Rates and Patterns of Mitochondrial DNA Sequence Evolution in Fringilline Finches (Fringilla spp.) and the Greenfinch (Carduelis chloris)

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Rates and patterns of evolution in partial sequences of five mitochondrial genes (cytochrome b, ATPase 6, NADH dehydrogenase subunit 5, tRNA^Glu, and the control region) were compared among taxa in the passerine bird genera Fringilla and Carduelis. Rates of divergence do not vary significantly among genes, even in comparisons with the control region. Rate variation among lineages is significant only for the control region and NADH dehydrogenase subunit 5, and patterns of variation are consistent with the expectations of neutral theory. Base composition is biased in all genes but is stationary among lineages, and there is evidence for directional mutation pressure only in the control region. Despite these similarities, patterns of substitution differ among genes, consistent with alternative regimes of selective constraint. Rates of nonsynonymous substitution are higher in NADH dehydrogenase subunit 5 than in other protein-coding genes, and transitions exist in elevated proportions relative to transversions. Transitions appear to accumulate linearly with time in tRNA^Glu, and despite exhibiting the highest overall rate of divergence among species, there are no transversal changes in this gene. Finally, for resolving phylogenetic relationships among Fringilla taxa, the combined protein-coding data are broadly similar to those of the control region in terms of phylogenetic informativeness and statistical support.

Introduction

Relative simplicity of characterization combined with a high rate of evolution and an often uniparental mode of inheritance have made the mitochondrial genome a uniquely well studied molecule from a variety of evolutionary perspectives. With complete sequences available from some 44 organisms, the organization and function of mitochondrial DNA (mtDNA) genes are largely understood, and sequence variation within them provides a rich source of genetic markers for phylogenetic and population studies. However, certain aspects of sequence evolution remain problematic for these applications. For example, variation in evolutionary rate among sequences can substantially affect the accuracy of phylogenetic inference, as in the “long-branch attraction” phenomenon (Hendy and Penny 1989). Additionally, strong bias in base composition compromises some methods used to correct sequence divergence for multiple substitutions (Saccone et al. 1990).

Underlying its popularity as a genetic marker is the widespread belief that mtDNA variation conforms to a neutral model, thereby exemplifying the “molecular clock” hypothesis that substitutions between amino acid or (by extension) nucleotide sequences occur approximately linearly with time (Zuckerkandl and Pauling 1965). However, even small amounts of positive selection can influence the evolutionary history of the entire mitochondrial genome; moreover, many studies never attempt to test the assumptions of the neutral model (Ballard and Kreitman 1995). One such assumption is that of mutation rate constancy, under which substitution rates are determined by selective constraint. A potential problem for evolutionary studies arises when unknown patterns of selective constraint, especially in conjunction with high rates of substitution, confound attempts to account for multiple substitutions (Kocher and Wilson 1991). Furthermore, mutation rates are thought to vary in different physiological environments (e.g., Martin and Palumbi 1993), and substitution rate may vary among lineages with different generation times (Li, Tanimura, and Sharp 1987).

The avian genus Fringilla comprises three closely related species, one of which (the common chaffinch, F. coelebs) is widely distributed throughout Europe, northern Africa, and the Atlantic islands, and comprises several morphologically and genetically distinct subspecies (Baker et al. 1990). The blue chaffinch (F. teydea) of the Canary Islands is the sister species to the common chaffinch, and these two form a sister group to the brambling (F. montifringilla) of Eurasia (Baker and Marshall 1997). Along with the closely related greenfinch (Car- duelis chloris), these species provide an opportunity to compare sequences from a range of taxonomic levels, an advantage when attempting to understand the dynamics of mtDNA evolution (Thomas and Beckenbach 1989). Herein, we examine rates and patterns of substitution in several mitochondrial genes in this group of finches to test the assumption of selective neutrality, to assess differences in patterns of selective constraint among genes and rate variation among lineages, and to evaluate the suitability of mtDNA sequences for phylogenetic studies of fringilline finches.

Materials and Methods

Mitochondrial Genes Examined

We amplified and sequenced 641 bp of cytochrome b (cyt b), 342 bp of NADH dehydrogenase subunit 5 (nd5), 300 bp of ATPase 6 (atp6), 918–932 bp of the control region (cr), and the tRNA^Glu gene (tglu; 71 bp). Cyt b was amplified and sequenced using two overlapping sets of internal primers, L14841 and H15149 (Kocher et al. 1989) and L15063 and H15506 (unpublished data), which together provide 56.1% of the gene 98 bp
downstream of the 5′ end. The nd5 primers (L12301: 5′-AGGAGCAATCCGTTGGTCTTAGG-3′, and H12766: 5′-GACATGATTCCTACTCCTTCTCA-3′) target a relatively small portion (19%) of this approximately 1,800-bp gene, located downstream of the rRNALeu, in which the forward primer was placed. The atp6 primers (L8552 and H8957; unpublished data) are situated within the gene and yield sequence for approximately half (43.9%) of it, closer to the 5′ end. The alphanumerical designation of each primer indicates whether it occurs in the heavy or light strand (H or L) and the position of its 3′ end. The nd5 primers target a relatively small portion of the gene, located downstream of the rRNALeu, in which the forward primer was placed. The atp6 primers are situated within the gene and yield sequence for approximately half (43.9%) of it, closer to the 5′ end. The alphanumerical designation of each primer indicates whether it occurs in the heavy or light strand (H or L) and the position of its 3′ end.

Collection of Samples and DNA

A total of 28 specimens representing six subspecies of common chaffinch, the blue chaffinch, the brambling, and the greenfinch were used in this study. Taxa, collection locality, and genes sequenced for each specimen are given in Table 1. At least two individuals from each taxon were sequenced for each gene, although the particular individuals vary among genes. Genomic DNA was extracted from liver, heart, or spleen using standard proteinase K–phenol–chloroform methods (Sambrook, Fritsch, and Maniatis 1989).

Polymerase Chain Reaction (PCR) and Sequencing

Double-stranded amplification reactions contained 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 50 μM of each dNTP, 0.4 μM of each primer, and 1 U Taq DNA polymerase (Boehringer Mannheim) in a 25-μl volume. Amplification was achieved with a thermal cycle of 93°C for 30 s, 48–50°C for 30 s, and 72°C for 60 s, repeated 35 times. Products were purified using agarose separation followed by binding to glass beads (Gene Clean; BIO 101), and were sequenced using the Sequenase 2.0 dideoxy sequencing kit (United States Biochemical) and 35 S radiolabel or the AmpliCycle cycle sequencing kit (Perkin Elmer) and 32P radiolabel, according to the manufacturer’s instructions. At least two individuals per taxon were sequenced, and portions of each gene were sequenced from both strands.

Sequence Analysis

Sequences were compared using the computer programs ESEE (Cabot and Beckenbach 1989) and MEGA.
(Kumar, Tamura, and Nei 1993). With the exception of the control region, sequences were aligned manually; the alignment of the control region is described in Marshall and Baker (1997). To describe the evolution of mtDNA in finches, we first evaluated conformity of the data to expectations under neutral theory using statistical tests. McDonald and Kreitman’s (1991) test was used to assess the distribution of synonymous and replacement changes within and between species for the combined protein-coding data. Genes were combined for this test to increase the number of variable sites and, therefore, the potential to reject the null hypothesis. This was considered valid because mitochondrial genes belong to a single linkage group. Tajima’s (1989) test was applied to samples of F. coelebs and F. montifringilla control region sequences in a previous study (Marshall and Baker 1997).

Compositional evolution was evaluated by calculating nucleotide bias, AT skew, and GC skew for each gene individually and at each codon position, and amino acid composition bias was estimated for the three protein-coding genes by the formulae described in Kocher et al. (1995). As an estimate of directional mutation pressure, we evaluated deviations from 50% GC content at third positions and in the two noncoding genes with the \( \chi^2 \) test. To determine whether sequences are compositionally stationary, compositional divergence was calculated for among-species pairwise comparisons of each gene according to the method of Gillespie (1986). To evaluate patterns of substitution, transition-to-transversion \( (ts/tv) \) ratios were estimated using the computer program PAML (Yang 1995). In addition, pairwise estimates of transition and transversion differences for each gene were calculated and plotted against third-position transversions from the three protein-coding genes combined. For coding genes, we confined this analysis to third-position changes, as they were most frequent. Third-position transversions were chosen to represent time, as they are thought to be the least saturated component of the sequence data (Kocher et al. 1995).

To compare rates of evolution among mitochondrial genes, pairwise estimates of divergence were calculated among species and among individuals within F. coelebs at all sites for each gene, and separately at synonymous and nonsynonymous sites for the three protein-coding genes. The Nei and Gojobori (1986) algorithm, which includes a Jukes and Cantor (1969) correction for multiple substitutions, was considered suitable for this purpose, despite certain shortcomings described by Muse (1996), as pairwise divergences were generally not high (except in comparisons with C. chloris). The significance of differences in divergences among genes was assessed using the Kolmogorov-Smirnov two-sample one-tailed test (D; as in Bielawski and Gold 1996), which does not assume a particular distribution, and/or the \( t \)-test with infinite degrees of freedom (as suggested in Kumar, Tamura, and Nei 1993).

Rate variation among lineages was evaluated for each gene and for the combined protein-coding sequence data using the likelihood values of the best tree produced with and without the imposition of equal branch lengths (Felsenstein 1988), assuming that the tree topology is the same under these two criteria. Differences in log-likelihoods for these trees were compared using the \( G \)-test of goodness of fit (Sokal and Rohlf 1981). The programs DNAML and DNAMLK implemented in Phylip 3.5 (Felsenstein 1993) were used to obtain the likelihood values. \( ts/tv \) parameters were estimated as already described. Felsenstein’s test was chosen because it does not depend on being able to accurately assess the phylogenetic relationships among the taxa. As a result, it identifies overall rate heterogeneity in the data set.

To assess the utility of these mitochondrial genes for phylogeny estimation, we constructed separate neighbor-joining trees (Saitou and Nei 1987) using (1) the control-region sequences and (2) the protein-coding genes. We used the Kimura two-parameter method (Kimura 1980) to estimate distances, as recommended by Kumar, Tamura, and Nei (1993) for situations in which distances are small \((d < 0.3)\) and/or \( ts/tv \) ratios are low \((ts/tv < 2)\). Branch length confidence probabilities and bootstrap confidence levels (500 replicates) were calculated for each tree.

### Results and Discussion

#### Sequences

A total of almost 2.3 kb was sequenced for one to three individuals from each taxon (table 1; GenBank accession numbers AF002879–AF002978). The cr sequences correspond to positions 110–411 and 611–1252 presented for these species in Marshall and Baker (1997). Approximately equal numbers of variable sites were identified for the protein-coding genes combined as for the noncoding tglu and cr sequences. In the former, 251 variable sites (89 potentially phylogenetically informative) were found, corresponding to 18.1%, 18.7%, and 23.1% of the total sequence for cytb, atp6, and nd5, respectively. As expected, most (192) are third-position changes; there are 44 first-position and 15 second-position variable sites. In the tRNA\(^{Glu}\) gene, 21 sites are variable, while the control region contains 226 variable positions (96 potentially phylogenetically informative), accounting for approximately 24%–25% of the sequence obtained. Consistent with a mitochondrial rather than a nuclear origin, all protein-coding sequences translate appropriately with the mitochondrial code and the tglu and cr sequences contain expected putative structural features (Marshall and Baker 1997).

According to the McDonald-Kreitman (1991) test, if sequence variation is neutral, the ratio of synonymous to replacement changes should be the same for within-species polymorphism as for between-species divergence. Here, 63 silent and 13 replacement sites were found within F. coelebs (15 individuals in six subspecies), and 39 silent and 6 replacement sites were found between F. coelebs and F. teydea (consensus sequence). According to the \( G \)-test of independence (Sokal and Rohlf 1981), these ratios are sufficiently similar to be consistent with the expectation under neutrality \((G = 0.31, P = 0.60)\). The fact that F. coelebs individuals were not sampled from a randomly mating population...
does not invalidate the result, as the test does not make this assumption. The test requires that the within-species sample of alleles have the same common ancestor; that the mutation rate not vary between species, and that the species be closely related, all features of these data. The acceptance of the null hypothesis of neutrality must nevertheless be considered conservative due to the fragmentary nature of the genes used. None of the other commonly used statistical tests of neutrality are appropriate for the coding-region data presented herein. However, Tajima’s (1989) tests applied to samples of F. coe- lebs and F. montifringilla cr sequences in a previous study (Marshall and Baker 1997) were consistent with neutrality.

Sequence Composition and Patterns of Substitution

Nucleotide and amino acid composition contain information related to patterns of selective constraint and, thus, are important descriptive features of genes. Here, composition bias is strongest in the atp6 gene (0.269) and weakest in tglu (0.094), while in the protein-coding genes it is strongest at the third codon position (0.497–0.563) and weakest at the first (0.027–0.294). The composite bias at third positions and in the noncoding genes reflects an elevated proportion of A and reduced G, resulting in positive AT (0.558–0.765) and negative GC (0.896–0.942) skew values at this position. First positions are characterized by slight positive AT skew (0.0208–0.290) and, except in cytb, moderate negative GC skew (0.333–0.587), while second positions exhibit moderate negative AT (0.334–0.424) and GC (0.228–0.600) skew. Only in the control region was the GC content significantly different from 50% ($\chi^2 = 16.18, P < 0.0001$). There was no evidence that base composition varies among lineages for any gene in finches, as indicated by plots of pairwise transition and transversion divergence estimates against total-third-position transitions (fig. 1). In all genes, transversions appear to accumulate linearly. The maximum transition difference is about 9% in the cr gene and between 12% and 14% at third positions of the coding genes. In contrast, transitions accumulate more slowly after third-position transversion differences reach 3%–5%, with saturation occurring most quickly in the nd5 gene. An exception is the tglu gene, which shows no evidence of transition saturation even at the maximum observed difference of approximately 25%. Maximum observed transition differences in all genes are higher than transversion differences, especially in cytb where third-position transition differences approach 20%.

Patterns of substitution (the relative probabilities of change from one nucleotide to another) provide another indication of the spectrum of mutations tolerated by a gene. Substitution bias is prevalent in each gene among the four finch species and within F. coelebs. For example, ts/tv ratios range from 1.42 (among species for the cr gene) to 14.53 (third positions in cytb within F. coelebs), substantially exceeding the unbiased expectation of 1/2. Differences in the accumulation of transitions and transversions among genes are apparent from plots of pairwise transition and transversion divergence estimates against total-third-position transversions (fig. 1). In all genes, transversions appear to accumulate linearly. The maximum transition difference is about 9% in the cr gene and between 12% and 14% at third positions of the coding genes. In contrast, transitions accumulate more slowly after third-position transversion differences reach 3%–5%, with saturation occurring most quickly in the nd5 gene. An exception is the tglu gene, which shows no evidence of transition saturation even at the maximum observed difference of approximately 25%. Maximum observed transition differences in all genes are higher than transversion differences, especially in cytb where third-position transition differences approach 20%. Differences in the accumulation of transitions among genes indicate different proportions of sites that are free to vary (Wills 1995) as well as different rates of substitution, as discussed below.

Evolutionary Rates of Mitochondrial Genes

Relative rates of substitution of the different partial genes were inferred from average pairwise divergence estimates, and similar patterns were observed in among-species comparisons relative to within-species comparisons (fig. 2). In all genes it was observed that transitions accumulate more slowly after third-position transversion differences reach 3%–5%, with saturation occurring almost completely in the control region under the assumption that the mutation rate not vary between species, and that the species be closely related, all features of these data. The acceptance of the null hypothesis of neutrality must nevertheless be considered conservative due to the fragmentary nature of the genes used. None of the other commonly used statistical tests of neutrality are appropriate for the coding-region data presented herein. However, Tajima’s (1989) tests applied to samples of F. coelebs and F. montifringilla cr sequences in a previous study (Marshall and Baker 1997) were consistent with neutrality.

Table 2

<table>
<thead>
<tr>
<th>Species Pair</th>
<th>cytb</th>
<th>atp6</th>
<th>nd5</th>
<th>tglu</th>
<th>cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. coelebs–F. teydea....</td>
<td>0.0883</td>
<td>0.0314</td>
<td>0.0901</td>
<td>0.0749</td>
<td>0.288</td>
</tr>
<tr>
<td>F. teydea–F. montifringilla........</td>
<td>0.0949</td>
<td>0.0238</td>
<td>0.00606</td>
<td>0.0940</td>
<td>0.0741</td>
</tr>
<tr>
<td>F. coelebs–Carduelis chloris........</td>
<td>0.258</td>
<td>0.332</td>
<td>0.254</td>
<td>0.648</td>
<td>0.380</td>
</tr>
<tr>
<td>F. teydea–C. chloris................</td>
<td>0.286</td>
<td>0.456</td>
<td>0.0911</td>
<td>0.499</td>
<td>0.371</td>
</tr>
<tr>
<td>F. montifringilla–C. chloris........</td>
<td>0.105</td>
<td>0.591</td>
<td>0.132</td>
<td>0.857</td>
<td>0.306</td>
</tr>
</tbody>
</table>

**Notes:** $d = \frac{1}{2} \sum (p_i - q_i)^2 / \sum (p_i + q_i)$, where the frequencies of the four bases (i = G, A, T, C) in the two sequences being compared are $p_i$ and $q_i$, $P_i = (p_i - q_i)^2$; and $n$ is the number of nucleotides in the comparison. cytb = cytochrome b; atp6 = ATPase; nd5 = NADH dehydrogenase subunit 5; tglu = tRNA Glu; cr = control region.
ing gene divergences are lower (although not significantly so) than those in the noncoding genes. However, the average protein-coding synonymous divergence is significantly higher \((t = 4.88, P < 0.0001; D = 5/6, P = 0.025)\) than the average cr divergence among species, while nonsynonymous differences in protein-coding genes are significantly lower than cr rates both within \((t = 2.08, P = 0.04)\) and among \((t = 2.08, P = 0.04; D = 1, P = 0.0005)\) species. Most rate differences among protein-coding genes are insignificant, with the exception that nonsynonymous differences are significantly higher in nd5 among species \((\text{nd5 vs. atp6: } t = 3.5, P = 0.0003; D = 1, P = 0.0005)\) and nd5 vs. cyt

For the combined protein-coding sequences, significant rate variation was found among species \((G = 11.8, P = 0.025)\), but not within F. coelebs.

With respect to rate variation among genes, the results for finches suggest that the intensity of selective constraint is weaker for nd5 and the cr than for the other protein-coding genes and that patterns of selective constraint may vary among genes. Rate variation among lineages only occurs for certain genes, so we suspect that it is also related to different selective constraints in different environments, as opposed to differences in metabolic rate or generation time. The species examined are all small passerines with similar weight-specific metabolic rates and generation times, but they occupy a range of habitats from hot and arid, to subtropical or temperate with high rainfall, to boreal. In support of the selective-constraint hypothesis, the nd5 gene of finches not only contains an unusually large number of replace-
mitochondrial DNA sequences, such as base composi-
tion bias and rate variation among lineages, which may
confound phylogenetic inferences in fringilline finches.
The results obtained must be interpreted with caution,
given that partial genes were examined. However, no
departures from compositional homogeneity, such as
those reported in sharks (Martin 1995) and cichlid fishes
(Kocher et al. 1995), were observed. There was no ev-
idence for positive selection, nor for variation in substi-
tution rate among genes or lineages due to forces other
than different intensities and patterns of selective con-
straint. Rate variation among lineages was apparent for
certain genes, but this can be circumvented by choosing
a phylogenetic method which does not assume equal
rates. Patterns of selective constraint do clearly vary
among genes. For example, nd5 contains an excess of
replacement substitutions relative to other protein-cod-
ing genes, while cytb exhibits high third-position tran-
sition differences. Tglu appears to evolve quickly, yet
transitions are unsaturated in this gene, and the cr con-
tains the lowest proportion of transitions relative to
transversions of any gene. In rigorous phylogenetic anal-
yses, such as will be performed in a subsequent study,
these patterns will need to be addressed by the incor-
poration of weighting schemes and rate parameters.
In summary, though, these sequences appear to be suitable
for phylogeny reconstruction.

In addition to examining relevant aspects of the
data, we are also interested in how the genes compare
in their ability to resolve the relationships among frin-
gilline finches. Because only small portions of certain
genes were examined, we decided to compare combined
protein-coding sequences with cr sequences. We feel
that combining genes is justified, because mitochondrial
genes are linked and thus share a phylogenetic history,
and because the major difference among protein-coding
genes appears to concern the intensity of selective con-
straint, which can ultimately be accounted for by a
weighting scheme. Superficially, both the cr gene and
the combined protein-coding genes show similar phy-
logenetic potential. Interestingly, the neighbor-joining
phylogenies recovered from the two data sets (fig. 3)
are different. Both trees share the same specific rela-
tionships ((F. coelebs, F. teydea) F. montifringilla) reported
previously for cr data (Marshall and Baker 1997). In the
protein-coding tree (fig. 3A), individuals from the Can-
ary Islands form a clade whose sister group contains
Madeiran birds, and island subspecies form a separate
group from continental subspecies. However, the cr tree
exhibits a within--F. coelebs topology in which the is-
land subspecies are polyphyletic (fig. 3B). Variation also
occurs in the arrangement of the different Canaries hap-
lotypes (F. c. canariensis and F. c. palmae individuals)
and the different continental haplotypes (F. c. coelebs
and F. c. africana individuals). Neither using a parsi-
mony method nor limiting the analysis to the same num-
er of lineages results in the same topology for both
data sets (results not shown). Similarly, trees constructed
from single genes do not provide better support for
poorly supported nodes (results not shown).

Topological differences between the two data sets
are reconcilable on examination of statistical support.
Clades appearing in both trees tend to be well supported both by bootstrap confidence levels and by branch length confidence probabilities, and values for these are similar in the two data sets. Topologies that differ between the analyses are quite poorly supported; for instance, the clade grouping all island subspecies has a bootstrap value of 54 for the protein-coding data, and the inclusion of the continental subspecies with two of the island subspecies in the cr tree is supported by only 42 bootstrap replicates. Similarly, support values are very low for some groupings within the Canaries clade, and within the continental clade. Thus, neither data set is capable of satisfactorily resolving certain relationships. This may indicate a paucity of variation, resolvable by collecting more data. However, combining the protein-coding and cr data does not result in greatly improved statistical support (data not shown). Alternatively, the explanation may be that irresolvable nodes result from an evolutionary polytomy, consistent with a rapid wave of colonization. The relationships among fringilline taxa will be addressed more thoroughly in a subsequent study pertaining to the colonization of the Atlantic islands by chaffinches.

The phylogenetic utility of the combined protein-coding genes therefore compares favorably with that of the cr among closely related taxa such as subspecies, and the cr actually performs well for resolving species-level splits. The cr is usually described as hypervariable (see Simon 1991 for a review), but this may result in part because only the more variable portions are usually targeted for analysis. Alternatively, because the taxa studied here are relatively recently diverged, large differences among genes may not have had time to accumulate. Nevertheless, this unexpected result underscores the importance of evaluating rates and patterns of mtDNA evolution prior to employing it as a phylogenetic marker.

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