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Can Weighting Improve Bushy Trees? Models of Cytochrome *b* Evolution and the Molecular Systematics of Pipits and Wagtails (Aves: Motacillidae)

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Abstract.— Among-site rate variation (α) and transition bias (κ) have been shown, most often as independent parameters, to be important dynamics in DNA evolution. Accounting for these dynamics should result in better estimates of phylogenetic relationships. To test this idea, we simultaneously estimated overall (averaged over all codon positions) and codon-specific values of α and κ, using maximum likelihood analyses of cytochrome *b* data from all genera of pipits and wagtails (Aves: Motacillidae), and six outgroup species, using initial trees generated with default values. Estimates of α and κ were robust to initial tree topology and suggested substantial among-site rate variation even within codon classes; α was lowest (large among-site rate variation) at second-codon and highest (low among-site rate variation) at third-codon positions. When overall values were applied, there were shifts in tree topology and dramatic and statistically significant improvements in log-likelihood scores of trees compared with the scores from application of default values. Applying codon-specific values resulted in yet another highly significant increase in likelihood. However, although incorporating substitution dynamics into maximum likelihood, maximum parsimony, and neighbor-joining analyses resulted in increases in congruence among trees, there were only minor improvements in phylogenetic signal, and none of the successive approximations tree topologies were statistically distinguishable from one another by the data. We suggest that the bushlike nature of many higher-level phylogenies in birds makes estimating the dynamics of DNA evolution less sensitive to tree topology but also less susceptible to improvement via weighting.[Character weighting; cytochrome *b*; maximum likelihood; Motacillidae; rate variation; systematics; transition bias.]

A principal goal of systematics is to generate the best possible estimate of the interrelationships of a group of organisms, and molecular techniques are increasingly providing systematists with the means for doing so. To test the strength of molecular phylogenies, systematists have often relied on congruence with other estimates of phylogeny (Miyamoto and Fitch, 1995). Frequently this has involved comparing molecular phylogenies with phylogenies based on morphological data (e.g., Austin, 1996; Nunn and Cracraft, 1996), but increasingly, tests of congruence are being used to compare several molecular estimates of phylogeny with one another (e.g., Hackett, 1996; Yoder et al., 1996). Unfortunately, most taxonomic groups do not have previous estimates of phylogenetic relationships, either morphological or molecular, to permit tests of congruence.

In lieu of congruence tests, many systematists use parsimony and likelihood scores, or bootstrapping (Felsenstein, 1985), as "internal" tests of the robustness. To improve a phylogenetic signal, as measured by either congruence or "internal" tests, a recent trend has been to apply a priori weighting schemes either to individual characters (e.g., downweighting fast-changing third positions) or individual transformations (e.g., downweighting transitions). Although the conditions under which weighting improves trees is not fully explored (Huelsenbeck et al., 1994), weighting can considerably improve the congruence and internal robustness of phylogenetic estimates (Chippindale and Wiens, 1994; Miyamoto et al., 1994; Allard and Carpenter, 1996; Yoder et al., 1996; Edwards and Arctander, 1997). However, two general concerns about the application of weighting schemes have been raised: (1) Many schemes are ad hoc and have not been based on empirical patterns derived from the data under study or from those of related groups, and (2) some weighting schemes do not take much information from the data into account (Allard

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and Carpenter, 1996; Wakeley, 1996; Yang, 1996a; Edwards, 1997; Purvis and Bromham, 1997; Sullivan and Swofford, 1997). These concerns have been particularly acute in avian molecular systematic studies, many of which apply arbitrarily chosen weights to particular characters or transformations (e.g., Lanyon, 1994; Ellsworth et al., 1996; Nunn and Cracraft, 1996; Richman, 1996; but see also Edwards and Wilson, 1990; Edwards et al., 1991; Cooper et al., 1992; Austin, 1996; Edwards, 1997; Houde et al., 1997). For example, many recent avian studies alternately include or exclude third positions, or third-position transitions, without considering intermediate weights or treatments for these sites.

Neglecting to incorporate details of sequence evolution, or taking an all-or-nothing approach to weighting, can be problematic; for example, completely ignoring some classes of data (e.g., third-codon positions) or transformations (e.g., transitions) can result in loss of phylogenetic information at some hierarchical levels (e.g., Cracraft and Helm-Bychowski, 1991; Simon et al., 1994; Yoder et al., 1996; Moore and DeFilippis, 1997). In the past, ignorance of the values to apply to these parameters slowed implementation of character and transformation weighting schemes. New maximum likelihood (ML) methods for estimating dynamics of gene regions (e.g., Sullivan et al., 1995; Yano, 1996b) may provide a solution to at least some of these concerns, as they can suggest weighting schemes that employ dynamics estimated directly from the data. An advantage of employing ML methods is that they provide statistically rejectable hypotheses of sequence evolution (Goldman, 1993) and tree topologies (Kishino and Hasegawa, 1989; Huelsenbeck et al., 1996; Sullivan and Swofford, 1997). For example, the Hasegawa–Kishino–Yano model with gamma distribution (HKY85Γ; Hasegawa et al., 1985), in which there is a single transition:transversion (ts:tv) ratio (κ) and a single parameter incorporating rate variation among sites (α), has proven a surprisingly versatile descriptor of DNA sequence evolution (Hasegawa et al., 1985; Goldman, 1993; Yang, 1996a). But in principle, ML methods permit rejection of this model in favor of more detailed models by such methods as the likelihood ratio test (LRT; Goldman, 1993) (Kishino and Hasegawa, 1989; Sullivan, 1996).

Even if phylogenetic estimates are not improved by accounting for differences in character evolution, measuring the dynamics of DNA sequences is interesting in its own right and can provide useful descriptions of the molecular evolutionary process. For instance, although the gene for cytochrome *b* is by far the most widely used gene in avian systematics, the dynamics of this gene have only begun to be described in birds (e.g., Griffiths, 1997). Thus, most bird systematists employ very general and ad hoc weighting schemes, typically based on estimates from other data sets. This tendency has contributed to questions about whether cytochrome *b* is a useful phylogenetic marker in a variety of taxa (Graybeal, 1993; Avise et al., 1994; Meyer, 1994).

We use the pipits, wagtails, and longclaws (Aves: Motacillidae) to explore the above ideas. Despite an essentially global distribution, no explicit morphological or molecular phylogeny exists for this group. Traditionally, five genera have been recognized within the family. *Anthus* (pipits) is the most diverse, including about 40 species (Clancey, 1990; Sibley and Monroe, 1990), and is also the most widespread, with species occurring on every major landmass except Antarctica. *Motacilla* (wagtails) includes 10 species and is distributed from Africa through Eurasia to Alaska. *Macronyx* (longclaws) consists of an African radiation of 10 species. Two monotypic genera, *Tmetothylacus* (golden pipit) and *Dendronanthus* (forest wagtail) are restricted to eastern Africa and eastern Asia, respectively. In addition to these genera, *Hemimacronyx* has recently been resurrected on the basis of limited morphological data (Cooper, 1985; Clancey, 1990) as a link between *Macronyx* and *Anthus*; two species, *Macronyx sharpei* and *Anthus chloris* have been united in this genus. The only statement of phylogenetic relationships among motacillid genera is the limited description of Cooper (1985). Although Cooper suggested that *Dendronanthus* and *Motacilla*

formed a sister clade to a clade in which *Tmetothylacus* and *Anthus* were most closely related and linked to *Macronyx* via *Hemimacronyx*, he provided no data or character matrix to support this.

In this paper, we use the approach of successive approximations, based on ML estimates of cytochrome *b* dynamics of our data, to determine the phylogenetic relationships of motacillid genera. Successive approximation approaches (Farris, 1969), in which character weights are determined by using some function of character consistency on an initial tree, are most often discussed in the context of parsimony but can in principle be applied in a ML context as well (Yang, 1994). We first apply an a priori weighting scheme to cytochrome *b* sequence data to estimate initial trees and then use these trees to estimate the dynamics of our data set, specifically the transitional bias (κ) and among-site rate variation (α). Using three different tree-building methods, we then incorporate these rate dynamics a posteriori to generate new trees of motacillid genera. Our approach can be termed successive approximations in so far as we incorporate information gleaned from an initial tree into subsequent phylogenetic analyses, although it differs from traditional successive approximation approaches because our emphasis is not directly on character weights but rather on transformation weights and distributions of rates among sites.

Materials and Methods

Taxa Investigated and DNA Extraction

We sequenced 1035 base pairs (bp) of the mitochondrial cytochrome *b* gene from 12 motacillid taxa, as well as from 6 outgroup taxa (Table 1). Frozen tissues were available for most of these taxa, and mitochondrial DNA (mtDNA) was isolated from

Table 1. Species, collection locality, and museum and voucher number for avian specimens used in this study.

Family, species	Origin	Collection[a] and voucher number
Troglodytidae		
Salpinctes obsoletus	Washington: Grant County	UWBM 56992
Cinclidae		
Cinclus mexicanus	Washington: Whatcom County	UWBM 56993
Prunellidae		
Prunella atrogularis	Russia: Gorno-Altay Republic	UWBM 46573
Turdidae		
Catharus fuscescens	Washington: Kittitas County	UWBM 56991
Motacillidae		
Dendronanthus indicus	Russia: Amurskaya Oblast'	UWBM 45241
Motacilla flava	Russia: Sakhalinskaya Oblast'	UWBM 47504
Motacilla cinerea	Kazakhstan: Alma-Ata Oblys	UWBM 46556
Motacilla alba	Russia: Gorno-Altay Republic	UWBM 46304
Motacilla capensis	South Africa: KwaZulu/Natal Province	UWBM 53145
Motacilla flaviventris	Madagascar	FMNH 352834
Tmetothylacus tenellus	Kenya: Coast Province	LACM 87914
Macronyx capensis	South Africa: KwaZulu/Natal Province	UWBM 52793
Macronyx croceus	South Africa: KwaZulu/Natal Province	UWBM 52806
Hemimacronyx chloris	South Africa: Eastern Cape Province	UWBM 52814
Anthus brachyurus	South Africa: KwaZulu/Natal Province	UWBM 52901
Anthus caffer	South Africa: KwaZulu/Natal Province	UWBM 52810
Bombycillidae		
Bombycilla garrulus	Alaska: Houston	UWBM 53989
Passeridae		
Passer griseus	South Africa: Orange Free State	UWBM 52748

[a]UWBM = University of Washington Burke Museum; FMNH = Field Museum of Natural History; LACM = Los Angeles County Museum.

these specimens via a cesium chloride gradient (Dowling et al., 1990) to minimize the chance of amplifying nuclear mitochondrial sequences. Tissues of *Motacilla flaviventris* were stored in buffer, and total genomic DNA was obtained by Chelex extraction (Walsh et al., 1991). Although our sample of *Tmetothylacus* was from a specimen preserved in alcohol/formalin, the DNA was successfully extracted via Chelex. We were able to amplify and combine smaller sections of cytochrome *b* from this specimen, each ~400 bp. *Dendronanthus* DNA was obtained by snipping a piece of foot pad from a museum skin and extracting the DNA with a commercial kit (Tissue Protocol, QiAmp, Qiagen; see Mundy et al., 1997).

Cytochrome b *Isolation, Amplification, and Sequencing*

For purified mtDNA samples, the segment of cytochrome *b* considered here was amplified as a single unit via the polymerase chain reaction (PCR) with use of primers L14841 (Kocher et al., 1989) and H16065 (Helm-Bychowski and Cracraft, 1993). These two primers were also used in various combinations with primers L15114, L15299, L15609, H15547 (Edwards et al., 1991), and H15299 (Hackett, 1996) to amplify DNA from the remaining taxa in overlapping segments. For sequencing, we used the above primers, the primer H15915 (Edwards and Wilson, 1990), and the following primers designed specifically for this study (identified by numbers corresponding to the 3′ position in domestic fowl sequence; Dejardins and Morais, 1990):

L15086 (5′-CTCTGTAGCTCACATATGCC-3′),
L15376 (5′-CTAGCAGAATGAGCCTGAGG-3′),
L15616 (5′-GTTGCCCTAACCCTATTCTC-3′),
L15811 (5′-CCCCTACTCCACACATCAAA-3′),
H15345 (5′-GTAATAACGGTAGCTCCTCA-3′),
H15671 (5′-GGTGTGAAGTTTTCTGGGTC-3′),
H15853 (5′-GGCGGAAGGTTATTGATC-3′).

We also used:

L15350 (5′-TTACAAACCTATTCTCAGC-3′)

designed by J. Klicka.

Fragments were amplified in 100-μl PCR reactions; amplification conditions were 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C repeated for 35 cycles. Amplified products were prepared as templates for automated sequencing by purification and concentration in 22 μl of water after three passes through Ultrafree-MC filters by centrifugation (Millipore). Two microliters of the purified and concentrated PCR product was used as a template in a 10-μl DyeDeoxy Cycle Sequencing reaction (ABI), along with one of the above primers, according to the manufacturer's instructions (Perkin-Elmer). After cycle sequencing, products were placed on coarse-grained Sephadex columns and cleaned of excess nucleotides via centrifugal passage through the columns (1500 rpm for 7 min). Both light and heavy strands of the entire 1035-bp fragment considered were read with an ABI model 373 automated sequencer. Sequences were aligned unambiguously by eye by using Genetic Data Environment (developed and maintained by S. Smith, with compilation of programs by various authors; available free from ftp.bio.indiana.edu, in molbio/unix/GDE).

Phylogenetic Analysis

Choice of outgroups was difficult because the pipits and wagtails have been allied with no fewer than 12 passerine families (see Sibley and Ahlquist, 1981). We therefore included in this study six of these potential outgroups, two of which, accentors (Prunellidae) and Old World sparrows (Passeridae), were suggested as being close to pipits and wagtails on the basis of DNA hybridization evidence (Sibley and Ahlquist, 1981, 1990). We arbitrarily rooted all trees to an American dipper (*Cinclus mexicanus*; Cinclidae), which represents a family that falls close to but has not been proposed as a sister group to pipits, and allowed all other potential outgroup families to "float" in the phylogenetic analyses.

Nucleotide composition for each codon position and for the entire region sequenced, as well as compositional bias, was determined by using MOLPHY (Adachi and Hasegawa, 1996). The α (among-site rate variation) and κ (transition bias) parameters (see Yang, 1996a) were determined for each codon position and for the entire se-

quence by using PAML (Yang, 1996b), and trees were determined by various methods (see below). In PAML, we used the HKY85Γ model of DNA evolution, which allows for two substitution rates (transitions vs. transversions), as well as unequal base frequencies (Hasegawa et al., 1985).

Maximum parsimony (MP) analyses were performed with PAUP* 4.0.054d (written by David L. Swofford; results published with permission). In the initial analysis, all sites were treated as unordered character states. Although κ was incorporated into subsequent analyses via step-matrices applied to the entire sequence (i.e., one ts:tv value for all sites) and to each codon position (i.e., a different ts:tv value for each codon position), α could not readily be incorporated into parsimony analyses. The heuristic search option with random addition and 10 replicates was used for each weighting scheme. Cladistic signal was determined for each clade by bootstrapping (Felsenstein, 1985).

Using PAUP*, a neighbor-joining (NJ; Saitou and Nei, 1987) method was used to fit a tree to a matrix of Tamura–Nei distances, which permit unequal base frequencies. Global or codon-specific α and κ values were incorporated into subsequent NJ analyses by using the ML distance option. For the codon-specific analysis, we generated a distance matrix for each codon position based on codon-specific α and κ values and then summed these distances to generate distances based on codon-specific parameters; direct calculation of such distances is currently unavailable in PAUP*.

An initial ML analysis was done with PHYLIP (Felsenstein, 1993), using default options (F84 model; Felsenstein, 1984) with three jumbles of taxon order. This tree was identical in topology to one generated in PAUP* with the closely related HKY85Γ model. We therefore used the HKY85Γ model in all subsequent ML analyses because it describes the dynamics of many genes well (Goldman, 1993; Yang, 1996a) and because the parameter estimates from PAML were determined under the same model. We used PAUP* to conduct further ML analyses. In the first analysis, we lumped together all codon positions and used the overall κ and α estimates to generate a new tree and log-likelihood score. In a second analysis, we used codon-specific estimates of κ and α to generate log-likelihood scores for each position by utilizing the tree derived from the first weighted analysis and then summed these scores for an overall log-likelihood score. There are currently no accessible methods for estimating ML trees by assigning specific values of κ and α to specific sites, so we instead confined our analyses to estimating the log-likelihood on other trees.

To test whether the log-likelihood scores provided by each level of analysis were significantly different, we employed the LRT (Goldman, 1993). This method tests whether more complex (or parameter-rich) models provide significantly better explanations of the observed data than do simpler models. The test statistic follows a chi-square distribution, with degrees of freedom dependent on the difference in the number of parameters used between models. Thus, according to our methodology, two tests are possible: the log-likelihood score from the initial estimate of phylogeny versus that from the estimate derived by using overall rates of κ and α, and the score with a single κ and α versus the score with codon-specific variables. These comparisons are nested models (J. Huelsenbeck, pers. comm.; J. Felsenstein, pers. comm.) and so are appropriately tested by using LRT. We used the Kishino–Hasegawa (1989) test to determine whether some tree topologies were significantly worse than others. We used Rohlf's (1982; as implemented in PAUP*) consensus indices to measure whether trees generated by using estimated parameters were more congruent than those trees generated by using default parameters.

Results

Mitochondrial Cytochrome b *Sequences and Parameters*

We aligned 1035-bp samples without insertions or deletions (sequences to be submitted for separate publication). Because mtDNA was isolated for most specimens, nuclear copies are less likely for these taxa

but have a greater chance of amplification from *Tmetothylacus* and *Dendronanthus*. However, because there were no alignment problems, and no nonsense codons were present, we are confident that the genes used in this study were mitochondrial.

For the 1035 nucleotide sites, 447 were variable, and 289 were parsimony-informative across all taxa. Across ingroup taxa, 299 sites were variable, and 159 were parsimony-informative. Of the parsimony-informative sites, 62 (21.4%) were at first-, 25 (8.6%) were at second-, and 202 (70%) were at third-codon positions. The uncorrected percent sequence divergence between motacillid taxa varied from 3.77% to 15.07%. Sequence divergence between motacillid taxa and outgroups ranged from 14.20% to 22.32%. First-codon positions showed the least amount of base-compositional bias, followed by the second- and third-codon positions (Table 2). Third-codon positions showed a moderate amount of base-compositional bias (0.192, G-T poor), which is similar in direction for all sequences (Table 2).

Overall κ and α values varied very little across the initial tree topologies generated by each method of analysis (Table 3; see below). As one would expect for a protein-coding gene, the analyses showed substantial rate heterogeneity among all sites. The low α values for first and second positions (Table 2) suggest that most sites have either very low substitution rates or are invariable, whereas a few sites exist with very high rates (Sullivan et al., 1995; Yang, 1996a). Third positions, however, showed a higher value of α (Table 2), suggesting a more uniform distribution, in which most sites have intermediate rates and few sites have very low or very high rates. Third positions also had much higher κ values relative to first- and second-codon positions (Table 2). These dynamics are illustrated in Figures 1 and 2. The leveling off of the curve of transitions versus transversions for third positions (Figure 1) implies saturation at these sites in some comparisons. Removal of all third-position sites from the data set resulted in 26 equally parsimonious trees and caused *Motacilla* and *Anthus* to become paraphyletic, suggesting that the third positions contain some signal. Indeed, third-position-only transversion parsimony (where third-position transversions are upweighted relative to other substitutions at all codon positions; see Yoder et al., 1996) retained *Anthus* and *Motacilla* as monophyletic genera.

TABLE 3. Values of α and κ estimated from the initial maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) tree topologies.

Method	α	κ
MP	0.305	4.580
NJ	0.307	4.556
ML	0.308	4.543

Phylogenetic Analyses

An equally weighted (i.e., unweighted) parsimony analysis resulted in three most-parsimonious trees of 1174 steps (Fig. 3a), which varied only in their placement of *Motacilla cinerea*, *flava*, and *alba* relative to each other. Passeridae is placed as the closest outgroup to Motacillidae in a very pectinate

TABLE 2. Overall and codon-specific dynamics of the cytochrome *b* gene across all taxa estimated by using PAML and the unweighted ML tree (Fig. 3c).

Dynamic	Codon position			
	First	Second	Third	All
Mean base composition[a]	24, 24, 22, 30	13, 20, 42, 25	3, 45, 6, 46	13, 28, 25, 34
Mean nucleotide bias	0.004	0.061	0.192	0.030
α	0.299	0.152	1.352	0.308
κ	4.493	3.175	21.512	4.543

[a]Base composition is presented as %G, %A, %T, %C averaged over all sequences.

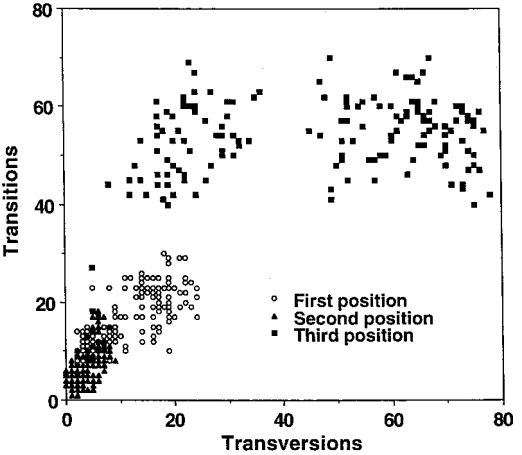

FIGURE 1. Codon-specific plots of observed numbers of transitions versus transversions for the motacillid mitochondrial cytochrome *b* gene.

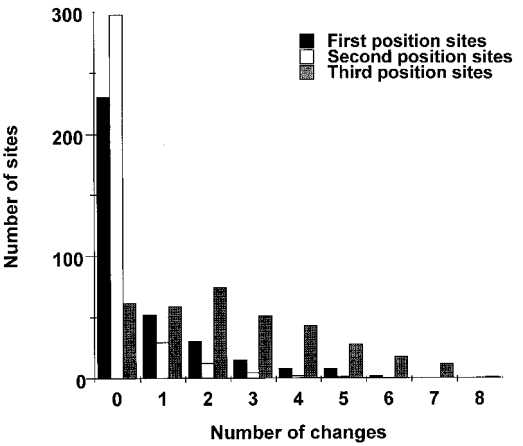

FIGURE 2. Codon-specific analysis of among-site rate variation in the motacillid cytochrome *b* sequence data.

(ladder-like) tree. This tree was not well supported, as indicated by bootstrap analysis, in which all intergeneric relationships were unresolved (i.e., < 50% bootstrap support [BS]), as were most familial relationships (Fig. 3a).

A MP step-matrix analysis that weighted tranversions by the overall κ value (Table 2) resulted in a single most-parsimonious tree, which differed from the equally weighted analysis (Fig. 3a) only in the switching of the *Macronyx* and *Tmetothylacus* nodes. There was slightly better bootstrap resolution of relationships, in that all pipit and longclaw genera were grouped together with moderate (60%) BS. Relationships between genera on this node, as well as between this node and *Dendronanthus* and *Motacilla*, nonetheless had low BS.

An MP analysis employing codon-specific step-matrices (i.e., weighting each position by its respective κ value) produced a single most-parsimonious tree whose topology was nonpectinate (Fig. 3d). The number of nodes supported at > 50% both within Motacillidae and among the closest outgroups was one greater than those of the equally weighted bootstrap analysis (Fig. 3a).

Results of the NJ analysis utilizing default values (Fig. 3b) strongly support the monophyly of Motacillidae. This result was robust to uncorrected, Kimura two-parameter (Kimura, 1980) and ML distance methods. Because of its placement with *Macronyx* and *Tmetothylacus* rather than *Anthus*, *Hemimacronyx* is supported as a valid genus in this analysis. The tree suggests that *Dendronanthus* and *Motacilla* are sisters. Prunellidae here is most closely related to Motacillidae. As in the MP analysis, there is strong support that Prunellidae and Passeridae are closer to Motacillidae than are the other outgroup taxa considered here.

An NJ analysis performed with overall rates of κ and α did not result in changes in tree topology, regardless of which distance measure was used. However, NJ analysis using distances generated from codon-specific κ and α values (Fig. 3e) changed the relationships of all *Motacilla* from those in the initial NJ analysis (Fig. 3b); unfortunately, the available programs did not permit a bootstrap test in this case. However, because species sampling can have a large impact on tree topology (Lecointre et al., 1993), and because we included only half of all *Motacilla* species in this analysis, neither tree topology can truly be considered a better estimate of relationships within this genus.

Initial ML analysis in PAUP* (HKY85Γ model) resulted in a tree with log likelihood of -7154.06. This tree (Fig. 3c) differed from

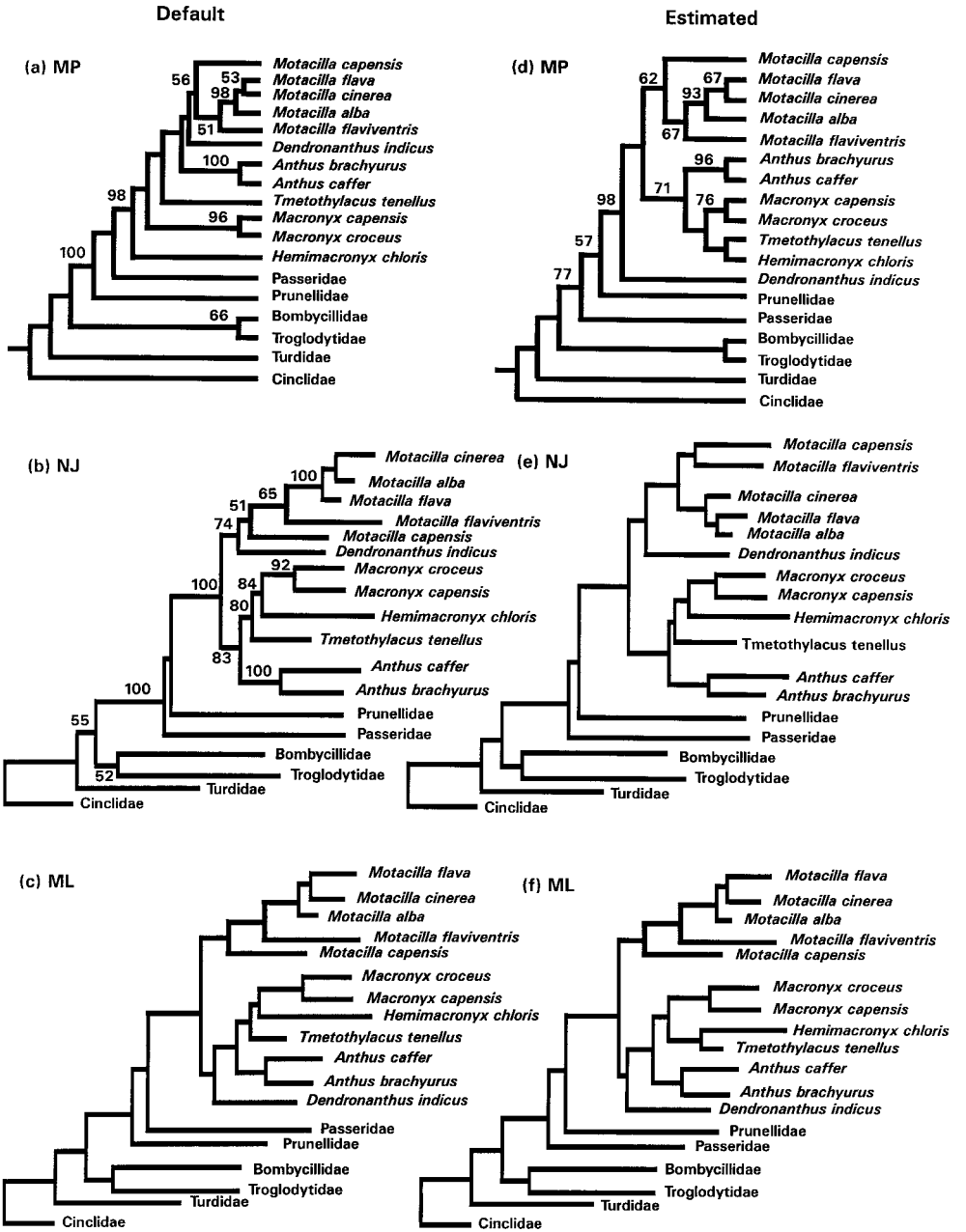

FIGURE 3. Phylogenetic relationships among motacillid genera based on default parameters (a–c) and on overall parameters estimated for cytochrome *b* (d–f). Numbers at nodes represent BS > 50% for 100 replicates.

the initial NJ tree only in that *Dendronanthus*, rather than *Motacilla*, was basal to the pipits and longclaws. Passeridae was placed as the closest outgroup to Motacillidae.

An ML analysis employing the overall α and κ values of 0.308 and 4.493 (Table 2), respectively, resulted in a change in tree topology (Fig. 3f) from the initial ML estimate

(Fig. 3c). *Hemimacronyx* was placed as sister to *Tmetothylacus*, and Prunellidae was placed as the closest outgroup to Motacillidae; this tree is otherwise consistent with the initial ML estimate, indicating robustness of ML to model of evolution. In addition to these tree changes, there was a significant improvement in likelihood score under the new model as compared with that for the default model on this new tree (Table 4).

Although this second ML tree is a significant improvement of relationships in comparison with the initial tree, we wondered whether an analysis utilizing an even more complex model of evolution, based on our picture of evolutionary dynamics of cytochrome *b* (Table 2), provided an improved fit of the data to this tree topology? To test this idea, we summed the likelihoods of each codon position on the second ML tree by using codon-specific dynamics; we obtained yet another highly significant improvement in log-likelihood score (Table 4). Unfortunately, we are unable to determine whether this codon-specific likelihood score could be coincident with a further change in tree topology with available programs; PAUP* does not yet accept multiple values of α (D. Swofford, pers. comm.), and PHYLIP is unable to assign specific values of α to specific sites (M. Kuhner, pers. comm.). Given the improvement in score when codons are analyzed independently from one another, a change in topology is entirely possible.

We were unable to reject statistically (via the Kishino–Hasegawa test) any of the tree topologies presented in Figure 3 as being significantly worse estimates of phylogenetic relationships. There is, however, an increase in congruence between default (or unweighted) and weighted tree topologies, as determined by Rohlf's (1982) consensus index. The average congruence between the unweighted MP, NJ, and ML topologies was 0.502 (Table 5), whereas the average congruence between the weighted MP, NJ, and ML topologies was 0.679 (Table 5). MP proved sensitive to which parameters were used; NJ and ML were not (Table 5).

Discussion

Cytochrome b *Dynamics*

Table 3 shows that the estimates of κ (transition bias) and α (among-site rate variation) were robust to the initial tree chosen; this is not always the case. Yang (1996a) and Wakeley (1996) showed that these rates could be seriously underestimated if the tree topology used to estimate the rates is completely wrong. Incorrect estimates may depend on how many wrong long branches are in the tree being used; in our motacillids and many other family-level trees (see below) there are few long internal branches. It remains to be seen how general the relationship between sensitivity to tree topology and branch lengths is.

As expected for a protein-coding gene like cytochrome *b*, there was ample evidence for variation in substitution rate among codon positions (Figs. 1 and 2; Table 2). Interestingly, we found substantial among-site rate variation within each codon position, the rate variation being most extreme within the first and second positions (Table 2). Within-codon-position rate variation is probably due to functional constraints on protein products; attempts to assess these functional constraints have only recently been undertaken for birds (e.g., Griffiths, 1997). κ also varied substantially between codon positions, with third positions showing very high bias compared with first and second positions (Table 2).

Table 4. Likelihood ratio tests of differences in log-likelihood estimates between increasingly more complex models of cytochrome *b* evolution utilizing Motacillidae and outgroup data.

Estimated values of κ and α	Log likelihood	Significantly better estimate?
None (default options)	-7154.06	
Overall	-6699.99	yes; $P << 0.001$
Codon-specific	-6195.41	yes; $P << 0.001$

TABLE 5. Measures of congruence among tree topologies in Figure 3, using Rohlf's (1982) consistency index.

	Default			Estimated		
	MP	NJ	ML	MP	NJ	ML
Default						
MP	—					
NJ	0.508	—				
ML	0.338	0.660	—			
Estimated						
MP	0.286	0.702	0.638	—		
NJ	0.460	0.915	0.596	0.638	—	
ML	0.286	0.702	0.870	0.761	0.638	—

Our exploration of among-site rate variation (Fig. 2) suggests that removing entire classes of characters may not be the best course of action, because some changes in those sites appear unsaturated and thus may be phylogenetically informative (e.g., third positions, Fig. 2). Some sites and changes within such classes can be evolving more slowly than some sites in nonsaturated classes of characters (Fig. 1). For example, 59 third-position sites with one change show less lability than do 82 first- and second-position sites with two or more changes per site (Fig. 2).

Although we showed (Table 4) that a model incorporating specific parameters was an improvement over the use of a single κ and α, the data in Figure 2 suggest other fruitful lines of analysis. A rate-specific model that could account for site-specific heterogeneity regardless of codon position might prove a better estimator of phylogeny and dynamics. We briefly explored this idea by placing all sites into one of three categories: slow sites (those changing 0 to 2 steps in Fig. 2), medium sites (3 to 4 steps), and fast sites (5 to 8 steps). We again used PAML with the ML tree to estimate κ and α for each category and then used these values to estimate likelihood scores on the tree in Figure 3f by using PAUP*. By summing these scores, we obtained a likelihood score of -6108.593, a reduction of 85 units from the codon-specific score (Table 2). Although this improvement may be nonsignificant (these scores are not comparable by use of the LRT), the trend does suggest that finer partitioning of sites would almost surely improve fit of models to data and possibly to tree topology.

The dramatic improvements of likelihood seen while using increasingly complex models (i.e., default values vs. overall dynamics vs. codon-specific dynamics) suggests that cytochrome *b* evolution cannot be described as either a "fast" or a "slow" gene (Meyer, 1994) nor as one with an overall high transition bias. Codon-specific dynamics thus represent a better description of cytochrome *b* evolution than do the overall dynamics employed in most studies with this gene.

We found differences in base composition between codon positions but little difference in base composition within any given codon position across motacillid species; interspecific variation in base composition of third positions was the most extreme (not shown). Our base composition findings were similar to those reported from other avian studies, and base composition at each codon position was found to vary little across many distantly related bird lineages, such as *Ramphocelus* tanagers (Hackett, 1996), birds-of-paradise (Nunn and Cracraft, 1996), and several suboscine and oscine passerine taxa (Edwards et al., 1991), as well nonpasserine taxa (e.g., some cranes [Krajewski and King, 1996] and woodpeckers [Cicero and Johnson, 1995]). This similarity in base composition among diverse avian lineages indirectly suggests similarity in cytochrome *b* dynamics (Sueoka, 1992; Jermiin et al., 1995). Equilibrium base compositions are probably due to patterns of directional mutation pressure (Sueoka, 1992), and the composition of sites with very biased compositions may reflect the underlying mutation spectrum more faithfully than sites with an even base composition (Jermiin et al., 1995). Thus

base composition can be a useful surrogate for substitution dynamics, unless patterns of selection vary widely across species, which is unlikely for cytochrome *b*. This similarity in base composition raises the exciting possibility that patterns of base substitution in cytochome *b* are similar across birds, a prediction that can be tested with further analyses.

The κ values estimated for other birds have varied by study and gene region (Cooper et al., 1992; Austin, 1996; Krajewski and King, 1996; Edwards, 1997), and inevitably these empirically derived values (1:3.8–1:9 for all codon positions in the cytochrome *b* segment examined) differ from those most commonly used in other studies (e.g., 1:2, 1:5, 1:10). Edwards (1997) found that estimates of κ and α varied, depending on the taxonomic level investigated and the depth of the tree. Because no bird studies have yet applied standard errors to estimates of κ, we do not know the extent to which these results are compatible with one another nor how much our estimates would change were we to examine, say, cytochrome *b* variation within *Anthus* alone. In addition, although previous ML estimates of κ in birds are to be commended, none of the previous estimates assumed among-site rate variation. Because Wakeley (1993) showed that κ and α are not independent and need to be estimated simultaneously, these earlier estimates are compromised.

Does incorporating these dynamics improve phylogenetic signal and consistency? Yes and no. NJ proved resistant to whether default or estimated parameters were employed (Fig. 3; Table 5). However, using estimated dynamics in ML analyses is clearly superior to using default values (Table 4), and in our MP analyses, the number of nodes supported at > 50% increased from 9, determined by using default values, to 10, determined by using overall rates or codon-specific rates (Fig. 3). Using overall rates provided less support within Motacillidae than did codon-specific rates (6 resolved nodes vs. 7). When we increased the criteria for resolution to 60% BS, overall or codon-specific rates also provided for higher levels of support for nodes within the Motacillidae than did default values (Fig. 3a: 3 nodes > 60%, Fig. 3d: 7 nodes > 60%), and total BS within Motacillidae improved from 583 when using default parameters to 673 and 656 with use of overall and codon-specific parameters, respectively. Incorporating the estimated κ value also increased congruence of MP to other tree topologies (Table 5) and resulted in greater average congruence among methods as well (although this does not necessarily imply correct relationships; Felsenstein, 1993). Our results suggest that a posteriori weighting has improved the detection of signal in our motacillid data.

Despite improvement in signal, we were unable to reject statistically any of the trees in Figure 3 via the Kishino–Hasegawa (1989) test. This may be a function of rapid evolution, rather than some inherent problem of using cytochrome *b* in phylogenetic analysis. Branch lengths linking *Dendronanthus* and the non-*Anthus* pipits to other genera are relatively short compared with other branches, perhaps suggesting that rapid evolution (similar to a star phylogeny) has occurred in this group and thus confounding any estimation of phylogenetic relationships from cytochrome *b*.

Our inability to determine whether Prunellidae or Passeridae is closer to Motacillidae (see below) may also be a function of rapid evolution. In their treatment of Passeridae, Sibley and Ahlquist (1990) placed Motacillinae between Prunellinae and Passerinae, stating that the divergences within Passeridae were so close together that they could not be certain of the exact sequence of branchings. Sibley and Ahlquist (1990) also suggested that these branchings of passerid lineages occurred within a period of 5 million years and that the branchings may be represented as a multifurcation. Other studies (Bleiweiss et al., 1994; Sheldon and Gill, 1996) have experienced similar difficulties in resolving higher-level avian relationships and have argued that short internodal distances suggests short time intervals between the divergences of major groups. Thus, the lack of resolution for the groups considered here should not be broadly interpreted as another strike against using cytochrome *b* in molecu-

lar systematics, despite the fact that poor resolution of many above-species-level trees in birds by cytochrome *b* is becoming commonplace. Indeed, we know that the poor resolution in our trees is not strictly a function of cytochrome *b*, because this gene has been shown to resolve branches that are older than or approximately equal to (e.g., Moore and DeFilippis, 1997) those in this study. A litmus test for poorly resolved groups will be to use a different, e.g., nuclear, gene on the same group to determine whether phylogenies derived from them are well-resolved and thus able to discriminate between bad gene or bad trees/groups in previous phylogenetic estimates.

The most frequently employed alternative to choosing an a priori weighting scheme is to use the default a priori weighting scheme of equal weight (i.e., unweighted) at all sites; thus, it bears repeating that the a priori assumption of equal weights is itself a strong assumption (Swofford et al., 1996) that can itself be more arbitrary than weights based on empirical and theoretical considerations (Simon et al., 1994). Many studies criticizing the use of cytochrome *b* are in effect saying that the methods of analysis and our understanding of cytochrome *b* dynamics are inadequate. Cytochrome *b* may well have complex dynamics that result in fewer sites that are parsimony-informative by standard methods, but ML estimates of ts:tv and other rates, obtained from the relevant sequence data and applied a posteriori, appear to be one way of improving estimates of phylogenetic relationships (e.g., Sullivan and Swofford, 1997; this study). Although not problem-free (e.g., Yang, 1996), additional benefits of using ML to estimate phylogenetic relationships from sequence data are its insensitivity to problems such as base composition inequalities, multiple substitutions, models of DNA substitution, and number of taxa—problems that are inherent in other methods (Huelsenbeck, 1995; Perna and Kocher, 1995; Schöniger and von Haeseler, 1995; Strimmer and von Haeseler, 1996; Swofford et al., 1996; see also Naylor and Brown, 1997). Even so, the ability to improve trees via weighting seems critically dependent on the nature of the trees themselves, and, if our pipit results are general, the short branches that seem to plague many avian phylogenies may prove resistant to improvement via weighting.

Phylogenetic Relationships

All methods of phylogenetic reconstruction considered here support three groups within the Motacillidae: *Dendronanthus*, wagtails (*Motacilla*), and the pipits plus longclaws (*Anthus, Tmetothylacus, Hemimacronyx, Macronyx*). Both NJ and ML strongly support (and MP weakly supports) the assertion from a morphological study (Cooper, 1985) that the yellow-breasted pipit is not an *Anthus*, and thus we suggest that it is reasonable to place this species in *Hemimacronyx*. Interestingly, removing this species from *Anthus* places all members of the pipit and longclaw group that are almost entirely yellow ventrally together as close relatives (*Tmetothylacus, Hemimacronyx, Macronyx*).

MP (Fig. 3a, d) fails to support, and NJ and ML (Figs. 3e, f) conflict regarding, the placement of *Dendronanthus*. The BS of the initial NJ tree is moderate (Fig. 3b). Despite a resemblance to *Motacilla* in overall appearance of plumage, most behavioral and morphological similarities support the placement of *Dendronanthus* near *Anthus*, as suggested by the ML tree. For example, when on the ground, *Dendronanthus* resembles a pipit (Ali and Ripley, 1973); also, like some *Anthus*, it spends much time in trees, is associated with woodlands, and is not closely associated with water, as *Motacilla* is (Neufeldt, 1961). Also, *Dendronanthus* eggs resemble those of *Anthus hodgsoni* (Neufeldt, 1961). Based on these resemblances to *Anthus*, we support the placement of *Dendronanthus* suggested by the ML tree (Fig. 3f).

All successive approximation analyses support Prunellidae as the nearest outgroup to Motacillidae, although Passeridae is closer in some default analyses (Fig. 3). Both of these findings are consistent with the findings from DNA–DNA hybridization, which suggested a close relationship between these three lineages (Sibley and Ahlquist, 1981, 1990; see above). All methods support Prunellidae and Passeridae as

being closer to Motacillidae than are other outgroups considered here, but a more-detailed analysis of familial relationships is necessary before a definitive statement can be made.

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