Body Size Effects and Rates of Cytochrome *b* Evolution in Tube-Nosed Seabirds

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Variation in rates of molecular evolution now appears to be widespread. The demonstration that body size is correlated with rates of molecular evolution suggests that physiological and ecological factors may be involved in molecular rate variation, but large-scale comparative studies are still lacking. Here, we use complete cytochrome *b* sequences from 85 species of tube-nosed seabirds (order Procellariiformes) and 5 outgroup species of penguins (order Sphenisciformes) to test for an association between body mass and rates of molecular evolution within the former avian order. Cladistic analysis of the 90 sequences estimates a phylogeny largely consistent with the traditional taxonomy of the Procellariiformes. The Diomedeidae, Procellariidae, and Pelecanoididae are monophyletic, while the Hydrobatidae are basal and paraphyletic. However, the two subfamilies within the Hydrobatidae (Hydrobatinae and Oceanitinae) are monophyletic. A likelihood ratio test detects significant deviation from clocklike evolution in our data. Using a sign test for an association between body mass and branch length in the seabird phylogeny, we find that larger taxa tend to have shorter terminal branch lengths than smaller taxa. This observation suggests that rates of mitochondrial DNA evolution are slower for larger taxa. Rate calibrations based on the fossil record reveal concordant body size effects. We interpret these results as evidence for a metabolic rate effect, as the species in this order exhibit large differences in metabolic rates, which are known to be highly correlated with body mass in this group. Our results support previous findings of body size effects and show that this effect can be significant even within a single avian order. This suggests that even lineage-specific molecular clocks may not be tenable if calibrations involve taxa with different metabolic rates.

Introduction

Rates of molecular evolution are known to vary widely among phylogenetic groups (Wu and Li 1985; Britten 1986; Hasegawa and Kishino 1989; Kocher et al. 1989; Krajewski 1990; Mindell et al. 1996; Fieldhouse, Yazdani, and Golding 1997). This has led to the rejection of a universal molecular clock and has prompted investigators to search for possible causes of rate heterogeneity. Although it is unlikely that rates of molecular evolution are determined by a single factor, body size has repeatedly surfaced in studies of rate variation as a correlate to rates of molecular evolution. In general, body size effects are thought to manifest themselves in three ways: (1) as generation time effects; (2) as population size effects; and (3) as metabolic rate effects, with smaller body size being associated with shorter generation times, larger population sizes, and higher metabolic rates. While other factors have also been associated with variation in rates of molecular evolution (e.g., Prager et al. 1974; Avise and Aquadro 1982; Britten 1986; Adachi, Cao, and Hasegawa 1993; Mindell et al. 1996), we focus on generation time, population size, and metabolic rate, since all have explicit mechanisms (Kohne 1970; Ohta 1972, 1992; Martin and Palumbi 1993)

and have received considerable attention in the literature.

The Generation Time Hypothesis

The generation time hypothesis (Kohne 1970) has gained widespread acceptance among evolutionary biologists. It has been used to explain higher substitution rates for monkeys than for humans (Li and Tanimura 1987; Chang et al. 1994) and higher rates for rodents than for primates (Wu and Li 1985; Bailey et al. 1991; Chang et al. 1994). Additional evidence for generation time effects has also come from avian studies (Moores and Harvey 1994). The basic tenet of the generation time hypothesis is that organisms with shorter generation times should have a greater number of germ cell divisions per unit time and, therefore, a concomitantly higher mutation rate. This holds if the majority of mutations are the result of errors during DNA replication, if the number of germ cell divisions are roughly similar per generation in most organisms, and if the majority of mutations are neutral (Kimura 1979).

Despite the support for the generation time hypothesis there is still much that needs to be established before we can accept it as a general explanation for rate heterogeneity. In particular, the assumption of equal numbers of germ cell divisions per generation across organisms remains to be tested outside of model mammalian systems. Thus, while evidence from humans and rodents is consistent with generation time effects, the ability of this hypothesis to explain rate variation outside such model systems has yet to be established.

Population Size Effects

The notion that population size can affect rates of molecular evolution derives from the nearly neutral theory (Ohta 1972, 1992). Specifically, the nearly neutral

theory predicts that populations with small effective sizes (i.e., N_e) will experience faster rates of evolution than populations with large effective sizes. This follows because slightly deleterious mutations, i.e., those that are nearly neutral, are more likely to be fixed in a smaller population. Thus, smaller populations will experience a higher fixation rate for slightly deleterious mutations than will larger populations due to the increased influence of drift over selection.

Evidence for the nearly neutral theory comes mainly from *Drosophila* and mammals. DeSalle and Templeton (1988) found that rates of mtDNA evolution for Hawaiian *Drosophila* that had undergone recent bottlenecks were faster than those for lineages which had maintained larger populations. Ohta (1992) found that the data published by Aquadro, Lado, and Noon (1988) and Aquadro (1990) for *D. melanogaster* and *D. simulans* fit the expectations of the nearly neutral theory. Patterns of DNA variation in mammals are also consistent with the nearly neutral theory (Ohta 1993, 1995; Nachman, Boyer, and Aquadro 1994; Nachman et al. 1996; Rand and Kann 1996). However, the generality of the nearly neutral theory is still an open question. If rates of molecular evolution are influenced by population size, then the majority of substitution events must involve slightly deleterious mutations, since the nearly neutral theory applies only to slightly deleterious mutations. In addition, Gillespie (1995) has argued that the nearly neutral theory critically depends on biologically unreasonable assumptions, both in its original (Ohta 1972) and modified (Ohta 1992) forms.

The Metabolic Rate Hypothesis

In recent years, many investigators have also found evidence for an association between rate heterogeneity of DNA sequence evolution and the thermal habits of various organisms (see Rand 1994 and references therein). Since metabolic rate is known to influence the thermal characteristics of vertebrate physiological environments, metabolic rate has been implicated in some studies of molecular rate heterogeneity. This led Martin and Palumbi (1993) to propose the metabolic rate hypothesis, which states that rates of molecular evolution should be positively correlated with metabolic rates. The logical basis of their hypothesis is twofold. First, increased rates of DNA replication and nucleotide replacement in organisms with higher metabolic rates should lead to higher mutation rates. Second, the increased concentrations of free oxygen radicals in cells with higher metabolic rates should be associated with a higher incidence of DNA damage. Support for this hypothesis comes from several observations. Rates of mitochondrial DNA synthesis are higher in tissues with higher metabolic rates (Gross, Getz, and Rabinowitz 1969). Similarly, small organisms have more mitochondria per unit body mass than large organisms, suggesting higher rates of mtDNA replication for smaller taxa. Finally, free oxygen radicals, the byproducts of metabolic respiration, are known mutagens (Shigenaga, Gimeno, and Ames 1989).

While the evidence supporting the metabolic rate hypothesis is compelling, problems remain. That whole-body metabolic rates are indicative of metabolic rates in female gonadal tissues, which are the relevent variable for mtDNA evolution, remains to be established (Mindell et al. 1996). In addition, the majority of studies which address metabolic rates and rates of molecular evolution have compared taxa from different classes (e.g., fish and amphibians vs. mammals [Thomas and Beckenbach 1989], sharks vs. mammals [Martin, Naylor, and Palumbi 1992], and turtles vs. mammals [Avise et al. 1992]). Complicating these comparisons are the many other factors that differ between such distantly related groups. More recently, Martin and Palumbi (1993) found evidence for a metabolic rate effect within primates. Taken together, these studies suggest that metabolic rate effects can manifest themselves at two different levels: (1) endotherms versus ectotherms, and (2) within endothermic lineages that vary in metabolic rate. However, detailed studies at this second level are still lacking.

Testing For Rate Heterogeneity

Since heterogeneity in rates of molecular evolution can have important consequences for studies that rely on DNA sequence data for molecular systematics and rate calibrations and studies of molecular evolution, insights into sources of heterogeneity have broad ramifications. Perhaps the most common method used to test for rate variation is the relative-rate test (Sarich and Wilson 1967; but see also Mindell and Honeycutt 1990; Muse and Weir 1992). Using this procedure, one can determine if rates of evolution are the same for two lineages by comparing how divergent they are from a known outgroup. While in some cases this can be a powerful tool for investigating rate heterogeneity, certain properties of this test make it less appropriate when large numbers of taxa are involved. When a large data matrix is analyzed, there will be many relative-rate tests possible, and it is unclear how many tests will need to be positive or negative to achieve statistical significance (Sheldon 1987; Gaut et al. 1992). Therefore, other approaches are necessary when many species are being compared.

One class of methods employed for detecting rate heterogeneity in large data sets involves comparing the fit of the data to a particular tree both with and without the assumption of a molecular clock. If relaxing the assumption of a molecular clock significantly improves the fit of the data based on some criterion, then clocklike evolution can be rejected. There are two primary methods available that utilize this approach: a maximum-likelihood method (Felsenstein 1988) and a least-squares method (Rohlf and Sokal 1981).

To specifically test for body size effects, one would ideally compare species that differ significantly in body size but are identical in other ways that might also contribute to rate variation (Rand 1994). In nature, the closest thing to this scenario is the existence of monophyletic and closely related species with significantly different body masses. Few such groups exist, since taxa with significantly different body masses tend not to be close relatives. However, the tube-nosed seabirds (order

Procellariiformes) provide an ideal opportunity among endotherms to test for body size effects, since species within this order display significant size variation. Additionally, metabolic rate is known to be highly correlated with body mass in many species (e.g., Nagy 1987 and references therein).

Here, we test for an association between rates of molecular evolution and body size in this avian order, a diverse radiation of approximately 110 species of pelagic seabirds. Our data set includes complete cytochrome *b* (cyt-*b*) genes from 85 species of tube-nosed seabird, representing all of the major groups within the order, and 5 penguin species (order Sphenisciformes) that serve as an outgroup. To test for rate heterogeneity in our data, we employ a maximum-likelihood ratio test to compare computed likelihoods with and without the assumption of a molecular clock. To assess how rate heterogeneity relates to body size, we use two different methods. First, we use a sign test on 23 phylogenetically independent comparisons to determine if rates of cyt-*b* evolution are associated with body size and to identify the nature of that association. Second, we compare fossil-calibrated rates for three lineages with different body sizes.

Materials and Methods
Sequence Data Collection

Our DNA extraction, PCR amplification, and DNA sequencing protocols for the majority of the new sequences presented here followed those of Nunn et al. (1996). Four exceptions involved *Halocyptena microsoma, Oceanodroma tethys, Oceanodroma melania,* and *Puffinus opisthomelas.* We collected the cyt-*b* sequences from these species using dye-terminator sequencing chemistry electrophoresed on a Model 377 DNA Sequencer (PE Applied Biosystems, Foster City, Calif.) in the manner described by Lee, Feinstein, and Cracraft (1997). Additionally, cyt-*b* sequences of 14 albatross taxa (GenBank accession numbers U48942–U48955) are taken from Nunn et al. (1996).

Phylogenetic Analysis

We conducted maximum-parsimony analyses as implemented in PAUP*, version 4.0d60 (D. Swofford, personal communication). We performed 500 heuristic searches with random addition of taxa to explore a large portion of the possible tree space and to minimize the effect of input order bias. Branch swapping was done using the tree bisection-reconnection (TBR) algorithm. We weighted all characters equally. We prefer equal weighting for several reasons. First, given the dense taxonomic sampling in our study, we feel that the need to differentially weight alternative character state transformations or character partitions is reduced. Second, given that we do not have a priori information with respect to which weighting scheme would be most appropriate for our data, we favor using equal weights in our analyses. Third, since we will use branch length information to test hypotheses concerned with the absolute number of mutational events, we feel that weighted branch lengths

would be meaningless in this context. Furthermore, our preference for equal weights derives from our opinion that any assumptions about evolutionary process in the initial assessment of a pattern in nature will lead to circularity if one then uses that pattern for subsequent investigations of process.

Analysis of Rate Variation

We tested for rate heterogeneity in our data set using a maximum-likelihood ratio test (Felsenstein 1988; Weir 1996; but see also Huelsenbeck and Rannala 1997). The test involves comparing an unconstrained likelihood score (L_i) to one under a null model with a molecular clock enforced (L_0) using the quantity $-2 \ln(L_0/L_i)$. The value is then used as a single-degree-of-freedom chi-square test statistic (Weir 1996, p. 371). The purpose of the test is to determine if a maximum-likelihood analysis that allows rates to vary between lineages provides a significantly better fit to the data than one that enforces a molecular clock. One advantage of this method over others, such as the least-squares method of Rohlf and Sokal (1981), is that rate variations across sites and between different types of character changes (i.e., transitions and transversions) can be taken into account during the likelihood computation. Utilizing the maximum-parsimony topologies found by heuristic search (see above), we constrained PAUP*, version 4.0d60 (D. Swofford, personal communication), to compute likelihood values for these topologies both with and without the assumption of a molecular clock. In these analyses, transition/transversion ratios were estimated from the data via a maximum-likelihood method. Additionally, rates were allowed to vary among sites using a gamma approximation with a shape parameter of 0.5 and four rate categories.

To investigate the specific effect of body size on rates of cyt-*b* evolution, we used a phylogenetic approach to test for an association between body mass and branch length in our procellariiform phylogeny. The body masses for the taxa in our analysis are from Dunning (1993, pp. 10–15), Marchant and Higgins (1990) or our own data collected in the field (see table 1). If weights were available from multiple individuals, we used mean weight. If only a range of weights was available (e.g., 100–150 g) we used the median value (i.e., 125 g). If weights were available for both males and females, we used female weight, since the maternal nature of mtDNA makes the metabolic rate of the female the relevant variable for our analysis. For branch length estimates, we used the number of unambiguous character state changes calculated in MacClade, version 3, on the maximum-parsimony tree (Maddison and Maddison 1992).

Using this information, we compared the relative body masses of phylogenetically independent pairs of taxa with the lengths of the branches leading to those taxa. To avoid subjectivity in selecting the taxa for each pair and to reduce possible saturation effects, we restricted our analysis to terminal paired sister taxa only. We further restricted all of our comparisons to pairs with nonzero branch lengths. This provided us with a total of 23 phylogenetically independent comparisons (table 2).

Table 1
Body Mass for 85 Species of Procellariiform Birds and Five Penguin Outgroups

Species	Body Mass (g)	Species	Body Mass (g)	Species	Body Mass (g)
Bulweria bulwerii[a]	99	*Pachyptila turtur*[a]	137	*Pterodroma neglecta*[c]	509
Calonectris diomedea[a]	535	*Pachyptila vittata*[a]	196	*Pterodroma nigripennis*[b]	185
Calonectris leucomelas[b]	500	*Pagodroma nivea*[a]	268	*Pterodroma phaeopygia*[a]	434
Daption capense[a]	428	*Pelagodroma marina*[a]	47	*Puffinus assimilis*[a]	226
Diomedea amsterdamensis[b]	6,270	*Pelecanoides garnoti*[c]	230	*Puffinus bulleri*[a]	380
Diomedea antipodensis[a]	6,900	*Pelecanoides georgicus*[a]	121	*Puffinus carneipes*[a]	568
Diomedea dabbenena[a]	6,900	*Pelecanoides magellanicus*[c]	134	*Puffinus creatopus*[a]	721
Diomedea epomophora[a]	8,200	*Pelecanoides urinatrix*[a]	141	*Puffinus gravis*[a]	849
Diomedea exulans[a]	6,900	*Phoebastria albatrus*[b]	6,900	*Puffinus griseus*[a]	787
Diomedea gibsoni[a]	6,900	*Phoebastria immutabilis*[a]	2,853	*Puffinus huttoni*[a]	364
Diomedea sanfordi[b]	6,670	*Phoebastria irrorata*[a]	3,040	*Puffinus lherminieri*[a]	168
Fregetta grallaria[b]	58	*Phoebastria nigripes*[a]	3,148	*Puffinus nativitatus*[a]	356
Fregetta tropica[b]	56	*Phoebetria fusca*[a]	2,500	*Puffinus opisthomelas*[a]	276
Fulmarus glacialis[a]	479	*Phoebetria palpebrata*[a]	2,785	*Puffinus pacificus*[a]	388
Fulmarus glacialoides[a]	1,000	*Procellaria aequinoctialis*[a]	1,213	*Puffinus puffinus*[a]	453
Garrodia nereis[a]	38	*Procellaria parkinsoni*[a]	675	*Thalassarche bassi*[b]	2,500
Halobaena caerulea[a]	202	*Procellaria westlandica*[a]	780	*Thalassarche bulleri*[b]	2,780
Halocyptena microsoma[a]	21	*Pterodroma cahow*[a]	246	*Thalassarche cauta*[b]	3,700
Hydrobates pelagicus[a]	25	*Pterodroma cookii*[a]	193	*Thalassarche chlororhynchus*[b]	2,200
Lugensa brevirostris[a]	357	*Pterodroma externa*[c]	436	*Thalassarche chrysostoma*[a]	3,264
Macronectes giganteus[a]	3,944	*Pterodroma feae*[a]	312	*Thalassarche eremita*[b]	3,770
Macronectes hallii[a]	4,000	*Pterodroma hasitata*[a]	278	*Thalassarche impavida*[c]	2,700
Oceanites oceanicus[a]	32	*Pterodroma hypoleuca*[a]	176	*Thalassarche melanophris*[a]	3,206
Oceanodroma furcata[a]	55	*Pterodroma incerta*[c]	520	*Thalassarche salvini*[b]	3,590
Oceanodroma leucorhoa[a]	40	*Pterodroma inexpectata*[a]	316	*Thalassoica antarctica*[a]	696
Oceanodroma melania[a]	59	*Pterodroma lessonii*[a]	698	Outgroups	
Oceanodroma tethys[a]	24	*Pterodroma longirostris*[c]	155	*Aptenodytes patagonicus*[a]	13,220
Oceanodroma tristrami[a]	84	*Pterodroma macroptera*[a]	505	*Eudyptes chrysocome*[a]	2,300
Pachyptila desolata[a]	147	*Pterodroma magentae*[b]	490	*Eudyptes chrysolophus*[a]	3,900
Pachyptila salvini[a]	164	*Pterodroma mollis*[a]	312	*Pygoscelis antarctica*[a]	4,150
				Pygoscelis papua[a]	5,500

NOTE.—Values are for adult birds and are for females if weights were available for both genders. Mean values are given when multiple weights were available for a single species. Median values are given when only a range of weights was available.

[a] Value taken from Dunning (1993).

[b] Value taken from Marchant and Higgins (1990).

[c] Value measured by us in the field.

We then counted both the number of times the larger taxon from each pair was associated with a longer branch (fig. 1*a*; represented by "+, +" in table 2) and the number of times the larger taxon in a given pair was associated with a shorter branch (fig. 1*b*; represented by "+, −" in table 2). We were interested in whether or not the "+, +" and "+, −" outcomes that we observed were both present in equal proportions. Since such a population of counts should be binomially distributed (Sokal and Rohlf 1981, p.449), we simply tested the hypothesis that $P = 0.5$. We used a one-tailed test, since we were testing whether or not there was a greater number of "+, −" counts (where the larger taxon is associated with a shorter branch) than expected, which is the predicted outcome if there is a body size effect consistent with the metabolic rate and generation time hypotheses.

As a second test for body size effects, we directly calibrated molecular evolutionary rates from first-appearance fossils for three monophyletic groups with significantly different body masses (orders Diomedeidae and Procellariidae and subfamily Oceanitinae) to see if the effect of metabolic rate on rates of evolution is consistent with the metabolic rate hypothesis. The body sizes of these groups vary from some of the largest known flying birds, the albatrosses (Diomedeidae), to the 'sparrow-sized' storm petrels (Oceanitinae). The first-appearance fossil dates used for this analysis are from Warheit (1992). To calibrate rates for these groups, we simply divided the maximum percentage of sequence divergence within each group using both uncorrected distances and a Kimura two-parameter (K-2) corrected distance (Kimura 1980) by the first-appearance fossil date for the group. While there are problems with this method of calibrating rates, certain aspects of our analysis prevent us from using more conventional methods (see below). As a result, we concentrate on the relative values over the absolute values for the rate estimates that we obtain.

Results and Discussion
DNA Sequences

Cyt-*b* sequences from 14 taxa in the Diomedeidae have previously been reported (Nunn et al. 1996). The additional sequences reported here, including the five penguin outgroup sequences, have been deposited in GenBank under accession numbers U74331–U74341, U74343–U74346, U74348–U74351, U74353–U74357, and AF076044–AF076095. Comparison of these cyt-*b*

Table 2
Sign Test Comparisons of Both Mass and Branch Length for 23 Pairs of Taxa

Species Pair	Body Masses (g)	Terminal Branch Lengths[a]	Signs of Body Mass vs. Branch Length Differences[b]
Calonectris diomedea/C. leucomelas	535/500	13/22	+, −
Diomedea dabbenena/D. sanfordi	6,900/6,670	12/15	+, −
Fregetta grallaria/F. tropica	58/56	31/45	+, −
Fulmarus glacialoides/F. glacialis	1,000/479	21/17	+, +
Macronectes hallii/M. giganteus	4,000/3,944	2/4	+, −
Oceanodroma furcata/Hydrobates pelagicus	55/25	42/37	+, +
Oceanodroma tethys/Halocyptena microsoma	24/21	37/23	+, +
Oceanodroma tristrami/O. leucorhoa	84/40	43/41	+, +
Pachyptila salvini/P. desolata	164/147	6/2	+, +
Pelagodroma marina/Garrodia nereis	47/38	45/56	+, −
Pelecanoides magellanicus/P. georgicus	134/121	30/31	+, −
Phoebastria nigripes/P. immutabilis	3,148/2,853	9/11	+, −
Phoebetria palpebrata/P. fusca	2,785/2,500	8/13	+, −
Procellaria aequinoctialis/P. parkinsoni	1,213/675	23/19	+, +
Pterodroma cookii/P. longirostris	193/155	27/32	+, −
Pterodroma externa/P. phaeopygia	436/434	10/17	+, −
Pterodroma lessonii/P. macroptera	698/505	5/7	+, −
Pterodroma mollis/P. cahow	312/246	14/18	+, −
Puffinus assimilis/P. lherminieri	226/168	11/16	+, −
Puffinus creatopus/P. carneipes	721/568	2/4	+, −
Puffinus pacificus/P. bulleri	388/380	18/13	+, +
Thalassarche cauta/T. bulleri	3,700/2,780	6/11	+, −
Thalassarche chrysostoma/T. impavida	3,264/2,700	8/13	+, −

[a] Branch lengths are based on one of four equally parsimonious trees and represent unambiguous character state changes as calculated in MacClade, version 3.06 (Maddison and Maddison 1992).

[b] Sign test scores for the relative body mass and branch length differences, respectively. A "+, +" score indicates that the larger of the two taxa was associated with a longer branch, and a "+, −" score indicates that the larger taxon was associated with a shorter branch.

sequences indicated no evidence of insertions or deletions, i.e., they are all of identical length (1,143 bp including stop codon). For a variety of reasons, we believe these sequences are of purely mitochondrial origin. In all cases, the entire cyt-*b* gene and flanking regions were initially amplified as one contiguous fragment by flanking primers described in Nunn et al. (1996). Further, the sequences can be fully translated using the chicken mitochondrial code (Desjardins and Morais 1990) without nonsense mutations or intervening stop codons. In addition, the colinearly aligned cyt-*b* gene sequences did not reveal an overabundance of first- and second-codon-position changes or a shift in the typical mtDNA transition bias, which can occur when mtDNA has been translocated into the nuclear genome (Arctander 1995). These issues have been discussed in more detail for the Diomedeidae sequences (Nunn et al. 1996), and apply to those sequences presented here as well.

Procellariiform Phylogeny

Our data set of 85 species of procellariiform birds and 5 penguin outgroup taxa contained 525 variable sites, 488 of which were parsimony-informative. Parsimony analysis produced four equally most parsimonious trees of 3,543 steps (consistency index [CI] [excluding uninformative characters] = 0.2241; retention index [RI] = 0.6696). However, the trees differed only in the arrangement of four species from the genus *Puffinus* (*P. opisthomelas, P. puffinus, P. gravis,* and *P. griseus*). The strict consensus of these four trees is highly concordant with many aspects of the traditional taxonomy of the group (fig. 2). Three of the four traditional families (Diomedeidae, Procellariidae, and Pelecanoididae) are monophyletic. The Diomedeidae form a sister group to a lineage which then bifurcates, forming the Procellariidae and Pelecanoididae—confirming relationships predicted by morphology (Warham 1996). The fourth family (Hydrobatidae) is basal to these and paraphyletic. However, the traditional subfamilies within Hydrobatidae (Hydrobatinae and Oceanitinae) each form monophyletic groups.
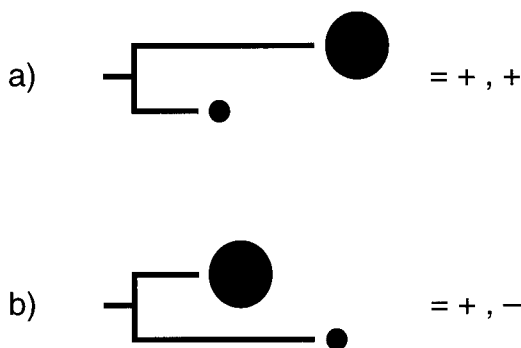

FIG. 1.—The graphic demonstrates how the 23 comparisons for the sign test were scored. Circles represent the relative body masses of the two taxa in a hypothetical example, with the branch lengths proportional to the number of character state changes. When the larger of the two taxa was also associated with a longer branch, i.e., condition *a*, it was scored as "+, +", and when the larger of the two taxa was associated with a shorter branch, i.e., condition *b*, it was scored as "+, −". The metabolic rate hypothesis predicts that condition *b* should prevail in comparisons.
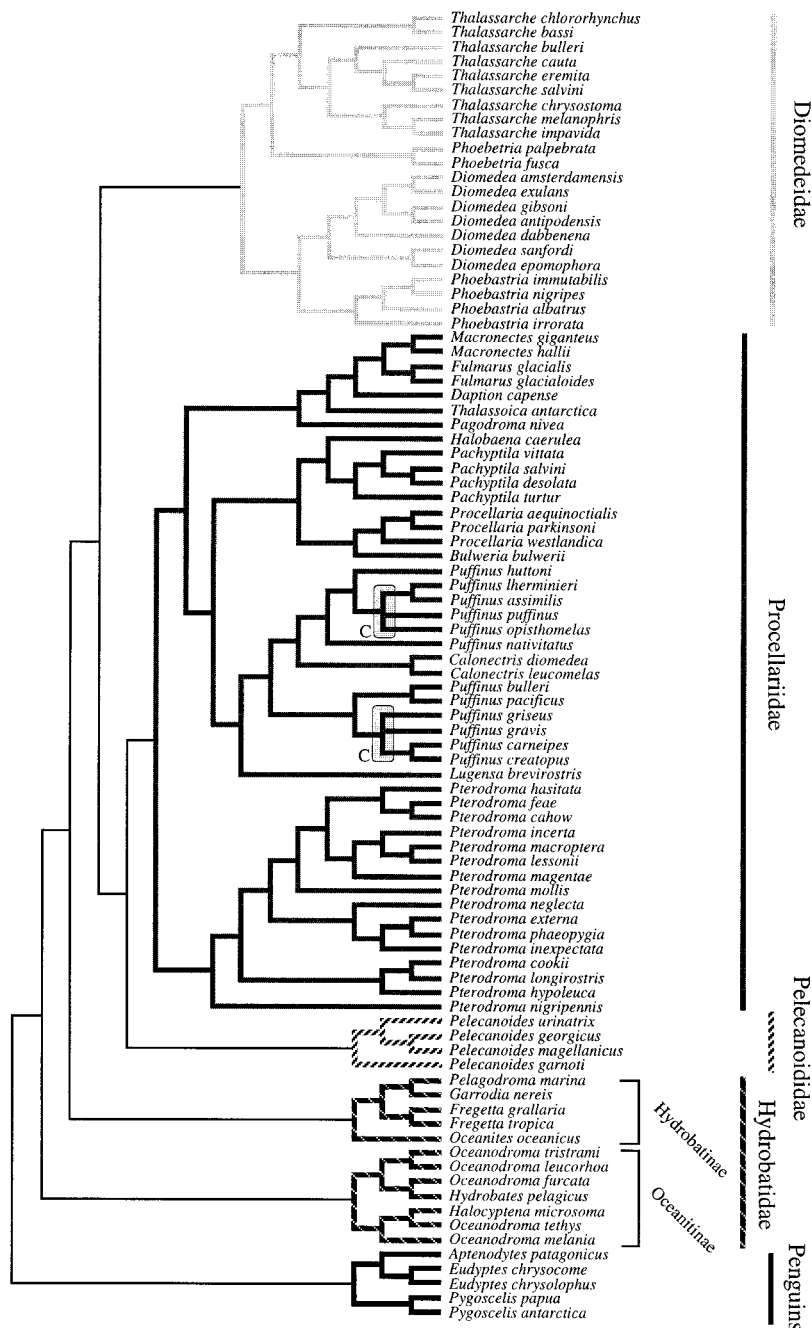
FIG. 2.—Cladistic analysis of 90 complete seabird cytochrome *b* genes, including those of five penguin outgroup species. The consensus tree of four equally most parsimonious trees is shown (length = 3,543 steps; CI [excluding uninformative characters] = 0.2241; RI = 0.6696). The consensus junctures are denoted by the letter C to the lower left of the shaded boxes. The four traditionally recognized families (e.g., see Warham 1996) are denoted by different hatching patterns on the tree and are labeled along the right side. The family Hydrobatidae is paraphyletic, but the traditional subfamilies recognized within it (in brackets) are monophyletic.

Rate Variation
Phylogenetic Approaches

A likelihood ratio test, topologically constrained to the parsimony trees, shows a significant improvement in the likelihood score when the constraint of clocklike evolution is relaxed (tree 1: $\chi^2 = 168$; tree 2: $\chi^2 = 154$; tree 3: $\chi^2 = 170$; tree 4: $\chi^2 = 168$; for all trees, df = 1, $P < 0.001$). Based on these results, we are able to

reject clocklike evolution, demonstrating the presence of rate heterogeneity in our data.

A cursory look at one of the four trees from our parsimony analysis with proportional branch lengths, together with the body masses of the species on the tree, suggests that rates of cyt-*b* evolution in these taxa are affected by body weight, since the smaller species tend to be associated with longer branches (fig. 3). The re-
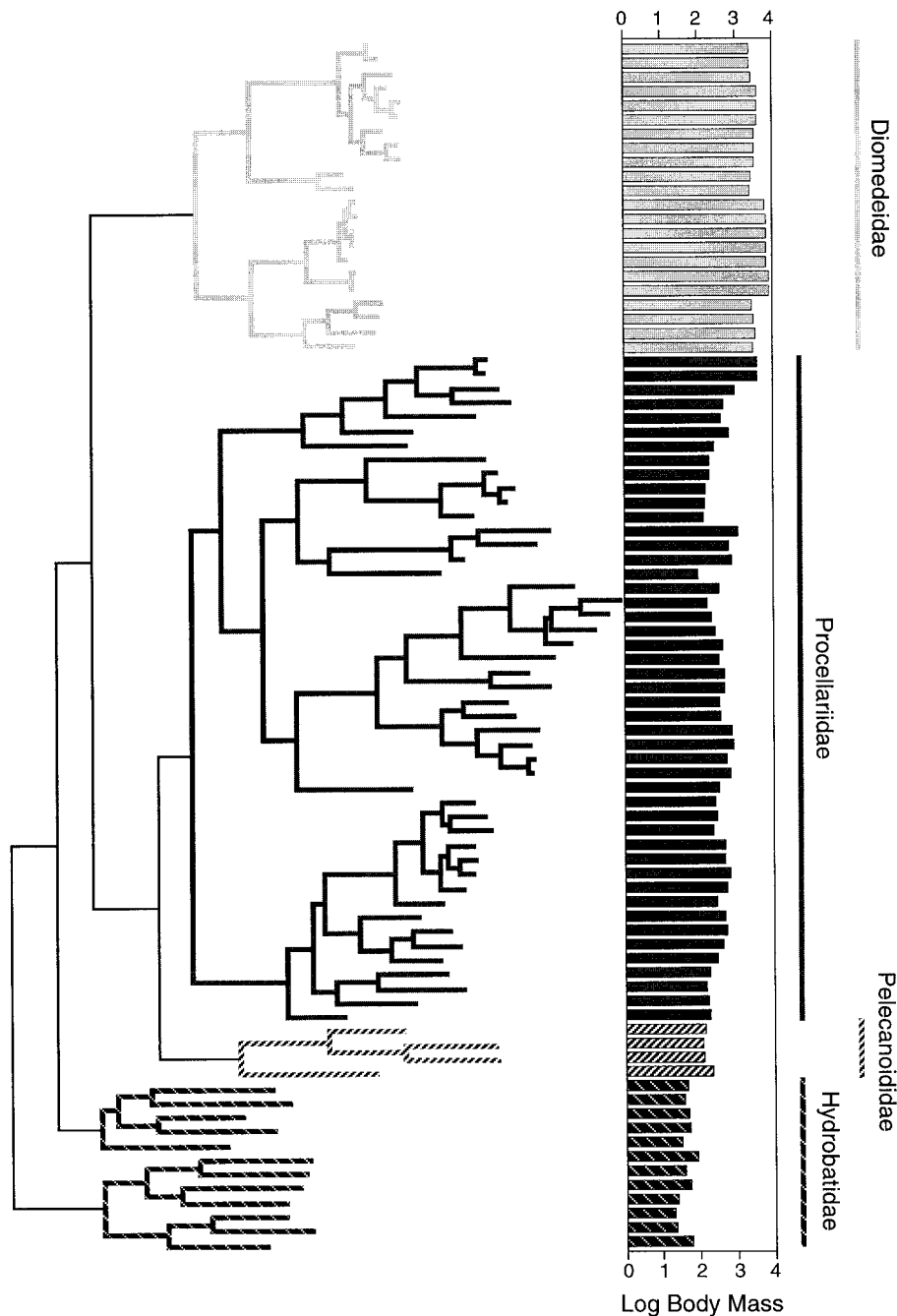
FIG. 3.—A section from one of the four equally most parsimonious seabird trees, shown with branch lengths proportional. For clarity, the outgroup penguin lineage is deleted from the figure because we did not make comparisons to, or within, this group (although topology and branch length computations include the penguin sequences). The bar graph represents the log of the body mass for each procellariiform species on the tree. Note that the branch lengths tend to be inversely proportional to the body masses of the species, and that the body masses vary over three orders of magnitude.

sults of the sign test support this observation. The association between larger taxa and shorter branch lengths (i.e., condition "+, −") is significant ($P < 0.05$), with 16 of 23 pairwise comparisons finding the larger species with the shorter branch (table 2). Since we compare only terminal sister taxa in our analysis, body size effects appear to manifest themselves at even the very tips of the procellariiform phylogeny.

Fossil Calibrations

Our fossil calibrations also indicate that body weight is correlated with rates of cyt-*b* evolution. The largest procellariiform birds have the slowest rate of divergence, 0.62% per Myr (0.88% using a K-2 correction). The intermediate-sized Procellariidae appear to be evolving at an intermediate rate, 0.78% per Myr (0.90% using a K-2 correction). Furthermore, the smallest mem-

bers of the order, those in the subfamily Oceanitinae, showed the fastest rate of evolution in our fossil calibrations at 0.92% per Myr (1.29% using a K-2 correction). Since we are dealing with single-point calibrations, we cannot determine how significant these differences are. In addition, we use a somewhat unconventional method for calibrating our rates, since we use the maximum divergence within each group as the genetic distance value for each calibration rather than comparing each group to its sister taxon. The danger here is that we might underestimate the amount of genetic divergence that has occurred within each group because some basal member has gone extinct or been excluded from our analysis. This would make our rate estimates biased in the direction of slower rates. However, we are interested in whether or not the rates of molecular evolution for each group differ from those of their sister taxa. The traditional method assumes that rates are the same for the group of interest and its sister taxon, and such an assumption would undermine our analysis. Our use of the maximum genetic distance for a given clade in our comparisons also carries the additional assumption that rates of speciation are similar for the three groups in our analysis, since differential speciation rates can affect rates of character change by preserving changes in newly formed species that might otherwise be lost. For these reasons, we present the fossil-calibrated rates simply as a first attempt to quantify the absolute substitution rate for cyt-*b* in these birds and we emphasize their relative values over their absolute values.

Base Composition

Since rate variation has been shown to correlate with shifts in nucleotide composition (Saccone, Pesole, and Preparata 1989), we also tested for differences in nucleotide composition bias in our data. For all 90 sequences in our analysis, we found no evidence of significant heterogeneity in nucleotide composition when all three codon positions were analyzed together ($\chi^2 = 72.38$, df = 252, $P > 0.05$). Nucleotide composition also appears to be homogeneous when we consider each codon position separately (first position: $\chi^2 = 35.7$, df = 252, $P > 0.05$; second position: $\chi^2 = 3.5$, df = 252, $P > 0.05$; third position: $\chi^2 = 217$, df = 252, $P > 0.05$). This suggests that the rate heterogeneity we observe cannot be explained by differences in nucleotide composition bias.

Since differences in nucleotide composition can escape detection in the above analysis if they are restricted to a small number of taxa, we also tested for nucleotide composition differences between the terminal sister taxa involved in our initial sign test for an association between branch length and body mass. We used the summary statistic for nucleotide composition described by Sidow and Wilson (1991) and compared it with branch length in the same manner as for body mass in table 2. This allowed us to determine if nucleotide composition bias could be responsible for the significant association we found between longer branches in our tree and smaller species. We found no significant association between

branch length and nucleotide composition (data not shown).

Body Size Effects
Metabolic Rate Effects

Given the apparent negative association between body mass and rates of cyt-*b* evolution in our data, we interpret our results as supporting the metabolic rate hypothesis. While in other vertebrates, body mass tends to be correlated with physiological and life history traits in addition to metabolic rate (e.g., generation time and population size), the extremely tight correlation between body mass and several measures of metabolic rate in many species of procellariiform birds makes metabolic rate a strong candidate for the cause of the body size effects we observe. Our confidence in this relationship is due to the fact that metabolic rate has been studied in detail for these birds (Ellis 1984), and different measures of metabolic rate are highly correlated with body mass. The regression for field metabolic rate versus body mass in a sample of procellariiform birds has an r^2 of 0.911 (Nagy 1987), and basal metabolic rate and body mass are also tightly correlated for sub-Antarctic Procellariiformes ($r^2 = 0.99$; Adams and Brown 1984). Unfortunately, we cannot directly test for metabolic rate effects, since the taxonomic sample in these studies is relatively small and does not overlap completely with ours, providing us with too few independent contrasts to be useful. However, several features of mtDNA in general, and procellariiform life history in particular, lead us to favor the metabolic rate hypothesis over both the generation time hypothesis and the population size hypothesis (see below).

Generation Time Effects

Since generation time and body size tend to be correlated in vertebrates, it is possible that the body size effects we see are also due to generation time effects. Unfortunately, generation time is not known for most procellariiform birds. Thus, it is difficult to determine the extent to which the relationship that we observe here between rates of evolution and body weight is due to generation time. In a review of the biology of petrels, Warham (1996, p. 18) suggests that body mass and age at first breeding (AFB) may be somewhat decoupled in these birds: ''Although smaller petrels do tend to breed for the first time when they are younger than do the larger ones, the trend is not constant.'' To determine if these two variables are correlated in the species in our analysis, we plotted AFB against the log of body weight (fig. 4) for the species for which AFB is known (Warham 1996, pp. 12–13), and we found that the correlation holds ($y = 2.721x - 0.139$; $r^2 = 0.458$; $P < 0.001$). Thus, it is possible that generation time effects are at least in part responsible for the rate variation that we detect. However, given the distribution of taxa for which AFB data exists, we can identify as many as seven phylogenetically independent comparisons to test for an association between generation time and branch length. When we apply the same sign test that we use above for body mass to those species with AFB data, we do
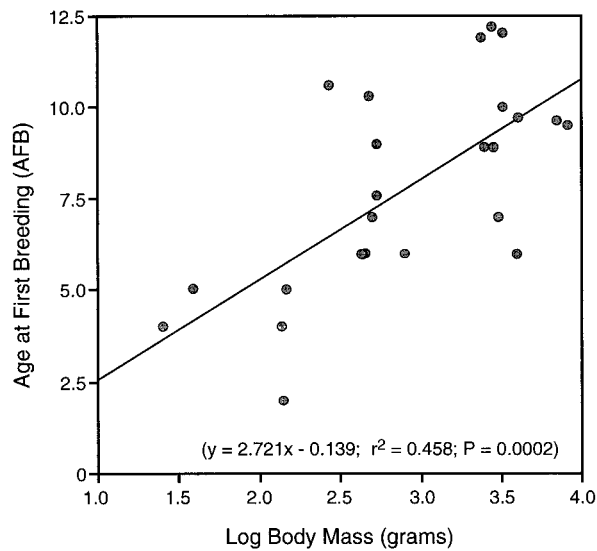
FIG. 4.—The plot of log body mass versus age at first breeding (AFB). Linear regression of the data is shown to the lower right in the graph.

not find evidence for a generation time effect. Of these seven comparisons, four run counter to the expectations of the generation time hypothesis (table 3), with the species that breeds later having a longer branch. This suggests that variation in branch lengths on our tree cannot be explained by differences in generation time in a manner consistent with the generation time hypothesis.

We also note that generation time is not expected to affect mitochondrial genes in the same way that it affects nuclear genes (Martin and Palumbi 1993). Nuclear genes are tied to the cell cycle, with one complete round of DNA replication per cell division. This relationship provides the basis for the generation time hypothesis. Unlike nuclear DNA, however, mitochondrial DNA replication is to some extent decoupled from the cell cycle (Rand 1994). While mtDNA does appear to double at the same rate as the cell generation time (Bogenhagen and Clayton 1977), mtDNA genomes often replicate independent of the cell cycle, with apparently higher turnover in tissues with higher metabolic rates (Gross, Getz, and Rabinowitz 1969). Thus, the genera-

tion time hypothesis may not apply to mtDNA, at least not in a predictable fashion. Unfortunately, the tight correlation between metabolic rate, generation time, and other physiological and ecological variables makes it difficult to know which of these is responsible for molecular rate heterogeneity.

The above considerations led Martin and Palumbi (1993) to conceptualize ''nucleotide generation time.'' They describe this as the average time before a nucleotide site is replicated or replaced through some DNA repair mechanism. This concept is relevant here, since we argue that the high turnover of mtDNA and the high levels of mtDNA damage relative to nuclear DNA damage led to a nucleotide generation time for procellariiform mtDNA that is very short compared to the actual generation time of the species involved. If this is true, then metabolic rate effects may essentially dilute generation time effects for mitochondrial genes, making the latter difficult to detect.

Population Size Effects

Accurate estimates of effective population size for procellariiform birds are few, despite the large number of population level studies that have been done on this group (see Warham 1996 and references therein). Part of the problem stems from the difficulty associated with accurately counting individuals of species that nest in burrows. For example, the majority of species in the Procellariidae exhibit this nesting behavior. It is also difficult to know exactly what unit (e.g., single nesting locality, single island, group of islands) makes up a population. In addition, there is no obvious relationship between body mass and population size where estimates for the latter exist and extremely large colonies of both small and large procellariiform birds are known (Warham 1996).

However, since body size and population size are correlated for some vertebrates and our analysis involved body mass, we tested for population size effects in order to determine the potential for this variable to explain rate heterogeneity in our data. Here we assumed, from general population biology principles, that body size and population size must be negatively correlated in procellariiform birds. If this is the case, then the re-

Table 3
Sign Test Comparisons of Age at First Breeding (AFB) and Branch Length for Seven Pairs of Taxa

Species Pair	AFB[a] (years)	Branch Lengths[b]	Signs of AFB vs. Branch Length Differences[c]
Diomedea antipodensis/D. epomophora	9.6/9.5	14/15	+, −
Macronectes giganteus/M. hallii	9.9/9.7	4/2	+, +
Pachyptila desolata/P. turtur	5.5/4.5	20/10	+, +
Phoebastria immutablis/P. irrorata	8.9/7	24/19	+, +
Phoebetria fusca/P. palpebrata	12.2/12	13/8	+, +
Puffinus puffinus/Calonectis diomedea	6.5/9	41/40	+, −
Thalassarche chrysostoma/T. melanophris	12/10	9/13	+, −

[a] AFB data are from Warham (1996).

[b] Branch lengths are patristic and based on unambiguous character state changes as calculated in MacClade, version 3.06 (Maddison and Maddison 1992), for one of four equally parsimonious trees.

[c] Sign test scores for the relative age at first breeding and branch length differences, respectively. A ''+, +'' score indicates that the species with a later AFB was associated with a longer branch, and a ''+, −'' score indicates that the late breeder was associated with a shorter branch.

Table 4
Sign Test Comparisons of Body Mass and Substitution Ratio for 14 Pairs of Taxa

Species Pair	Body Masses (g)	Ratio of Silent to Replacement Substitutions[a]	Signs of Body Mass *vs.* Ratio Differences[b]
Diomedea gibsoni/D. sanfordi	6,900/6,670	0.08/0.25	+, −
Fregetta grallaria/Garrodia nereis	58/38	0.10/0.22	+, −
Macronectes giganteus/Daption capense	3,944/428	0.07/0.12	+, −
Oceanodroma melania/Halocyptena microsoma	59/21	0.20/0.06	+, +
Oceanodroma tristrami/O. furcata	84/55	0.15/0.02	+, +
Pelecanoides garnoti/P. urinatrix	230/141	0.20/0.10	+, +
Phoebastria irrorata/P. immutabilis	3,140/2,853	0.58/0.09	+, +
Procellaria westlandica/Bulweria bulwerii	780/99	0.03/0.13	+, −
Pterodroma hypoleuca/P. longirostris	176/155	0.07/0.18	+, −
Pterodroma lessonii/P. cahow	698/246	0.18/0.04	+, +
Pterodroma neglecta/P. phaeopygia	509/343	0.09/0.04	+, +
Puffinus griseus/P. pacificus	787/388	0.08/0.04	+, +
Puffinus huttoni/P. opisthomelas	364/276	0.08/0.16	+, −
Thalassarche cauta/T. bulleri	3,590/2,780	0.14/0.22	+, −

[a] The numbers of silent and replacement substitutions were calculated using MacClade, version 3.06 (Maddison and Maddison 1992). Replacement substitutions were calculated by translating the DNA sequence data to protein data and mapping the number of protein substitutions on the tree in figure 3. Silent substitutions were calculated by subtracting the number of replacement substitutions from the total number of changes on the tree in figure 3, which was based on all of the DNA data.

[b] Sign test scores for the relative body mass and silent to replacement ratio differences, respectively. A "+, +" score indicates that the larger of the two taxa was associated with a larger silent-to-replacement ratio, and a "+, −" score indicates the larger taxon was associated with a smaller ratio.

sults of our original sign test contradict the expectations of the nearly neutral theory (see table 2). In this analysis, we determined that rates of cyt-*b* evolution tend to be faster for species with smaller body masses and, based on the above assumption, larger population sizes. Clearly, the rate differences we detected are counter to basic tenets of the nearly neutral theory (Ohta 1992, 1993).

It could be argued that this is a weak test of the nearly neutral theory, since we looked at closely related species that are likely to differ mostly at silent sites. The primary signature of population size effects is thought to be an increase in the amount of amino acid replacement substitutions relative to silent substitutions in taxa with small population sizes (Ohta 1995). This expectation derives from the fact that replacement substitutions, if they are slightly deleterious, will accumulate faster in smaller populations than in larger ones, since drift will lead to a higher fixation probability. We repeated our sign test to determine if such an effect could be detected deeper in our phylogeny where replacement substitutions would have had the opportunity to accumulate. In this analysis, we restricted our comparisons to pairs of species that differed by at least one replacement substitution across the phylogeny. We then compared the ratio of replacement to silent substitutions along each lineage with the body mass of each species involved (table 4). This provided us with 14 comparisons. The results of this analysis do not support the nearly neutral theory, and they show no signs of population size effects. In 7 of the 14 contrasts, the larger species (with an assumed smaller population size) actually had fewer replacement substitutions per silent substitution. Again, the results of our analysis run counter to the expectations of the nearly neutral theory.

Conclusions

The assumption that rates of molecular evolution are constant across lineages has not stood up to recent empirical observations. Thus, attention has turned to possible causes of rate heterogeneity. Unfortunately, identifying sources of rate heterogeneity has proven to be a difficult task. Studies of molecular rate variation require DNA sequence data from many taxa for multiple independent tests. These data-rich analyses are essential for detecting correlations between variation in rates of DNA sequence evolution and the contributing factors which generate that variation. Unfortunately, in nature, correlation among the different factors that might be associated with rate variation make it difficult to establish precise causation. In addition, no single factor is likely to explain all of the variation in rates of molecular evolution for a given data set. Even body size, which has repeatedly been found to correlate with rate heterogeneity, is likely to account for only a fraction of the total variation in rates of molecular evolution as measured by branch length. This is not surprising given the many sources of error involved in estimating the relevant variables, the uncertain history of those variables along any particular lineage, and the possibility of complex interactions between multiple variables that may ultimately determine rates of evolution. As a result, we must proceed with caution when investigating possible sources of rate heterogeneity.

Previous comparisons between distantly related endotherm and ectotherm lineages demonstrated that rate differences occur that are consistent with the metabolic rate hypothesis. Here, we present evidence for an association between rates of molecular evolution and body mass within a more closely related monophyletic endothermic group, the procellariiform birds. The size of our taxonomic sample provided us with many phylogenetically independent comparisons with which to search for correlates to molecular rate variation. Fortuitously, there is a relatively large amount of physiological and natural history information available for this group of birds. Such sources of key information provid-

ed us with an ability to investigate the relative contributions of potential causative variables to differences in rates of cyt-*b* evolution within this avian order. Assimilating this information, we conclude that patterns of mitochondrial cyt-*b* gene evolutionary rate variation in our data support the metabolic rate hypothesis over alternative explanations. The implication is that lineage effects relating to rate heterogeneity can operate at a scale that has largely been ignored to date. This suggests that even rate calibrations within vertebrate orders may not be tenable, especially if the lineages involved exhibit significant differences in body mass.

Acknowledgments

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