ORIGINAL ARTICLE

doi:10.1111/j.1558-5646.2007.00089.x

BODY MASS AND TEMPERATURE INFLUENCE RATES OF MITOCHONDRIAL DNA EVOLUTION IN NORTH AMERICAN CYPRINID FISH

G. F. Estabrook,[1] G. R. Smith,[2,3] and T. E. Dowling[4]

[1]*Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan 48109*

[2]*Museum of Paleontology, University of Michigan, Ann Arbor, Michigan 48109*

[3]*E-mail: grsmith@umich.edu*

[4]*School of Life Sciences, Arizona State University, Tempe, Arizona 85287*

Received May 17, 2006
Accepted December 15, 2006

The mass-specific metabolic rate hypothesis of Gillooly and others predicts that DNA mutation and substitution rates are a function of body mass and temperature. We tested this hypothesis with sequence divergences estimated from mtDNA cytochrome *b* sequences of 54 taxa of cyprinid fish. Branch lengths estimated from a likelihood tree were compared with metabolic rates calculated from body mass and environmental temperatures experienced by those taxa. The problem of unknown age estimates of lineage splitting was avoided by comparing estimated amounts of metabolic activity along phyletic lines leading to pairs of modern taxa from their most recent common ancestor with sequence divergences along those same pairs of phyletic lines. There were significantly more pairs for which the phyletic line with greater genetic change also had the higher metabolic activity, when compared to the prediction of a hypothesis that body mass and temperature are not related to substitution rate.

KEY WORDS: **Body size, fish, metabolic rate, molecular clock, mtDNA, substitution rate, temperature.**

The rate of evolution of mitochondrial DNA is sufficiently constant, among taxa and within genes, to permit estimates of branching ages in evolutionary studies (Li 1997; Rambaut and Bromham 1998; Bromham and Penny 2003). Accuracy of age estimates, however, is compromised by heterogeneity of rates (Britten 1986; Welch and Bromham 2005), among other factors (Smith and Peterson 2002; Graur and Martin 2004; Near et al. 2005). Estimated overall pairwise rates for mitochondrial cytochrome *b* (cyt*b*) vary from about 0.5%/million years to 5.0%/million years among Cenozoic freshwater fish (Smith et al. 2002; Smith and Dowling, in press) and from 0.3%/million years to 1.4%/million years in a broad sampling of cyt*b* rates among vertebrates (Gillooly et al. 2005). Rate heterogeneity and heterotachy (Lopez et al. 2002) are hypothesized to be caused by processes that influence mutation and substitution rates, summarized by Mindell and Thacker (1996). Some early suggestions of factors that could influence mutation rate include exposure to free radicals and mutagens, replication frequency, replication efficiency, and inefficient repair mechanisms (Gillespie 1991; Li 1997). [Substitution rate may be further influenced by natural selection, synonymous versus nonsynonymous sites (Fitch et al. 1991), effective population size (Ohta 1976; DeSalle and Templeton 1988; Chao and Carr 1993), identity of neighboring bases (Siepel and Haussler 2004), secondary or tertiary structure (Wang and Lee 2002), and conserved regions of the molecule influenced by complexity of development or function (Aris-Brosou 2005).] Additional factors, operating at the level of life-history ecology and organismal biology, include generation time (Chao and Carr 1993; Martin and Palumbi 1993; Ohta 1993; Bromham 2002), homeothermy (Adachi et al. 1993), thermal habit (Bargelloni et al. 1994; Rand 1994), environmental

change (Li and Graur 1991), and variation conserved in diverging lineages (Futuyma 1987; Bousquet et al. 1992; Mindell 1996).

Interest in metabolic rate as a causal mechanism influencing rate variation was stimulated especially by Martin and Palumbi (1993) and Rand (1994) who documented effects of body size, temperature, and correlated variables, including generation time. The mass-specific metabolic rate model of Gillooly et al. (2001) used equations from thermodynamics to relate temperature and body size to metabolic rate. Gillooly et al. (2005) demonstrated that body mass, temperature, and metabolic rate explain a significant fraction of the variance in nucleotide substitution rates in a broad sample of organisms (Wilke 2005). Specifically, analysis of 71 datasets, including invertebrates, fish, amphibians, reptiles, birds, and mammals, that vary in body mass by 10 orders of magnitude and in body temperature from 0°C to 40°C, indicated that substitutions accumulate at a constant rate per unit of mass-specific metabolic energy flux rather than per unit time (Gillooly et al. 2005).

Gillooly et al. (2001) present physical, chemical, and biological arguments for the appropriateness of the following formula to estimate metabolic rate:

$$B = b_o M^{-1/4} \quad e^{-E/kT}, \tag{1}$$

where B is the metabolic rate, b_o is a coefficient independent of mass and temperature, M is body mass, $e^{-E/kT}$ is the Boltzmann factor, which underlies the temperature dependence of metabolic

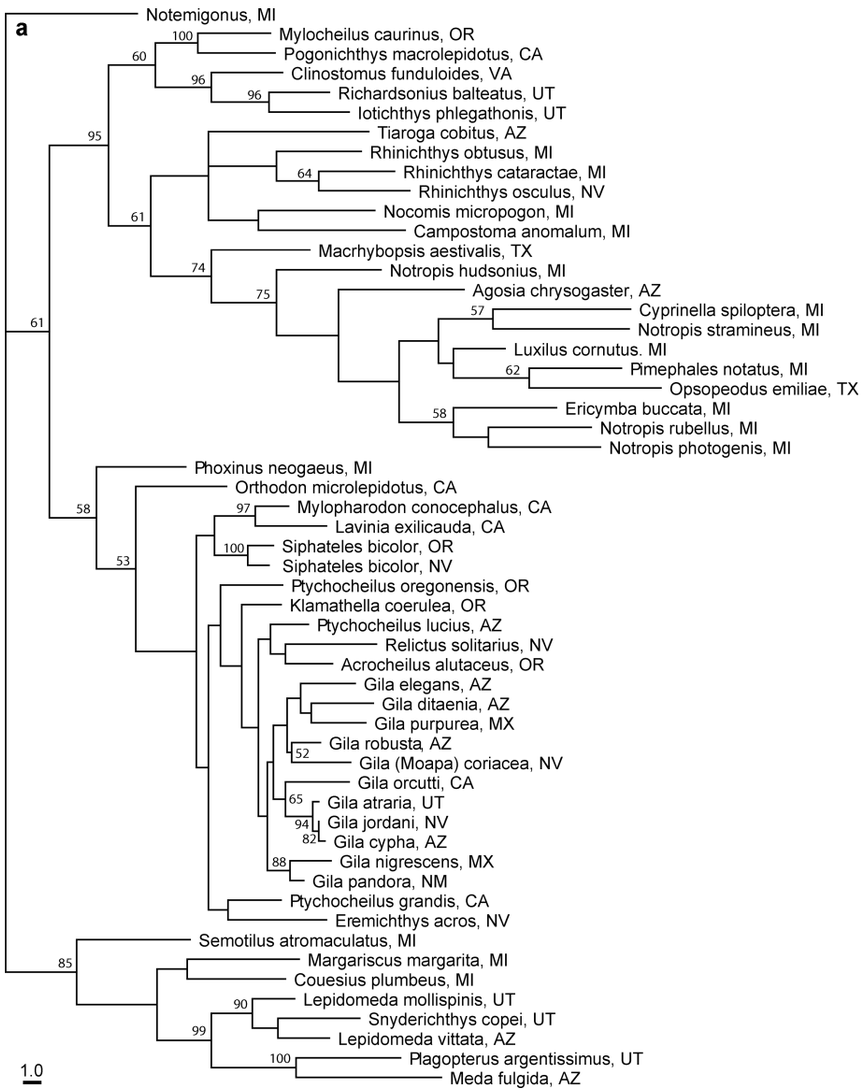

Figure 1. (a) Phylogenetic tree estimated with maximum-likelihood methods in PAUP* (Swofford 2000) with bootstrap values for nodes. Ln likelihood = 16822.58. (b) Strict consensus of five parsimony trees based on the same sequence as in (a) and estimated in PAUP*.

rate (E is the activation energy of the rate-limiting biochemical reactions of metabolism [0.65 eV], k is Boltzmann's constant [8.62×10^{-5} $eV\,K^{-1}$], and T is absolute temperature [K]).

In this paper we test whether body mass, environmental temperature, and metabolic rates estimated with this formula vary monotonically with molecular evolutionary rates of lineages, using comparisons of sister pairs among 54 species of North American Cyprinidae (minnows). This group of fish is well suited to test this hypothesis because mitochondrial DNA sequences (cytochrome *b* gene, cyt*b*) have been determined for most genera and many species from North America, and phylogenetic relationships are beginning to become repeatable and consistent. We present methods to test the general hypothesis of Gillooly et al. (2005) at the level of individual lineages and apply them to these taxa. If evidence supports a predictive relationship between metabolic rate and mutation/substitution rates it might be appropriate to evaluate such information in rate studies, as an alternative to construing variation as error.

Materials and Methods

The family Cyprinidae is the most species-rich group of freshwater fish. Most of the nearly 300 North American species live east of the Rocky Mountains, but 36 species in Pacific drainages are diverse morphologically and one third of them are represented by late Cenozoic fossils (Smith et al. 1982, 2002). The record indicates that genera appear to have achieved their modern body plans and ecological roles by middle or late Miocene

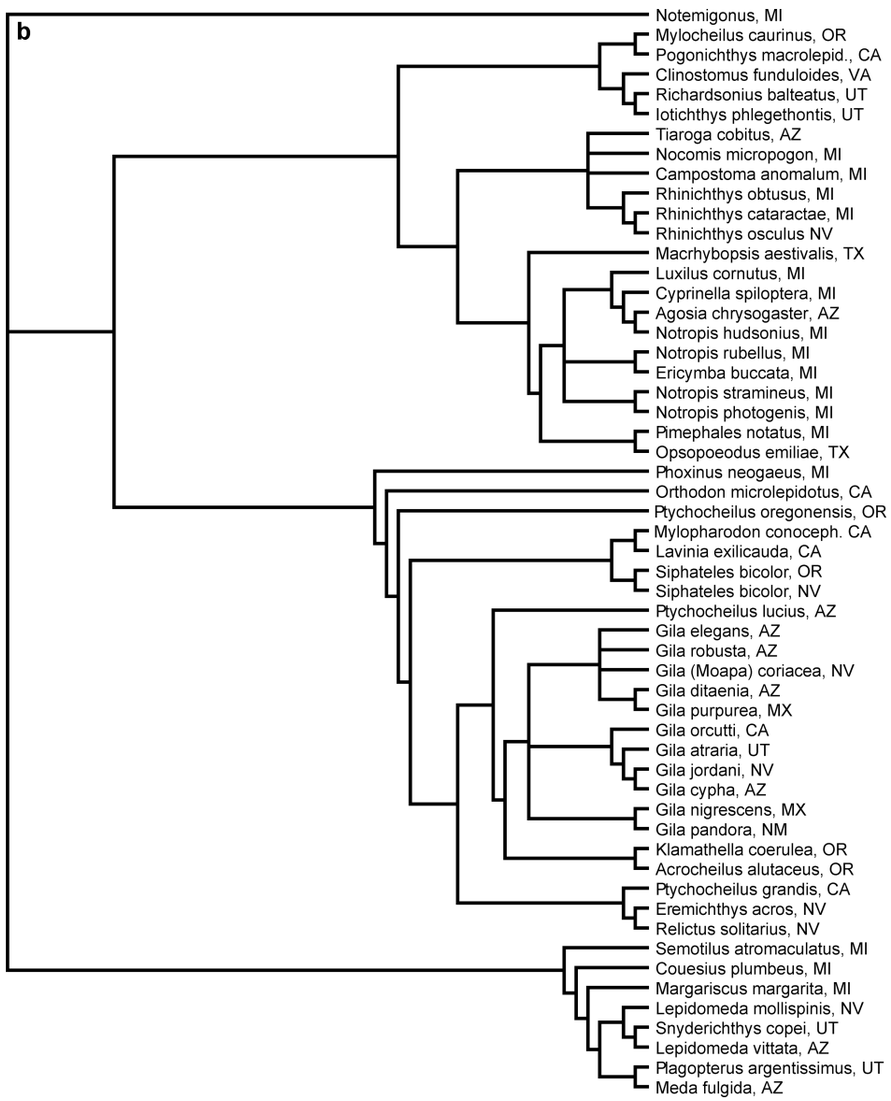

Figure 1. continued

(Smith 1981, Cavender 1986). Phylogenetic hypotheses for western forms and eastern outgroups were presented by Smith et al. (2002) and Dowling et al. (2002) based on cyt*b*. These phylogenies disagreed with previous morphology-based phylogenies (Cavender and Coburn 1993; Coburn and Cavender 1993), which are relatively unresolved and possibly compromised by convergence, introgression, and other homoplasy (Smith 1992; Smith et al. 2002).

We use a phylogenetic tree estimated by maximum likelihood (Swofford 2000) from 1140 base pair sequences of cyt*b* (Dowling et al. 2002) as the basis of this study. This tree (Fig. 1a) is similar to a consensus of parsimony trees (Fig. 1b) and both trees are generally consistent with other phylogenies estimated by Dowling et al. (2002) and Smith et al. (2002).

Body mass estimates for species were taken from length-weight equations given by Carlander (1997) where applicable, and otherwise from length-weight graphs constructed from restored live weights and body lengths from museum records, state monographs (e.g., LaRivers 1962; Minckley 1973), or the *Atlas of North American Freshwater fish* (Lee et al. 1980). Each taxon is represented in Table 1 by a mass estimate midway between the female mass at first reproduction and maximum size.

We assumed that the body temperatures of these fish were the temperatures of the water in which they lived (geographic distributions in Lee et al. 1980, and museum records), which we approximate with ambient temperature (data from the *Climatic Atlas of the United States*, U.S. Department of Commerce, Washington, D.C., 1968). Temperature varies with time of year: between first and last frost we take the minimum water temperature to be 4°C; we assume that June, July, and August are the warmest months and take the water temperature during these months to be the average air temperature; and we approximate the water temperature between last frost and 1 June by starting at 4°C and attributing equal increments up to the average June-July-August temperature to each day until 1 June, and similarly from 1 September until first frost. We then calculate an annual total metabolic rate for each taxon as the total over all the days of the year of the metabolic rate on each day.

To estimate amounts of evolutionary change that took place along the phyletic (phylogenetic) lines from the most recent common ancestor for a pair of taxa, we use the number of nucleotide substitutions along each phyletic line segment (between nodes), estimated by the maximum-likelihood tree that we use as the basis for our study. Although we do not know the absolute times when lineages diverged from the common ancestors, we do know that each member of a pair of lineages has been evolving from the common ancestor for the same amount of time. Thus, for any pair of taxa, the one that accumulated more cyt*b* substitutions since the most recent common ancestor (call it x) has had a faster average rate of evolutionary change.

Because we identify the faster rate by the greater number of molecular substitutions over the two phyletic lines from the common ancestor, x, we must compare these estimates of genetic change with estimates of the total amount of metabolic activity over the same two phyletic lines. To integrate metabolic activity over any phyletic line, we would need to estimate annual metabolic rate for each ancestral node in the phylogenetic tree. To estimate annual total metabolic rate for each ancestor, we estimate body size and thermal regime for each ancestor, starting with those for which descendants were extant taxa with known body mass and thermal regimes. Webster and Purvis (2002) demonstrated that the average of the observed data provides a better estimate of the set of ancestral values than do any of several more sophisticated methods for estimating ancestral values. For thermal values we assign to an ancestor the average of the values of immediate descendants, but for body mass, following the relation between metabolic rate and body mass in equation (1), we assign the fourth power of the average of the fourth roots of the body masses of the immediate descendants. By the same means, we then assign values to any ancestor for which values were known for all of its immediate descendants. We repeat this process until all ancestral nodes have values from which to calculate an estimated annual metabolic rate. We then assume that metabolic rate increased or decreased at a uniform rate from ancestor to ancestor, equally spaced temporally, along the phyletic line segments from the most recent common ancestor to each member of the pair of taxa in question (Webster and Purvis 2002).

If the amount of genetic change over the life of each lineage varied monotonically with the amount of metabolic activity for each pair of taxa, the taxon with more genetic change since the most recent common ancestor, x, would be the taxon with more metabolic activity since x. To quantify the extent to which greater metabolic activity tends to be associated with greater genetic change, we count the number of pairs for which this pattern was observed.

To discuss this more succinctly: For each pair of taxa (a, b) we calculate the values that we want to compare: First we discover the most recent common ancestor x of a and b. Then we discover the amount of sequence divergence $D(x, a)$ from x to a and $D(x, b)$ from x to b, taken from branch lengths on the maximum-likelihood tree. Then we estimate metabolic activities $M(x, a)$ by integrating annual metabolic rate over the phyletic line segments from x to a, and $M(x, b)$ by integrating annual total metabolic rate over the phyletic line segments from x to b. If all the estimating procedures are reasonably accurate, a general prediction of the mass-specific metabolic rate hypothesis of Gillooly et al. (2005) would be

$$\text{if } M(x, a) > M(x, b) \quad \text{then} \quad D(x, a) > D(x, b) \quad \text{and}$$

$$\text{if } M(x, a) < M(x, b) \quad \text{then} \quad D(x, a) < D(x, b).$$

Table 1. Maximum-likelihood tree file with taxa, nodes, linkages, and branch lengths and their standard errors, body mass, temperature, frost-free days, and annual metabolic rate (AMR). Analyzed pairs are located by connections to the same node in the second column. Less significant nodes are marked with an asterisk next to the standard error. *Notemigonus* is the North American outgroup (not included in rate comparisons).

Taxon node	Connected to node	Branch length	Standard error	Mass grams	Temperature	Frost-free days	AMR
Notemigonus	108	0.307	0.042				
100	108	0.021	0.014*	45.2	19.9	165.9	33.9
76	100	0.114	0.024	22.4	21.2	182.0	44.6
59	76	0.043	0.017	63.4	19.2	168.1	29.9
56	59	0.067	0.019	253.9	18.3	180.0	20.5
Mylocheilus	56	0.079	0.017	263.0	15.5	120.0	15.7
Pogonichthys	56	0.087	0.017	245.0	21.0	240.0	27.4
58	59	0.070	0.019	7.4	20.1	156.3	52.5
57	58	0.052	0.015	4.3	19.0	147.5	55.6
Richardsonius	57	0.048	0.012	12.0	18.7	165.0	44.5
Iotichthys	57	0.088	0.015	1.1	19.2	130.0	76.1
Clinostomus	58	0.116	0.020	12.0	21.3	165.0	50.5
75	76	0.065	0.019	5.5	23.1	195.9	73.3
64	75	0.020	0.012*	6.6	21.8	176.3	62.9
61	64	0.029	0.014	2.9	21.0	150.0	69.1
60	61	0.025	0.012	3.3	20.7	135.0	63.8
Rhinichthys cataract.	60	0.086	0.016	4.5	17.4	100.0	45.6
Rhinichthys osculus	60	0.096	0.017	2.5	24.0	170.0	90.2
Rhinichthys obtusus	61	0.146	0.023	3.0	21.3	165.0	74.8
63	64	0.0	0.015*	12.9	22.6	202.5	59.2
Tiaroga	63	0.270	0.035	3.0	24.0	240.0	98.6
62	63	0.068	0.021	37.6	21.3	165.0	38.0
Nocomis	62	0.170	0.029	44.0	21.3	165.0	36.5
Campostoma anom.	62	0.272	0.039	32.0	21.3	165.0	39.5
74	75	0.067	0.020	4.6	24.4	215.6	85.5
73	74	0.054	0.019	8.2	20.9	161.2	54.5
72	73	0.017	0.013*	4.0	21.9	202.5	75.2
Agosia	72	0.283	0.037	4.6	22.0	240.0	78.4
71	72	0.029	0.014	3.5	21.7	165.0	71.7
68	71	0.005	0.006*	4.4	22.1	165.0	68.4
65	68	0.030	0.015*	3.1	21.3	165.0	70.7
Cyprinella spil.	65	0.229	0.031	4.0	21.3	165.0	66.5
Notropis stram.	65	0.173	0.026	2.4	21.3	165.0	75.6
67	68	0.003	0.006*	6.0	23.0	165.0	66.6
Luxilus cornutus	67	0.072	0.015	15.8	21.3	165.0	47.2
66	67	0.082	0.021	1.7	24.6	217.5	101.9
Pimephales prom.	66	0.125	0.024	2.7	21.3	165.0	73.4
Opsopoeodus	66	0.256	0.036	1.0	27.9	270.0	176.8
70	71	0.029	0.013	2.7	21.3	165.0	73.5
69	70	0.019	0.011*	5.9	21.3	165.0	60.3
Notropis rubellus	69	0.136	0.022	2.5	21.3	165.0	74.8
Notropis photogenis	69	0.128	0.021	12.0	21.3	165.0	50.5
Ericymba	70	0.214	0.030	1.0	21.3	165.0	94.0
Notropis hudsonius	73	0.243	0.034	15.0	20.0	120.0	40.5
Macrhybopsis	74	0.258	0.035	2.3	27.9	270.0	143.5
99	100	0.035	0.016	81.9	18.6	149.9	26.6
98	99	0.036	0.014	471.3	19.8	199.7	20.2
97	98	0.053	0.015	344.0	18.7	159.4	19.0
79	97	0.006	0.005*	214.5	19.4	172.5	22.3
77	79	0.049	0.011	216.8	21.0	240.0	28.2
Mylopharodon	77	0.023	0.008	363.0	21.0	240.0	24.8

continued

Table 1. continued

Taxon node	Connected to node	Branch length	Standard error	Mass grams	Temperature	Frost-free days	AMR
Lavinia	77	0.068	0.012	120.0	21.0	240.0	32.7
78	79	0.035	0.009	212.2	17.7	105.0	18.0
Siphateles (Klamath)	78	0.020	0.006	178.0	15.5	90.0	16.3
Siphateles (Pyramid)	78	0.014	0.005	251.0	20.0	120.0	20.0
96	97	0.006	0.005*	524.9	18.0	146.4	15.9
94	96	0.008	0.005*	665.7	16.5	117.7	13.0
93	94	0.015	0.006	292.0	17.4	115.5	16.6
92	93	0.007	0.004*	389.6	19.3	140.9	18.0
81	92	0.002	0.002*	1621.8	17.2	100.0	10.4
Ptychocheilus lucius	81	0.042	0.008	9484.0	17.4	90.0	6.6
80	81	0.002	0.005*	63.6	17.1	110.0	23.7
Relictus	80	0.108	0.016	10.0	18.6	100.0	39.8
Acrocheilus	80	0.039	0.008	224.0	15.5	120.0	16.3
91	92	0.006	0.003*	41.6	21.4	181.9	38.4
89	91	0.001	0.002*	69.7	21.8	213.8	37.6
85	89	0.011	0.004	82.1	23.8	210.0	40.4
83	85	0.001	0.002*	94.6	24.0	225.0	40.4
Gila elegans	83	0.054	0.009	468.0	22.0	210.0	23.3
82	83	0.006	0.004*	6.3	26.0	240.0	92.7
Gila ditaenia	82	0.055	0.010	4.5	26.0	240.0	101.0
Gila purpurea	82	0.048	0.009	8.7	26.0	240.0	85.6
84	85	0.003	0.003*	70.9	23.5	195.0	39.0
Gila robusta	84	0.022	0.006	398.0	21.0	180.0	21.5
Gila (Moapa)	84	0.055	0.010	3.2	26.0	210.0	103.5
88	89	0.007	0.004*	58.8	19.8	217.5	35.3
87	88	0.021	0.006	178.2	19.6	135.0	22.3
Gila atraria	87	0.005	0.003*	251.0	18.7	120.0	18.8
86	87	0.003	0.002*	122.5	20.5	150.0	26.1
Gila jordani	86	0.0		46.0	20.0	120.0	30.6
Gila cypha	86	0.004	0.002*	269.0	21.0	180.0	23.3
Gila orcutti	88	0.058	0.010	12.6	20.0	300.0	60.2
90	91	0.018	0.006	30.6	23.0	195.0	46.6
Gila nigrescens	90	0.031	0.007	40.0	24.8	240.0	53.9
Gila pandora	90	0.012	0.004	23.0	21.0	150.0	41.2
Klamathella	93	0.039	0.008	214.0	15.5	90.0	15.6
Ptychocheil. oregon.	94	0.073	0.012	1318.0	15.5	120.0	10.5
95	96	0.010	0.007*	407.7	19.5	175.0	19.2
Ptychocheil. grandis	95	0.061	0.011	3388.0	21.0	240.0	14.2
Eremichthys	95	0.115	0.017	3.4	18.0	110.0	51.7
Orthodon	98	0.129	0.021	631.0	21.0	240.0	21.6
Phoxinus neogaeus	99	0.181	0.028	3.4	17.4	100.0	48.9
107	108	0.067	0.020	33.9	20.7	148.7	36.9
106	107	0.020	0.015*	7.0	20.2	132.5	50.8
101	106	0.032	0.016*	9.8	17.4	90.0	36.7
Semotilus atromac.	107	0.258	0.036	105.0	21.3	165.0	29.4
Margariscus	101	0.159	0.026	4.8	17.4	90.0	43.9
Couesius plumb.	101	0.117	0.022	18.0	17.4	90.0	31.6
105	106	0.100	0.023	4.8	23.0	175.0	72.2
103	105	0.052	0.017	8.6	23.3	167.5	61.9
102	103	0.006	0.007*	5.5	22.5	135.0	61.5
Snyderichthys	102	0.084	0.014	6.6	21.0	120.0	52.5
Lepidomeda vit.	102	0.045	0.010	4.5	24.0	150.0	73.7
Lepidomeda moll.	103	0.052	0.011	13.0	24.0	200.0	63.1
104	105	0.151	0.028	2.4	22.6	182.5	86.4
Plagopterus	104	0.076	0.021	2.3	24.0	200.0	97.3
Meda	104	0.238	0.033	2.4	28.0	300.0	147.7
Sum		7.846					

We will refer to this prediction as the monotone (monotonic) prediction, and to pairs of taxa consistent with it as monotone pairs.

To determine the significance of the number of monotone pairs we compare the observed number with the number of monotone pairs predicted by a null hypothesis that metabolic rate (estimated with body mass and temperature) is not related to DNA substitution rate. We state this null hypothesis by describing a random probability process that generates a data structure that is identical in form and total content to the one observed, but that separates body mass and temperature data (as an integrated unit) from amount of genetic change so that any data structure generated by the hypothesized null process samples body mass and temperature independently of amount of sequence divergence. The random process we use to represent the null hypothesis chooses with equal probability one of the 54 factorial ways to reassign the observed body mass and temperature data to the 54 taxa, while leaving the phylogenetic tree and the genetic distances of its phyletic line segments unchanged. Once a data structure has been randomly generated in this way, we integrate metabolic activity along phyletic lines from most recent ancestors, as described earlier. The number of monotone pairs is counted, and that number is saved in a table. A thousand random data structures, each sampling the null hypothesis, are simulated, and for each the number of monotone pairs is counted and saved in the same table. The table is plotted with possible numbers of monotone pairs on the X-axis and number of simulated data structures with less than or equal that number of monotone pairs on the Y-axis. This plot represents the cumulative probability distribution for the number of monotone pairs predicted by the null hypothesis. The observed number of monotone pairs is then compared with this probability distribution. The number of simulated data structures for which the number of monotone pairs is greater than or equal to the observed number estimates the realized significance that the observed number of monotone pairs is too large to be consistent with the null hypothesis. If this realized significance (number of such simulated data structures divided by 1000, the total number of simulated data structures) is very small, then the observed data structure can be considered inconsistent with the null hypothesis and the null hypothesis is rejected.

There are two methodological shortcomings of this approach. (1) We do not actually know the body size or thermal regimes of the ancestors. Thus, estimates of metabolic rates of ancestors, based, as they are, on successive averages of body sizes and thermal regimes of extant taxa, are probably wrong (possibly egregiously wrong) for some (possibly many) ancestors. (2) Slower metabolic rates and slower substitution rates may not be causally related but instead simultaneously inherited from common ancestors so that convex areas of the phylogenetic tree would contain taxa with slow metabolism and slow substitution rate. Similarly, faster metabolic rates and faster substitution rates may not be causally related but instead simultaneously inherited from common ancestors so that convex areas of the phylogenetic tree would contain taxa with fast metabolism and fast substitution rate. Pairs of taxa with one member chosen from a slow area and the other chosen from a fast area would be monotone but not constitute evidence that metabolism and rate of sequence divergence were causally related. If much of a phylogenetic tree comprised such slow or fast areas, then many pairs would be monotone but have no causal relationship between metabolic rate and substitution rate. Harvey and Pagel (1991) explain this phenomenon more generally, and discuss various approaches to minimizing its effect on statistical arguments.

To address these methodological problems, first we restrict consideration to pairs of sister taxa only, of which there are 17 or 18 (one sister pair was so weakly supported that it is questionable whether it can be construed as a sister pair). If we consider only sisters, this eliminates problem (2). For a sister pair (a, b) we make no estimate of metabolic rate for the most recent common ancestor x, and set $M(x, a)$ to be the metabolic rate of extant taxon a, similarly for $M(x, b)$. This eliminates problem (1). We use the same null hypothesis to determine significance.

A problem with this approach is that it uses data for only 36 (or 34) of the 54 taxa. From Figure 1 one can see that there are three major clades arising from ancient ancestors. Because of their antiquity, the body masses and thermal regimes of the two ancestors are likely to be among those least accurately estimated. Phylogenetically dependent areas are convex areas of the phylogenetic tree with taxa for which slower (or faster) metabolic rates and evolutionary rates were inherited from common ancestors. If there were phylogenetically dependent areas in different areas of the three major clades, then many pairs with one member from a slow area and one from a fast area would also be a pair with one member from one clade and the other member from another clade. Therefore, our second approach is to consider only pairs of taxa for which members belong to the same major clade. This still involves estimating metabolic rates for some of the more recent ancestors, and if a single major clade contained a phylogenetically dependent fast area as well as a phylogenetically dependent slow area, then some monotone pairs within that major clade still would not evidence a causal relationship between metabolic rate and evolutionary rate. Thus, our second approach reduces but does not eliminate either of the two methodological difficulties.

Some of the internal branch lengths on the tree are short relative to their standard error (Table 1). To avoid the possibility that results depend on the least significant internal branch lengths, we also perform the same two analyses using a tree in which all internal branch lengths less than twice their standard error are collapsed (branches indicated with asterisks in Table 1).

All computations were carried out using ECERFODM, a computer program written for the purpose by G.F. Estabrook, and

available for download, together with instructions and the data analyzed here, from http://www-Personal.umich.edu/~gfe/.

Results

The structure of the maximum-likelihood tree used in these analyses, together with taxon names and abbreviations, ancestral node numbers, divergence estimates to immediate ancestors, mass, growth temperatures, numbers of frost-free days per year for taxa, and estimates of the same for ancestral nodes, with resulting estimates of annual metabolic activity are shown in Table 1.

Fourteen of the 18 sister pairs are monotone. The realized significance of this under the null hypothesis that metabolic rate and substitution rate are unrelated is $P = 0.0154$. If we eliminate the dubious sister pair, then 14 of 17 sister pairs are monotone, which has a realized significance of $P = 0.0064$. These results are not confounded by questionable estimates of metabolic rates of ancestors, or by phylogenetic dependencies. Even though they are based on only part of the data, the high level of realized significance indicates that faster rates of sequence divergence tend to occur with faster metabolic rates. A similar analysis using the tree with less significant nodes collapsed enabled 35 pairs to be compared, of which 26 are monotone with a realized significance of $P = 0.006$.

Of the 535 possible pairs of taxa both members of which belong to the same major lineage, one pair has a most recent common ancestor, x, from which a positive distance to one of the taxa did not result from the maximum-likelihood estimates of branch lengths, and for that reason we do not include it in our comparisons. For the remaining 534 pairs (a, b) we calculate the distances $D(x, a)$ and $D(x, b)$ from the most recent common ancestor x, and estimate amounts of metabolic activity $M(x, a)$ and $M(x, b)$ along the phyletic lines from the common ancestor x.

To illustrate the scatter of the relationship between relative amounts of genetic change and relative levels of metabolic activity we plot these 534 pairs of taxa in Figure 2, where the X-axis is $\ln[M(x, a)/M(x, b)]$, below denoted *MaMb*, and the Y-axis is $\ln[D(x,a)/D(x,b)]$, below denoted *DaDb*. We plot ln ratios because the absolute magnitude does not change with the arbitrary choice of which taxon plays the role of a, and which plays the role of b. If substitution rates were equal, then the expected value of *DaDb* would be $\ln(1.0) = 0.0$. If amount of genetic change were strictly proportional to amount of metabolic activity then $MaMb = DaDb$ (within the limits of appropriate stochastic variation). The line $MaMb = DaDb$ is shown in Figure 2.

In viewing Figure 2, it is important to realize that the coordinates of the 534 points have been calculated from 534 pairs of taxa from a phylogenetic tree. Thus these points may not be independent samples of the same random process, but may have phylogenetic dependencies, as explained above. Therefore, it is not appropriate to interpret trends as regressions.

The hypothesis we are testing asserts that the taxon with more metabolic activity along the phyletic line from x tends to be the taxon with greater genetic change from x. Pairs that are consistent with this hypothesis, monotone pairs, plot in the upper right or lower left quadrants in Figure 2. Pairs that are not monotone plot in the lower right or upper left quadrants of Figure 2.

Of the 534 pairs considered, 380 are monotone. There are only 11 simulated data structures with at least 380 monotone pairs. Thus, the realized significance of the observed 380 monotone pairs (and 154 nonmonotone pairs) is 0.011, which rejects the null hypothesis because there are improbably too many monotone pairs observed. With less significant nodes collapsed, 533 pairs are compared, of which 373 are monotone with a realized significance of $P = 0.014$.

Discussion

Molecular substitution rates in this sample of North American cyprinid fish co-occur with, and may be influenced by, body mass, temperature, and metabolic rate as well as other factors. Smaller fish and fish that grow in warmer waters have faster metabolic and substitution rates. Even though the data significantly indicate that faster metabolic rates tend to occur with more rapid substitution rates in these fish, there is evidence that other factors are involved. Six other factors are (1) inaccurate estimates of ancestral body mass, (2) inaccurate estimates of temperatures in the paleo-environments of growth, (3) unresolved or wrong branching pattern in the phylogeny, (4) inaccurate estimates of amount of sequence divergence, (5) mutagenic effects of free oxygen radicals, and (6) small population size.

Mass is estimated from modern populations, which are themselves variable depending on geographic location, age structure, and life history. Use of fossils has not yet been applied to estimate mass of the ancestral populations. Contrast of estimated mass and predicted substitution rates in the five very large species of minnows in the dataset allow a comparison of the hypothesized effects of body mass and mutagenic free radicals of oxygen, because the predictions of these alternative hypotheses differ. Mutation rates are predicted to decrease with increasing size because metabolic rate decreases with greater body mass; mutations caused by free radicals of oxygen are predicted to accelerate as a function of longevity and metabolic products (Schmidt-Nielsen 1984), for example, in annual production of enormous volumes of gametes (a correlate of size and longevity). In our study, three species of pike minnows (*Ptychocheilus lucius*, *P. oregonensis*, *P. grandis*) have existed over the past 15.5 million years, whereas *Mylocheilus* and *Acrocheilus* are known over the past 6–8 million years. These five species were significantly larger than other minnows in the

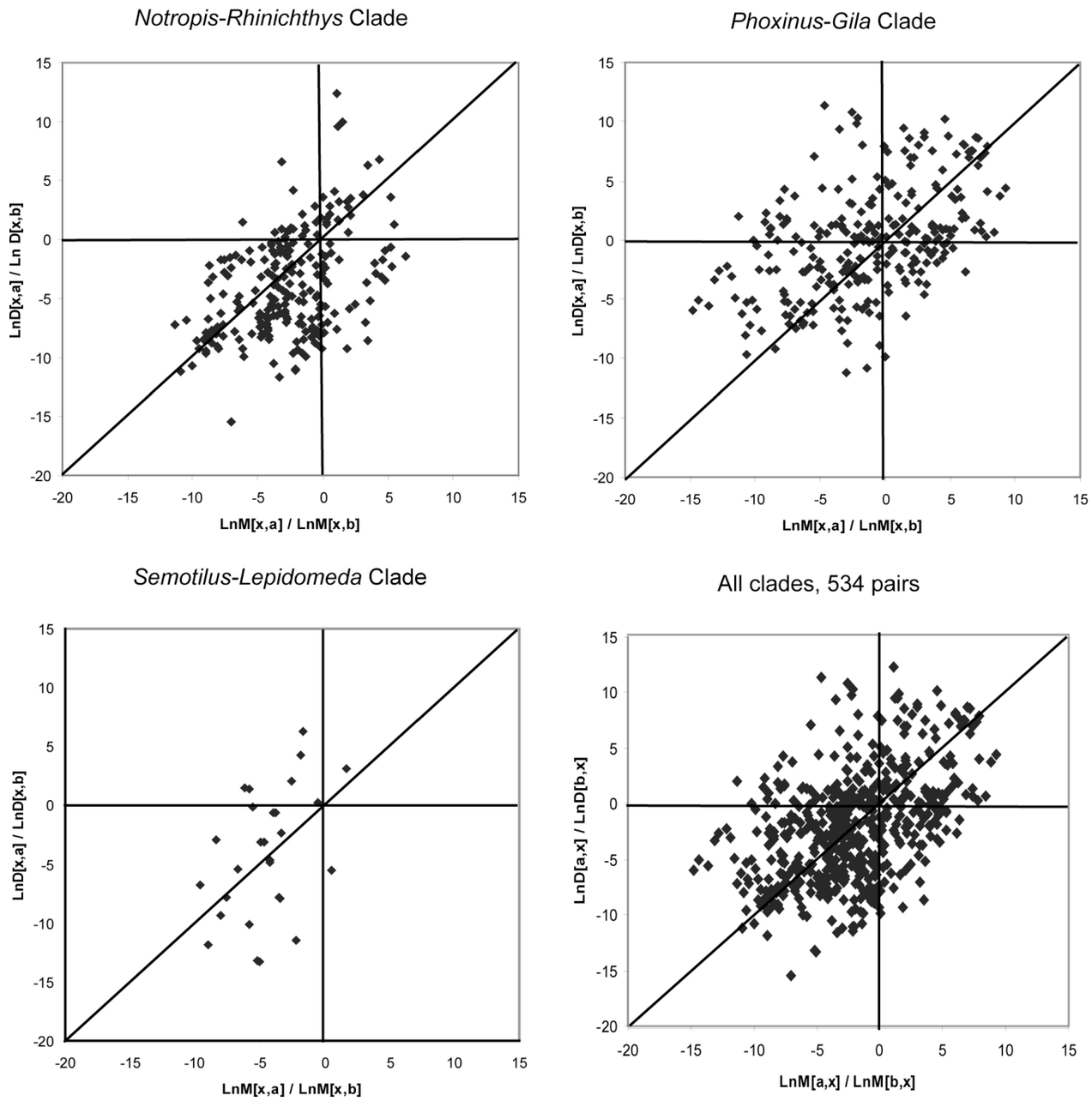

Figure 2. **Scatter plots of points for which coordinates are determined by the 534 pairs of taxa from within the three major clades of 54 taxa of western North American cyprinid fish. For each pair, its X coordinate is 10 times the Ln of the ratio of the amounts of metabolic activity since the common ancestor, x, and its Y coordinate is 10 times the Ln of the ratio of the amounts of genetic change since the common ancestor x. Points in the upper right and lower left quadrants of each plot represent monotone pairs.**

dataset in the Miocene and Pliocene, reaching lengths of 1–1.8 m (Smith et al. 2000; Spencer et al., in press), with correspondingly large investment in gametes (every 1–2 years) in individuals that reached 20–30 years of age (Smith, unpubl. ms.). Therefore these fish should show low substitution rates compared to smaller species (Fig. 1; Table 1), but higher substitution rates than predicted from modern body mass. The estimated modern metabolic rates (AMR, Table 1) with deviations from predicted mtDNA substitution rates (in brackets), are *Mylocheilus* (10.5 [+< 0.2]), *Acrocheilus* (16.3 [+0.3]), and *Ptychocheilus lucius* (6.6 [+0.4]; *P. oregonensis* 10.5 [+0.4]; and *P. grandis* 14.2 [+0.2]) (Table 1). All five of these large species have low substitution rates compared

to smaller fish, but higher substitution rates than predicted from their modern size, suggesting combined effects of body mass and mutagenic oxygen free radicals.

If large and small species are sisters, their ancestor may be assigned the wrong mass by the simple averaging method (see Webster and Purvis 2002); however, this does not apply to our analysis of 18 sisters because no metabolic rates for ancestors were used.

Erroneous estimates of environmental temperature are caused by local habitat temperatures that are different from regional ambient temperatures, for example, cold springs, climates different from the present for ancestors that lived in the Pliocene and Pleistocene. Overestimation of temperature causes overestimation of metabolic rate, which results in anomalously slow estimates of rate of molecular evolution. Possible examples appear in *Rhinichthys cataractae*, *R. osculus*, *Phoxinus*, and *Gila pandora*; for these small species, estimates of sequence divergence are lower than expected from modern temperatures and body mass (Table 1).

Relictus shows an inordinately rapid estimate of substitution rate (Fig. 1, Table 1), which may be an example of rapid substitution caused by small population size. *Relictus* is restricted to a few small spring-fed ponds in Nevada; other species are more abundant and widespread.

Introgressive transfer of DNA resets the molecular clock in the recipient species. Introgression is known to have occurred in the genus *Gila* (Smith 1992; Dowling and DeMarais 1993). This probably explains the underestimated mtDNA distances among *Gila* that have diverged in other respects. Sequence divergences among *Gila cypha*, *G. jordani*, and *G. atraria* (Fig. 1; Table 1) are unexpectedly low, given the Miocene fossil record of the most derived species, *Gila cypha* (Spencer et al., in press). These examples of suspected introgressive transfers among mtDNA lineages (Dowling and DeMarais 1993) are a source of mismatched metabolic rates and substitution rates. Any mismatch introduces random noise, unlikely to bias the results. Introgressively diminished mtDNA distances are not expected to be correlated with any other variable, such as low temperature, large body size, long generation time, or low metabolic rate.

General agreement between the maximum-likelihood and parsimony trees suggests that tree estimates might not be a source of large error, but much of this agreement may be a result of having based both of these estimates on the same data. Trees based on morphology were less resolved than those based on mtDNA cytochrome *b* sequences, so comparison of those results does not contribute more than our test of the collapsed maximum-likelihood tree (above) to the test of the mass-specific metabolic rate hypotheses.

Establishment of timing of evolutionary events with molecular divergence estimates requires that sources of intrinsic error be known and incorporated into estimates of rate and age (Gillespie 1991; Li 1997). If differences in rates are influenced by lineage effects caused by deterministic mechanisms, such as metabolic rates, the influence of those mechanisms on substitution rates should be considered in estimation of ages. Since the elucidation of rate heterogeneity by Ohta and Kimura (1971) and Langley and Fitch (1974), increasingly elegant methods have been constructed to model different molecular rates and evolution of rate changes, based on nucleotide distributions among branches (e.g., Sanderson 1997, 2002; Rambaut and Bromham 1998; Thorne et al. 1998; Huelsenbeck et al. 2000; Yoder and Yang 2000; Kishino et al. 2001; Thorne and Kishino 2002; Douzery et al. 2004). Using different measures of success, these methods variously exclude aberrant rates, estimate local rates, smooth to favor small steps in rate change, or employ Bayesian methods to estimate different rates (Welch and Bromham 2005). Assumptions underlying these models range from rate changes being rare and large to rate changes being common but small. Some smoothing methods model changes as a random walk, assuming widespread noise in substitution rates. Performance of rate estimators using relaxed (local) clock models has been evaluated by Kishino et al. (2001), Ho et al. (2005), Welch and Bromham (2005), and Linder et al. (2005).

If rates result from deterministic mechanisms, however, estimation of local rates requires more than random walk models aimed at overcoming presumed noise. Martin et al. (1992) showed possible effects of body size and poikilothermy and Martin and Palumbi (1993) demonstrated strong correlations between molecular rates and body sizes, metabolic rates, and generation times. They also hypothesized a plausible causal mechanism—that metabolic rate could affect mutation rate by a mutagenic effect of free radicals that are by-products of aerobic respiration (Schmidt-Nielsen 1984). Further demonstration of faster molecular rates in animals with shorter generation time (Larson and Wilson 1989; Schlotterer et al. 1991) suggested that examination of more causal mechanisms of correlations among body size and metabolic rate could be informative. Martin and Palumbi (1993) suggested multiple linear regression as a means of teasing apart effects of generation time and body size on molecular rate. Gillooly et al. (2001) used general principles from thermodynamics to model effects of body mass and temperature on metabolic rate, and Gillooly et al. (2005) demonstrated likely causal influence of metabolic rate on the rate of molecular evolution, although Thomas et al. (2006) found no influence of body size on substitution rates in invertebrates.

A test of the Gillooly et al. (2005) model within a family of North American freshwater fish is advanced here. Because this sample of species demonstrates a significant co-occurrence of temperature and body size with rates of molecular substitution in mtDNA, it strengthens the contention that the mass-specific metabolic rate hypothesis may help explain molecular substitution rate in poikilotherms and perhaps other organisms.

Interactions between heterogeneity in substitution rate and phylogenetic estimation are rapidly becoming tractable (Swofford et al. 1996; Susko et al. 2002; Douzery et al. 2004; Seipel and Haussler 2004; Gadagkar and Kumar 2005). More accurate estimation of recent and ancestral mass and thermal conditions, using physiological ecology, the fossil record, and phylogenetic modeling, will provide more accurate estimates of metabolic rates and substitution rates in studies of coalescence, phylogeography, biogeography, and evolution.

ACKNOWLEDGMENTS

We thank R. Bailey, W. Barber, D. Buth, M. Belk, C. Latta, and P. Marsh for providing specimens. Federally listed fish were collected or otherwise obtained under federal endangered-species permits from U.S. Fish and Wildlife Service (Region 1, Portland, OR; Region 2, Albuquerque, NM; and Region 6, Denver, CO), and appropriate state collecting permits. We thank these agencies for these and other courtesies, colleagues and associates for help in obtaining specimens and other assistance, and a number of organizations, primarily National Science Foundation, U.S. Fish and Wildlife Service, and U.S. Bureau of Reclamation for monetary support. John Megahan helped with the figures.

LITERATURE CITED

Adachi, J. Y., Y. Cao, and M. Hasegawa. 1993. Tempo and mode of mitochondrial DNA evolution in vertebrates at the amino acid sequence level: rapid evolution in warm-blooded vertebrates. J. Mol. Evol. 36:270–281.

Aris-Brosou, S. 2005. Determinants of adaptive evolution at the molecular level: the extended complexity hypothesis. Mol. Biol. Evol. 22:200–209.

Bargelloni, L. P., A. Ritchie, T. Patarnello, B. Battaglia, D. M. Lambert, and A. Meyer. 1994. Molecular evolution at sub-zero temperatures: mitochondrial and nuclear phylogenies of fish from Antarctica (suborder Notothenioidei), and the evolution of antifreeze glycopeptides. Mol. Biol. Evol. 11:854–863.

Bousquet, J., S. H. Strauss, A. H. Doerksen, and R. A. Price. 1992. Extensive variation in evolutionary rate of rbcL gene sequences among seed plants. Proc. Natl. Acad. Sci. USA 89:7844–7848.

Britten, R. J. 1986. Rates of DNA sequence evolution differ between taxonomic groups. Science 231:1393–1398.

Bromham, L. 2002. Molecular clocks in reptiles: life history influences rate of molecular evolution. Mol. Biol. Evol. 19:302–309.

Bromham, L., and D. Penny. 2003. The modern molecular clock. Nat. Rev. Genet. 4:216–224.

Carlander, K. D. 1997. Handbook of freshwater fishery biology. Iowa State Univ. Press, Ames, Iowa.

Cavender, T. M. 1986. Review of the fossil history of North American freshwater fish. Pp. 699–724 *in* C. H. Hocutt and E. O. Wiley, eds. The zoogeography of North American freshwater fish. Wiley Interscience Publications, New York.

Cavender, T. M., and M. M. Coburn. 1993. Phylogenetic relationships of North American Cyprinidae. Pp. 293–327 *in* R. L. Mayden, ed. Systematics, historical ecology, and North American freshwater fish. Stanford Univ. Press, Stanford, CA.

Chao, L., and D. E. Carr. 1993. The molecular clock and the relationship between population size and generation time. Evolution 47:688–690.

Coburn, M. M., and T. M. Cavender. 1993. Interrelationships of North American cyprinid fish. Pp. 328–373 *in* R.L. Mayden, ed. Systematics, historical ecology, and North American freshwater fish. Stanford Univ. Press, Stanford, CA.

DeSalle, R., and A. R. Templeton. 1988. Founder effects and the rate of mitochondrial DNA evolution in Hawaiian *Drosophila*. Evolution 42:1076–1084.

Douzery, E. J. P., E. A. Snell, E. Bapteste, F. Delsuc, H. Philippe. 2004. The timing of eukaryotic evolution: Does a relaxed molecular clock reconcile proteins and fossils? Proc. Natl. Acad. Sci. USA 101:15386–15391.

Dowling, T. E., and B. D. DeMarais. 1993. Evolutionary significance of introgressive hybridization in cyprinid fish. Nature 362:444–446.

Dowling, T. E., C. A. Tibbets, W. L. Minckley, and G. R. Smith. 2002. Evolutionary relationships of the plagopterins (Teleostei: Cyprinidae). Copeia 2002:665–678.

Fitch, W. M., J. M. E. Leiter, X. Li, and P. Palese. 1991. Positive Darwinian evolution in human influenza viruses. Proc. Natl. Acad. Sci. USA 88:4270–4274.

Futuyma, D. J. 1987. On the role of species in anagenesis. Am. Nat. 130:465–473.

Gadagkar, S. R., and S. Kumar. 2005. Maximum-likelihood outperforms maximum parsimony even when evolutionary rates are heterotachous. Mol. Biol. Evol. 22:2139–2141.

Gillespie, J. H. 1991. The causes of molecular evolution. Oxford Univ. Press, New York, and Oxford, U.K.

Gillooly, J. F., J. H. Brown, G. B. West, V. M. Savage, and E. L. Charnov. 2001. Effects of size and temperature on metabolic rate. Science 293(5538):2248–2251.

Gillooly, J. F., A. P. Allen, G. B. West, and J. H. Brown. 2005. Metabolic rate calibrates the molecular clock: reconciling molecular and fossil estimates of evolutionary divergence. Proc. Natl. Acad. Sci. USA 102:140–145.

Graur, D., and W. Martin. 2004. Reading the entrails of chickens: molecular timescales of evolution and the illusion of precision. Trends Genet. 20:80–86

Harvey, P. H., and M. D. Pagel. 1991. The comparative method in evolutionary biology. Cambridge Univ. Press, Cambridge, UK.

Ho, S. Y. W., M. J. Phillips, A. Cooper, and A. J. Drummond. 2005. Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. Mol. Biol. Evol. 22:1561–1568.

Huelsenbeck, J. P., B. Larget, and D. Swofford. 2000. A Poisson compound process for relaxing the molecular clock. Genetics 154:1879–1892.

Kishino, H., J. L. Thorne, and W. J. Bruno. 2001. Performance of a divergence time estimation method under a probabilistic model of rate evolution. Mol. Biol. Evol. 18:352–361.

Langley, C. H., and W. M. Fitch. 1974. An estimation of the constancy of the rate of molecular evolution. J. Mol. Evol. 3:161–177.

LaRivers, I. 1962. Fish and fisheries of Nevada. Nevada State Fish and Game Comm., Carson City.

Larson, A., and A. C. Wilson. 1989. Patterns of ribosomal RNA evolution in salamanders. Mol. Biol. Evol. 6:131–154.

Lee, D. S., C. R. Gilbert, C. H. Hocutt, R. E. Jenkins, D. E. McAllister, and J. R. Stauffer, Jr. 1980. Atlas of North American freshwater fish. N. C. Biol. Surv. Publ. 1980–12, i–x,1–848.

Li, W.-H. 1997. Molecular evolution. Sinauer, Sunderland, MA.

Li, W.-H., and D. Graur. 1991. Fundamentals of molecular evolution. Sinauer, Sunderland, MA.

Linder, H. P., C. R. Hardy, and R. Rutschmann. 2005. Taxon sampling effects in molecular clock dating: an example from the African Restionaceae. Mol. Phylogenet. Evol. 35:569–582.

Lopez, P., D. Casane, and H. Philippe. 2002. Heterotachy, an important process of protein evolution. Mol. Biol. Evol. 19:1–7.

Martin, A. P., and S. R. Palumbi. 1993. Body size, metabolic rate, generation time, and the molecular clock. Proc. Nat. Acad. Sci. USA 90:4087–4091.

Martin, A. P., G. J. P. Naylor, and S. R. Palumbi. 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. Nature 357:153–155.

Minckley, W. L. 1973. fish of Arizona. Arizona Dept. Game and Fish., Phoenix, AZ.

Mindell, D. P. 1996. Positive selection and rates of evolution in immunodeficiency viruses from humans and chimpanzees. Proc. Natl. Acad. Sci. USA 90:4087–4091.

Mindell, D. P., and C. E. Thacker. 1996. Rates of molecular evolution: phylogenetic issues and applications. Ann. Rev. Ecol. Syst. 27:279–303.

Near, T. J., P. A. Meylan, and H. B. Shaffer. 2005. Assessing concordance of fossil calibration points in molecular clock studies: an example using turtles. Am. Nat. 165:137–146.

Ohta, T. 1976. Role of slightly deleterious mutations in molecular evolution and polymorphisms. Theor. Popul. Biol. 10:254–275.

———. 1993. An examination of the generation-time effect on molecular evolution. Proc. Natl. Acad. Sci. USA 90:10676–10680.

Ohta, T., and M. Kimura. 1971. On the constancy of the evolutionary rate of cistrons. J. Mol. Evol. 1:18–25.

Rambaut, A., and Bromham, L. 1998. Estimating divergence dates from nuclear sequences. Mol. Biol. Evol. 15:442–448.

Rand, D. M. 1994. Thermal habit, metabolic rate and the evolution of mitochondrial DNA. Tree 9:125–131.

Sanderson, M. J. 1997. A non-parametric approach to estimating divergence times in the absence of rate constancy. Mol. Biol. Evol. 14:1218–1242.

———. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol. Biol. Evol. 19:101–109.

Schlotterer, C., B. Amos, and D. Tautz. 1991. Conservation of polymorphic simple sequence loci in cetacean species. Nature 354:63–65.

Schmidt-Nielsen, K. 1984. Scaling: why is animal size so important? Cambridge Univ. Press, New York.

Siepel, A., and D. Haussler. 2004. Phylogenetic estimation of context-dependent substitution rates by maximum-likelihood. Mol. Biol. Evol. 21:468–488.

Smith, G. R. 1981. Late Cenozoic freshwater fish of North America. Ann. Rev. Ecol. Syst. 12:163–193.

———. 1992. Introgression in fish: significance for paleontology, cladistics, and evolutionary rates. Syst. Biol. 41:41–57.

Smith, G. R., and T. E. Dowling. Correlating divergence times and hydrography in speckled dace (*Rhinichthys*: Pisces, Cyprinidae) in the Colorado River drainage. M. Reheis, R. Hershler, and D. Miller, eds. Geol. Soc. America Special Vol. *In press*.

Smith, A. B., and K. J. Peterson. 2002. Dating the time of origin of major clades: molecular clocks and the fossil record. Ann. Rev. Earth Planet. Sci. 30:65–88.

Smith, G. R., K. Swirydczuk, P. G. Kimmel, and B. H. Wilkinson. 1982. Fish biostratigraphy of late Miocene to Pleistocene sediments of the western Snake River Plain, Idaho. Pp. 519–542 *in* B. Bonnichsen and R. M. Breckenridge, eds. Cenozoic geology of Idaho. Idaho Bur. Mines and Geol. Bull, Moscow, Idaho. 26, 405 p.

Smith, G. R., N. Morgan, and E. Gustafson. 2000. fish of the Mio-Pliocene Ringgold formation, Washington: Pliocene capture of the Snake River by the Columbia River. Mus. Paleontol Pap. Paleontol. 32:1–47.

Smith, G. R., T. E. Dowling, K. W. Gobalet, T. Lugaski, D. Shiazawa, and R. P. Evans. 2002. Biogeography and timing of evolutionary events among Great Basin fish. Pp. 175–234 *in* R. Hershler, D. B. Madsen, and D. R. Currey, eds. Great Basin aquatic systems history. Smithsonian Contr. Earth Sci., Washington, DC. 33, 405 p.

Spencer, J. E., G. R. Smith, and T. E. Dowling. Middle and late Cenozoic evolution of topography and late Cenozoic evolution of fish in the American Southwest. M. Reheis, R. Hershler, and D. Miller, eds., Geol. Soc. Am. Special Vol. *In press*.

Susko, E., Y. Inagaki, C. Field, M. E. Holder, and A. J. Roger. 2002. Testing for differences in rates-across-sites distributions in phylogenetic subtrees. Mol. Biol. Evol. 19:1514–1523.

Swofford, D. S. 2000. PAUP* 4.0. Phylogenetic analysis using parsimony (* and other methods, ver. 4b2). Sinauer Associates, Sunderland, MA.

Swofford, D. S., G. Olsen, P. Waddell, and D. Hillis. 1996. Phylogenetic inference. Pp. 407–514 *in* D. Hillis, C. Moritz, and B. K. Mable, eds. Molecular systematics. Sinauer, Sunderland, MA.

Thomas, J. A., J. J. Welch, M. Woolfit, and L. Bromham. 2006. There is no universal molecular clock for invertebrates, but rate variation does not scale with body size. Proc. Natl. Acad. Sci. USA 103:7366–7371.

Thorne, J. L., and H. Kishino. 2002. Divergence time and evolutionary rate estimation with multilocus data. Syst. Biol. 51:689–702.

Thorne, J. L., H. Kishino, and I. S. Painter. 1998. Estimating the rate of evolution of the rate of molecular evolution. Mol. Biol. Evol. 15:1647–1657.

Wang, H.-Y., and S.-C. Lee. 2002. Secondary structure of mitochondrial 12S rRNA among fish and its phylogenetic applications. Mol. Biol. Evol. 19:138–148.

Webster, A. J., and A. Purvis. 2002. Testing the accuracy of methods for reconstructing ancestral states of continuous characters. Proc. R. Soc. Lond. B Biol. Sci. 269:143–149.

Welch, J. J., and L. Bromham. 2005. Molecular dating when rates vary. Trends Ecol. Evol. 20:320–327.

Wilke, C. O. 2005. The heavy, the cold, and the slow. Heredity 95:115.

Yoder, A. D., and Z. Yang. 2000. Estimation of primate speciation dates using local molecular clocks. Mol. Biol. Evol. 17:1081–1090.

Associate Editor: R. Harrison