional Troglodytidae (exclusive of *Donacobius*), both with and without the addition of *Polioptila* as the only outgroup (Fig. 4). The nodal posterior values clearly indicate that placement of the outgroup is responsible for a great deal of the basal irresolution observed in analysis of the complete data set. Specifically, posterior probabilities for the analysis of wrens alone exceed $1 - \alpha = 0.95$ at a number of nodes which were not reliably resolved in analysis of the complete taxon sample. Revealingly, posterior probabilities for placement of the outgroup *Polioptila* on this unrooted ingroup tree indicate substantial ambiguity (Fig. 4). The majority of Markov chain samples which had the ingroup topology shown in Fig. 4 placed *Polioptila* on the branch between *Cistothorus*/*Troglodytes* and the remaining wrens. However, substantial numbers of sampled trees placed the root on internodes among *Catherpes*/*Hylorchilus*, *Microcerculus*, *Salpinctes*, and *Odontorchilus* (Fig. 4). Thus, it seems likely that the weak support for basal wren relationships in analysis of the complete data set is attributable to ambiguous rooting of a relatively robust ingroup network. In fact, pruning of *Polioptila* from trees obtained in Bayesian analysis of wrens plus this outgroup yielded nodal posterior probabilities essentially identical to those obtained from analysis of wrens alone. Analysis of base substitution data alone seems insufficient to root the network of wren relationships conclusively.

3.6. Indels in β-fibrinogen intron 4 and provisional rooting of wren phylogeny

Alignment of the intron sequences obtained required a number of insertion/deletion events which appear phylogenetically informative (indels showed ensemble $CI = 0.74$ and $RI = 0.89$ in equally-weighted parsimony analysis of intron 4 alone), although inclusion of coded indels in phylogenetic analysis did not improve resolution or support (results not shown). Since taxon sampling was focused on the wrens, it is unsurprising that the

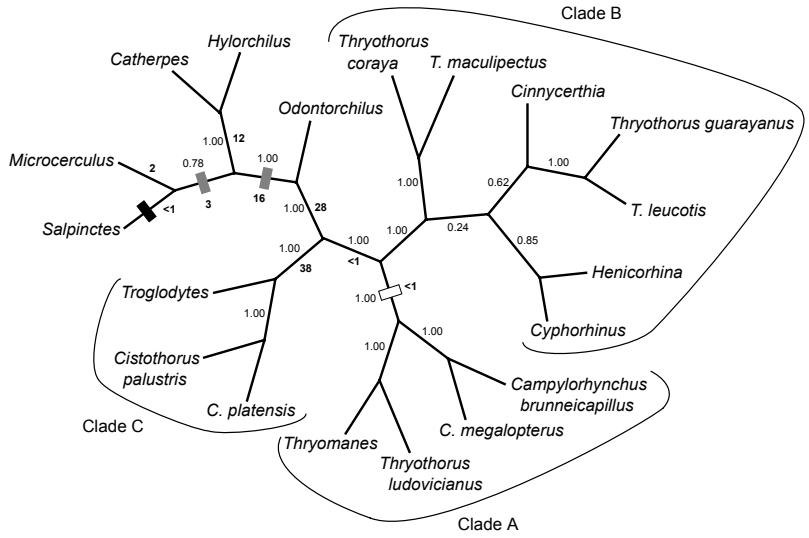

Fig. 4. Results of heterogeneous-model Bayesian analysis of cytochrome *b* and β-fibrinogen intron 4 from the Troglodytidae alone. Decimal values near each branch indicate estimated posterior probabilities (19,800 sampled generations). Integer values in bold indicate the percentage of generations where the root fell on the associated branch of this unrooted tree, in a Bayesian analysis including the single outgroup *Polioptila* (19,900 sampled generations). The single black hash mark indicates the optimal placement of the indel in region 6 of the intron 4 alignment, the white hash mark indicates optimization of the indel in region 16, and the two gray hash marks indicate two alternative optimizations of the indel in region 12 (see Appendix). Clades A–C are defined for discussion in the text.

majority of these informative indel events occur within the group. In particular, three taxon partitions presented in Fig. 4 were supported by indel events in intron 4 (Appendix A). Towards the tips of the tree, the placement of the (*Thryomanes*, *Thryothorus ludovicianus*) clade as sister-group to *Campylorhynchus*, was supported by a single base pair deletion (Fig. 4; Appendix A, region 16). Two other indel events within wrens provide potentially critical information regarding the most appropriate rooting of wren ingroup relationships. First, *Salpinctes* is separated from all other wrens by a single unambiguous indel event (Fig. 4; Appendix A, region 6). Based on outgroup comparison, this is clearly an insertion which occurred in the common ancestor of all wrens excepting *Salpinctes*, suggesting that this species may be the sister group to all other wrens. However, a second partially overlapping deletion in this same region of intron sequence from *Thryothorus coraya* suggests that *Salpinctes* having reversed this synapomorphic indel cannot be discounted entirely. A second, less clearly interpretable indel event in region 12 of the alignment (Fig. 4, Appendix A) separates either (*Salpinctes*, *Microcerculus*) or (*Salpinctes*, *Microcerculus*, *Catherpes*, *Hylorchilus*) from all other wrens. Based on outgroup comparison, this region appears to have experienced a six base pair deletion in the common ancestor of oscines (all sampled taxa except *Pipra*), followed by an overlapping three base pair deletion in all wrens excepting *Salpinctes*, *Microcerculus*, and possibly *Catherpes* and *Hylorchilus*. The ambiguity in reconstruction of this latter indel is caused by an overlapping 15 bp deletion event in the lineage leading to *Catherpes* and *Hylorchilus*. Parsimony-based reconstruction of indel evolution in both of these latter two regions of the intron suggest that basal relationships of wrens involve divergences among *Salpinctes*, *Microcerculus*, *Catherpes*/*Hylorchilus*, and the remaining wren genera. This result is consistent with the unrooted consensus ingroup tree obtained in Bayesian analysis, and with the distribution of optimal outgroup placement on this tree (see above).

4. Discussion

4.1. Monophyly of the traditional Troglodytidae

Based upon DNA hybridization results (Sibley and Ahlquist, 1984), and an unpublished study of pterylosis by Clench et al. (1982; cited in Kiltie and Fitzpatrick, 1984, Olson in Wetmore et al., 1984, p. 55), Sibley and Ahlquist (1990) suggested placement of the monotypic genus *Donacobius* within the Troglodytidae. This conclusion was adopted by the American Ornithologists' Union in its list of North American Birds (1983 and subsequent). *Donacobius atricapillus* is a widely distributed South American marsh-nesting bird which cooperatively breeds (as do some wrens) and performs vocal duets (as do most wrens). Traditionally, the genus has been associated with the Mimidae (e.g., Mayr and Greenway, 1960), though often noted as an aberrant member of the family (e.g., Ridgway, 1907). Though the species does exhibit some behavioral characteristics found in wrens, in others it is quite distinctive (e.g., it constructs a cup nest, whereas wrens typically construct

globular or pouch-like nests). Since the data on pterylosis are unpublished, they are impossible to evaluate relative to other evidence. Additionally, the genetic evidence cited (a list of hybridization distances; Sibley and Ahlquist, 1984) does not actually indicate a relationship between *Donacobius* and the wrens, rather demonstrating that *Donacobius* is just as genetically distant from the mimic-thrushes as it is from the wrens. Thus, the decisions by Sibley and Monroe (1990), and the A.O.U. Check-list committee (A.O.U., 1983) to refer the genus to the family Troglodytidae, are based upon evidence that is either unavailable for evaluation or equivocal in interpretation.

The analyses presented here, of both data sets separately and in combination, strongly support monophyly of the traditional Troglodytidae. Specifically, the genus *Donacobius* is excluded from membership within the family, and cannot even be parsimoniously (or probabilistically) placed as its sister-group. All analyses unequivocally place *Polioptila* as the sister-group to the wrens, to the exclusion of *Donacobius*, strongly contradicting the conclusions of Clench et al. (1982), as well as current taxonomies based on their result (A.O.U., 1998; Sibley and Monroe, 1990). Also of note is that *Donacobius* is consistently excluded from relationship with the traditional family Mimidae (Sibley and Ahlquist, 1990, subfamily Miminae), the other group to which the genus has been commonly assigned. The affinities of *Donacobius* appear to lie with the Old World sylvioid passerine radiation, as it consistently clusters with *Zosterops* and *Prinia* in analyses presented here. However, because of sparse taxon sampling, no particular hypothesis of relationship can be postulated for the genus based upon this result.

Beyond the *Polioptila*/wren relationship, both the cytochrome *b* and intron 4 data support monophyly of Sibley and Ahlquist's (1990) Certhiidae (as modified by exclusion of *Donacobius*), in that *Certhia* is found as the sister-group to the *Polioptila*/wren clade, though support for this relationship was only strong in analyses of intron 4 or the combined data (Figs. 1 and 3). No significant support was found for Sibley and Ahlquist's proposed grouping of *Sitta* with this broad conception of the Certhiidae, though this relationship appeared in the maximum likelihood analysis of the intron data (Fig. 2B; 71% bootstrap), and in the majority rule consensus of trees obtained in the Bayesian analyses of both the intron and combined data sets (0.93 and 0.87 posterior probabilities; Figs. 2B and 3). However, this latter relationship is well-supported by other nuclear sequence data (Barker et al., 2002).

4.2. Relationships among wrens

The pattern of relationships among genera within the Troglodytidae presented here is the first proposed for this family, beyond linear taxonomic series. Interpretation of this pattern is somewhat complicated by ambiguity of the placement of the root node on the wren ingroup network, but a number of phylogenetic conclusions can be drawn regardless of the precise rooting. Notably, the relationships proposed here conflict sharply with previous conceptions regarding the sequence from "primitive" to "derived" taxa within the family. Traditional taxonomic series generally begin with the genus *Campylorhynchus*, which has been placed in its own subfamily (e.g., Baird, 1858). However, this genus is clearly nested well within the wrens (Figs. 3 and 4). An assemblage of genera which tend toward a terrestrial behavioral and morphological habit (*Thryorchilus*, *Henicorhina*, *Microcerculus*, and *Cyphorhinus*) is typically listed at the end of the family. This assemblage is clearly polyphyletic, with *Thryorchilus* belonging to Clade C (ff. Rice et al., 1999), *Henicorhina* and *Cyphorhinus* as sister-taxa within Clade B, and *Microcerculus* as part of a probable basal radiation of the family (see Fig. 4 for group definitions).

The single species of *Catherpes*, the canyon wren (*C. mexicanus*) has been considered a close relative of the similarly petrophilous *S. obsoletus*, and even placed in the latter genus (e.g., Mayr and Greenway, 1960). This notion is clearly falsified by the analyses presented here, as *Catherpes* and *Hyorchilus* are sister taxa. A close relationship between *Catherpes* and *Hylorchilus* was previously suggested based on similarity of the songs of *C. mexicanus* and *H. sumichrasti* (Hardy and Delaney, 1987). The recently discovered differentiation of *H. navai* songs from the former two species (Atkinson et al., 1993; Gómez de Silva, 1997), probably represents a case of secondary modification. In itself, this relationship does not argue against placing all three genera, which together comprise four petrophilous species, within a single genus. However, data on indel events from the intron indicate that *Salpinctes* may actually represent the sister-group to all other wrens (see above). Until additional data are obtained, retaining all three genera seems warranted.

A more controversial hypothesis of relationship proposed by these data is the placement of *Thryothorus ludovicianus* and *Thryomanes bewickii* as sister taxa to the genus *Campylorhynchus*. Finding paraphyly of *Thryothorus* per se would not be particularly surprising. For instance, finding the genus *Thryomanes* (or *Henicorhina*, etc.) nested within *Thryothorus*, given the behavioral and morphological similarities of these genera, would not profoundly disturb traditional notions of relationship. However, the placement of *Thryothorus ludovicianus* as separate from other members of the genus, yielding polyphyly of *Thryothorus*, merits some careful consideration. Unfortunately, evidence for this relationship is from the intron data alone. Comparison of the cytochrome *b* maximum likelihood trees with the best trees found under the constraint of *Thryothorus* (or *Thryothorus* + *Thryomanes*) monophyly with the Shi-

modaira–Hasegawa test (Shimodaira and Hasegawa, 1999) failed to reject this alternative (1000 bootstrap RELL replications, $\delta = 6.47$ $p = 0.18$), whereas the same test with the intron 4 data is highly significant ($\delta = 64.00$, $p < 0.01$). One immediate objection which might be raised is the quality or nature of these sequence data; however, two individuals of *Thryothorus ludovicianus albinucha* from the Yucatán yielded intron sequences identical to that presented here (including the unique deletion event which unites them with *Thryomanes* and *Campylorhynchus* [Fig. 4; Appendix A, indel 16], unpublished data). Extensive hybridization between the common ancestor of *Campylorhynchus* and that of *T. ludovicianus* and *Thryomanes* could provide an alternative explanation for this pattern, though hybridization among wren genera has not been reported: this explanation seems unlikely at best. Only evidence from additional genetic loci (preferably nuclear) will resolve the issue definitively.

Future work on relationships of wrens will be necessary in order to resolve a number of issues. Most critically, resolution of basal relationships of wrens will require collection of additional sequence data and possibly more dense taxon sampling. Additionally, acquiring nuclear data from additional outgroups (specifically the gnatwrens *Ramphocaenus* and *Microbates*) might help stabilize placement of the root on the wren ingroup network. Resolution of these relationships will allow biogeographic analysis of the family, potentially resolving the question of the continental origin of wrens (Mayr, 1946). This analysis would also benefit greatly from sampling of the remaining genera of wrens, the Central American *Uropsila* and *Thryorchilus*, and (especially) the Cuban *Ferminia*. Finally, additional sampling from the taxonomically diverse genus *Thryothorus* and its close relatives may reveal additional evidence for paraphyly of the genus, and help resolve the history of morphological and behavioral diversification within the group.

4.3. Conflict between data partitions and combined analysis

Substantial incongruence was observed between estimates of relationship derived from analyses of the cytochrome *b* and intron 4 data (Fig. 1). This observed incongruence appears to have a number of sources. Comparison of trees derived from various parsimony weighting schemes and maximum likelihood analysis strongly suggests that one significant factor contributing to apparent incongruence is the contrasting evolutionary dynamics of the two loci. Cytochrome *b* and intron 4 differ substantially in their rates and patterns of sequence evolution (Tables 4–6). In particular, cytochrome *b* does not evolve in a clock-like fashion (see Section 3), accrues substitutions at a much higher rate than intron 4, and has a very different pattern of among-site rate heterogeneity (Table 4). When analyses which take some of these differing dynamics into account are employed (parsimony weighting and likelihood), observed incongruence between the loci is reduced (Fig. 1). As measured by bootstrap values and Bayesian posterior probabilities, the most congruent of these analyses (likelihood-based) indicates significant conflict at only one node (Fig. 2). This conflict, rather than due to conflicting history of the genes sampled, is very likely attributable to among-taxon heterogeneity in the dynamics of sequence evolution (as measured by base composition, see Section 3).

Likelihood evaluation of the optimal model for use in analysis of the two data sets suggests that they differ significantly in every parameter tested (Table 6). Demonstrably, the assumption of identically distributed site pattern probabilities can be rejected for these data, and homogeneous-model likelihood analysis is unwarranted. Previously, some authors have attempted to evaluate a limited number of hypotheses using heterogeneous-model maximum likelihood, with parameters independently estimated for each partition (Caterino et al., 2001; DeBry, 1999; Wilgenbusch and de Quieroz, 2000). Additionally, limited searches using heterogeneous models are possible in some available software (NNI branch swapping and star decomposition in PAML, Yang, 1997). These approaches are unsatisfying in that they provide little assurance that global optima have been obtained, and more importantly in that estimating branch support using resampling (i.e., the bootstrap) is intractable. Efficient search options with heterogeneous likelihood models will become available in the future (subsequent versions of PAUP*; Wilgenbusch and de Quieroz, 2000). Heterogeneous-model Bayesian analysis with uniform interval priors provides a reasonable and efficient alternative approach to such problems, and offers the additional advantages associated with Bayesian methods (Huelsenbeck et al., 2002). Methods for addressing among-taxon heterogeneity in evolutionary process have also been developed, but have yet to be implemented with efficient search algorithms (Galtier and Gouy, 1998; Yang and Roberts, 1995). As among-taxon compositional heterogeneity in this data set was limited, these approaches were not pursued. Bayesian analysis of other types of among-taxon process heterogeneity has proven useful (e.g., the rate of sequence evolution; Huelsenbeck et al., 2000; Kishino et al., 2001; Thorne et al., 1998), and may be a fruitful approach to addressing the problem of base composition as well (Huelsenbeck et al., 2002).

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Appendix A

Location of potentially informative gapped regions in the alignment of β-fibrinogen intron 4 sequences (EMBL Accession ALIGN_000588), and coding of indel data. All regions were coded separately as phylogenetic characters, except regions in italics which were included in the matrix but uncoded, and the region in bold which was excluded from analysis.

Locations. 1: 68-97, 2: 100, 3: 134-137, **4**: 153-165, 5: 169-170, 6: 209-221, 7: 255, 8: 328, 9:344-345, *10*: 359-371, *11*: 361-363, 12:487-507, *13*: 509-523, *14*: 524-526, *15*: 563-573, 16: 601, 17: 627-629, 18: 639, 19: 655-656.

Coding

	00000001111111
	12367891226789
Pipra pipra	00000000000000
Meliphaga gracilis	00001000000010
Dasyornis broadbenti	00001000000010
Sialia sialis	10000000000101
Sturnus vulgaris	10000100000100
Dumetella carolinensis	10000100000100
Sitta pygmaea	10000010000100
Certhia familiaris	10000000000100
Donacobius atricapillus	10000011000101
Campylorhynchus brunneicapillus	11010000011100
C. megalopterus	11010000011100
Odontorchilus cinereus	10010000010100
Salpinctes obsoletus	10000010000100
Catherpes mexicanus	100100001?0100
Hylorchilus sumichrasti	100100001?0100
Cinnycerthia peruana	10010000010100
Cistothorus platensis	10010000010100
C. palustris	10010010010100
Thryomanes bewickii	10010000011100
Thryothorus coraya	10010000010100
T. maculipectus	10010000010100
T. ludovicianus	10010000011100
T. leucotis	10010000010100
T. guarayanus	10010000010100
Troglodytes aedon	10010000010100
Henicorhina leucosticta	10010000010100
Microcerculus marginatus	10010010000100
Cyphorhinus arada	10010000010100
Polioptila caerulea	10000000000100
Prinia bairdii	1000????000100
Zosterops senegalensis	10000001000100
Acanthidops bairdii	10100000000100
Zeledonia coronata	10100000000100

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