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Evolution

Slow rate of molecular evolution in high-elevation hummingbirds

(Andes/DNA hybridization/molecular clock/oxygen metabolism)

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ABSTRACT **Estimates of relative rates of molecular evolution from a DNA-hybridization phylogeny for 26 hummingbird species provide evidence for a negative association between elevation and rate of single-copy genome evolution. This effect of elevation on rate remains significant even after taking into account a significant negative association between body mass and molecular rate. Population-level processes do not appear to account for these patterns because (*i*) all hummingbirds breed within their first year and (*ii*) the more extensive subdivision and speciation of bird populations living at high elevations predicts a positive association between elevation and rate. The negative association between body mass and molecular rate in other organisms has been attributed to higher mutation rates in forms with higher oxidative metabolism. As ambient oxygen tensions and temperature decrease with elevation, the slow rate of molecular evolution in high-elevation hummingbirds also may have a metabolic basis. A slower rate of single-copy DNA change at higher elevations suggests that the dynamics of molecular evolution cannot be separated from the environmental context.**

The initial view that molecular evolution proceeds at a steady or clock-like rate has given way to an appreciation that such rates may vary widely among organisms (1–3). A number of intrinsic biological attributes are known to be associated with deviations from clock-like rates of molecular evolution, including body mass, generation time, and population structure (4–7). Herein I present an analysis of published DNA hybridization data for hummingbirds (8) that indicates that rates of DNA evolution are slower in species living at higher elevations. Molecular adaptation to high elevations has been documented for a variety of molecules with specific physiological functions, hemoglobin, for example (9). As DNA hybridization measures change across the entire single-copy genome, however, the response of such a broad feature as rate of molecular change to an environmental parameter supports the generalization that DNA evolution is qualitatively similar to morphological evolution in that its form cannot be separated from the environmental context.

MATERIALS AND METHODS

Absolute rates of genetic evolution are difficult to obtain for hummingbirds because their fossil record is extremely limited (8). However, relative rates can be estimated by computing distances (fitted path lengths on the topology) from a designated "outgroup" taxon to members of a monophyletic "ingroup" with which it shares a common ancestor [relative rate test (10)], a procedure that avoids errors inherent in fossil calibrations of absolute rate. As a member of the sister group to hummingbirds (11, 12), the swift *Chaetura pelagica* provides an outgroup for relative-rate estimates among hummingbird species in both the hermit (Phaethornithinae) and nonhermit (Trochilinae) subfamilies (Fig. 1). In turn, any member of one hummingbird subfamily can serve as an outgroup for estimation of rates among species in the other subfamily.

As described in more detail elsewhere (8), the complete set of reciprocal median melting temperatures (T_m) for the 26 hummingbird species and outgroup swift were calculated from raw melting curves and then corrected in several steps to obtain the most accurate estimates of genetic distance (ΔT_mH-C) and phylogeny [ref. 8 and Fig. 1]. The hummingbirds included in the phylogeny represent all known principal lineages (Fig. 1) and reside at different elevations from sea level to over 5,000 m, reflecting the exceptional elevational diversification of hummingbird species during their evolutionary radiation (13). Of the two basal sister groups, nonhermits are an order of magnitude more diverse and occur over a much wider range of elevations than do the predominantly lowland tropical hermits (13). Consequently, nonhermits are better represented in the phylogeny (24 versus 2 species, respectively).

RESULTS

Associations. Previous comparisons of relative rates among the species examined herein have indicated significant molecular rate variation (14). More detailed comparisons based on the swift reveal that relative rates of evolution for the 24 nonhermits are significantly faster than for the two hermits (Wilcoxon two-sample test, $Z = 2.261$, $P < 0.0237$). The rates for hermits fall well outside both the normal distribution of rates for nonhermits (both subfamilies, Shapiro–Wilk $W = 0.835$, $P < 0.0005$; nonhermits only, Shapiro–Wilk $W = 0.967$, $P > 0.59$) and the 95% confidence interval of the regression of relative rates on elevation for nonhermits (Fig. 2); therefore, hermits appear to be distinct outliers for rates among hummingbirds and are analyzed separately.

For the 24 nonhermits, each of three measures of a species' elevational occurrence demonstrates a significant negative association with relative rates measured from the swift or from either of the two hermits (Table 1 and Fig. 2). The pattern is not caused by contributions from extreme outliers but expresses a consistent trend across the range of elevations occupied by hummingbirds. The same tendency obtains as well between the two hermits (Fig. 2) for relative rates measured from each of the 24 nonhermits (sign test, $x = 0$, $n = 24$, $P \ll 0.001$; Fig. 2). These consistent associations between elevation and rate are striking given the conservative estimates provided by relative rates, which discriminate only the independent terminal segments along the paths from the outgroup to the various ingroups.

A direct effect of elevation on rate of molecular evolution could be obscured by confounding variables such as generation

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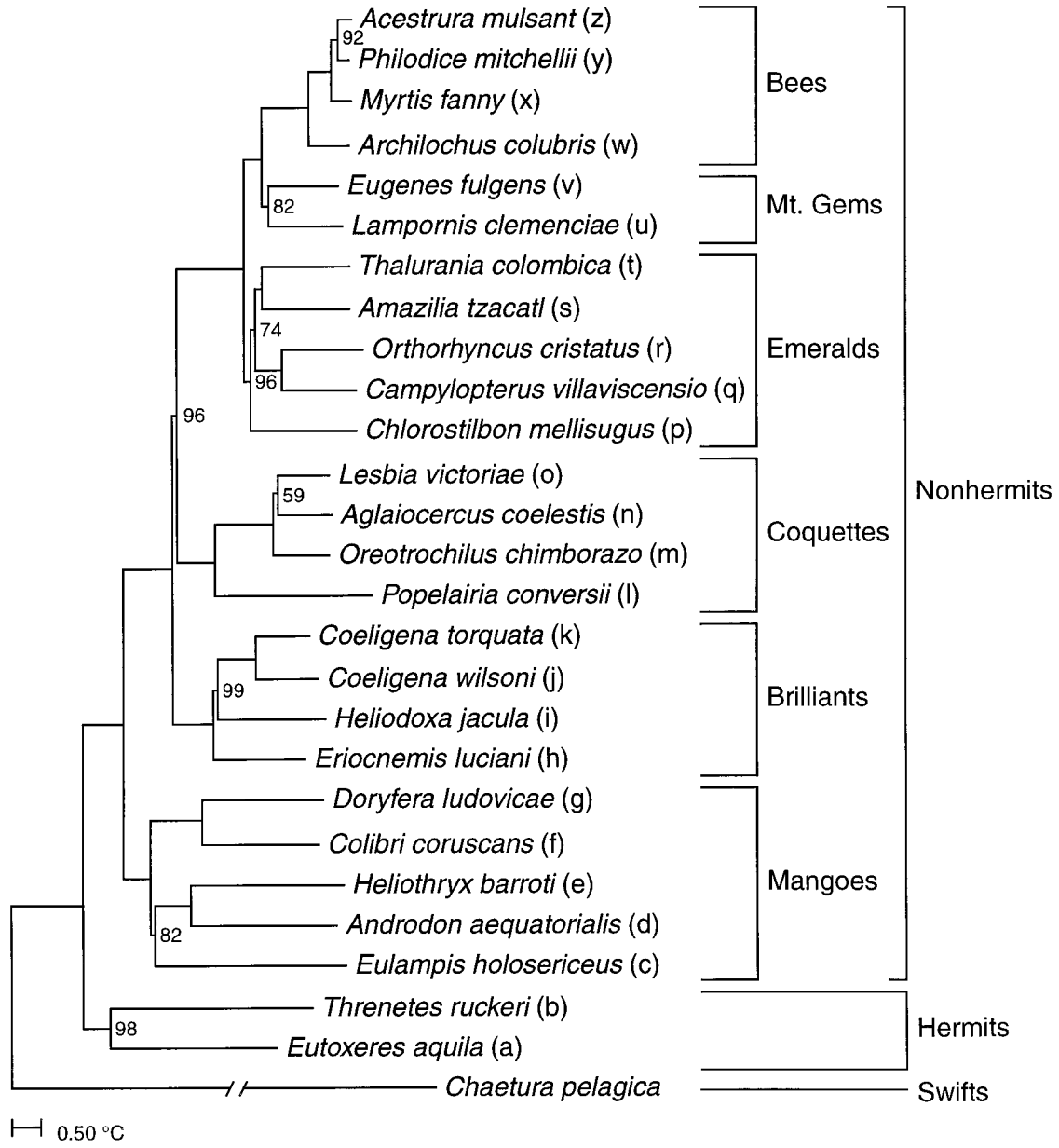

FIG. 1. Consensus unweighted least-squares FITCH topology obtained (8) from a complete matrix of symmetrized ΔT_mH-C values rooted with the outgroup swift *Chaetura pelagica*; names refer to principal nonhermit lineages and relevant subfamilies and families of the Apodiformes (hummingbirds and swifts), and letter codes to species as plotted in Fig. 2. The ΔT_mH-C index was obtained through several steps that minimize inaccuracies in distance measures (8). First, the T_{50}H index was obtained by correcting raw median melting temperatures (T_m) for normalized percentage hybridization (NPH) through application of the second-order polynomial found to fit observed values of T_{50}H regressed on T_m so as to avoid the excessive experimental error inherent in raw measures of NPH. The resulting T_{50}H values were multiplied by the empirically determined scaling factor of 1.2 for percentage sequence divergence (27) and then corrected for homoplasy (28). Finally, these distances were converted to so-called delta (Δ) values by standardizing the melting temperatures of different-species (heterologous) hybrids to the melting temperatures of same-species (homologous) hybrids (8). After symmetrization (29), average path lengths (12) for the resulting ΔT_mH-C values were estimated from 1,000 unweighted least-squares FITCH topologies (30) generated for a corresponding number of bootstrap pseudoreplicate matrices drawn from the complete matrix of 2,025 reciprocal genetic distances (three, rarely fewer, replicates per comparison). Internode support as indicated by bootstrap percentages (out of 1,000, if $<100\%$) suggests strong support for the symmetrized topology, which was stable to multiple-deletion jackknifing (12). [Reproduced with permission from ref. 8. (Copyright 1997, Society for Molecular Biology and Evolution).]

time or body mass, both of which demonstrate negative associations with molecular rates in some vertebrate groups (5–7). With respect to generation time, all hummingbirds breed within their first year and variation in breeding age within this time frame is not significantly associated with rate variation (14). Alternatively, the decrease in rate with elevation could be an indirect consequence of selection for greater body mass in species living at higher and, hence, colder elevations (15). However, elevational occurrence is not correlated with body mass for hummingbirds in this ($\log_e$ midpoint of elevational occurrence with $\log_e$ male body mass, $r = 0.290$, $P = 0.1509$) or much larger (16) samples. Even after calculating the residuals of elevational occurrence regressed on (male) body mass to remove the effect of the latter, and then entering both variables in a general linear model (Table 2), partial F values support a statistically significant contribution to rate by elevation over one also made by body mass (Table 2).

Sources of Error. Statistically based comparative methods to account for nonindependence caused by phylogenetic relat-

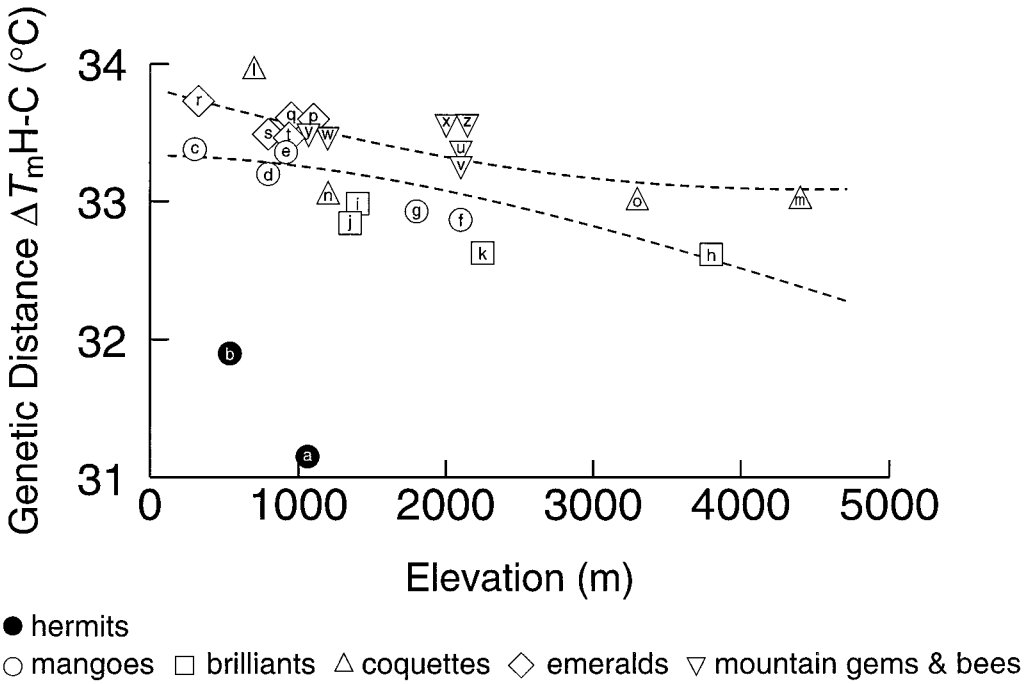

FIG. 2. Scatter plot of average path lengths to ingroup hummingbirds [Fig. 1; measured from outgroup swift (*Chaetura pelagica*)] versus midpoint of elevational occurrence. Taxa coded by principal lineage (symbol) and species (letter codes as indicated in Fig. 1). Overlapping symbols moved to reveal letter codes. Dashed lines indicate the 95% confidence intervals for least-squares regression of nonhermits. Folded F tests indicate significantly less variation in fitted path lengths measured from swift compared with those measured from either hermit [$F' = 9.93$ (*Threnetes*), $F' = 11.48$ (*Eutoxeres*); $df = 23, 23$; $P < 0.0001$], consistent with autocorrelation and saturation effects for the more distant swift comparison. The two hermits give virtually identical results except that distances are uniformly shortened when the more slowly evolving *Eutoxeres aquila* is used as the reference taxon for relative-rate estimates.

edness are not developed for relative-rate tests (17). Consequently, I treated each taxon in the analysis as an independent data point, which artificially inflates the degrees of freedom for hypothesis testing. However, I failed to detect any significant difference in the association between rate and elevation among the different hummingbird lineages [when principal nonhermit lineage (as defined in Fig. 2) is added as a factor to the above model, interaction terms are not significant; for elevation, swift as outgroup, $F = 0.63$, $P > 0.65$; either hermit as outgroup, $F = 0.64$, $P > 0.64$]. Evidence that the negative association between rate and elevation occurs in different phyletic lines (Fig. 2) suggests that the overall significance of the pattern is not biased by relatedness among the taxa examined.

Table 1. Pearson correlation coefficients of ($\log_e$) elevational occurrence (14) with ($\log_e$) genetic distance [average path lengths, based on ΔT_mH-C (Fig. 1)] for the swift (*Chaetura pelagica*) and two hermits (*Threnetes ruckeri* and *Eutoxeres aquila*) as outgroups and nonhermits as ingroups (8)

	Elevational occurrence*		
Outgroup	Minimum	Maximum	Midpoint
Swift			
Chaetura pelagica	−0.6295	−0.5473	−0.5937
	0.0010†	0.0056	0.0022
Hermits			
Threnetes ruckeri	−0.6321	−0.5511	−0.5977
	0.0009	0.0053	0.0020
Eutoxeres aquila	−0.6322	−0.5510	−0.5977
	0.0009	0.0053	0.0020

All probabilities are two-tailed.

*Minimum, lowest elevational occurrence; maximum, highest elevational occurrence; midpoint, midpoint between minimum and maximum.

†Analyses were performed in SAS for UNIX on a SPARC station 20. All P were less than 0.05 after Bonferroni correction for $n = 3$ simultaneous comparisons.

Both empirical and analytical considerations also suggest that observed variation in path lengths reflects variation in rates and not biases in experimental error or differences in genome structure. Melting temperatures of same-individual hybrids of each species used to calculate genetic difference (ΔT_mH-C) values for distance-matrix construction (see caption to Fig. 1) are free from variation caused by rate or evolutionary relationship, thereby providing a direct measure of the variation contributed by fragment length or base composition. Lack of significant correlations between same-individual hybrid melting temperatures and independent variables (e.g., $\log_e$ male body mass, $r = -0.0134$, $P = 0.948$; $\log_e$ midpoint of elevational occurrence, $r = 0.1306$, $P = 0.525$) suggests, therefore, that contributions by these other sources of variation are small and/or random with respect to the focal rate patterns. Furthermore, scaling of the data to the average homologous melting temperature (symmetrization; see caption to Fig. 1) before estimation of path lengths ameliorates the effects of variation in homologous melting temperatures [e.g., compression of distances (8)] and of unequal Δ values between reciprocal comparisons, which may arise from differences in genome size. Thus, the negative association between elevation and rate of molecular evolution appears robust to possible confounding influences.

DISCUSSION

Evidence for slower rates of single-copy DNA evolution in higher-elevation hummingbirds has a number of important implications for studies of organismal and molecular processes. The effect of elevation on rate implies that general features of molecular evolution depend on the physical environment, a connection attributed traditionally only to morphological traits. Moreover, calculations of divergence times based on the assumption of a molecular clock will underestimate the ages of

Table 2. General linear models with residual midpoint of elevational occurrence (RMIDEL; ref. 14) and male body mass (MALEM; ref. 14) as independent variables and genetic distance from outgroup to nonhermit ingroups [average path lengths, based on ΔT_mH-C (Fig. 1)] as dependent variable

Outgroup Source	*df*	Type III sum of squares	Mean square	*F* value	$P > F$
Swift					
Chaetura pelagica					
RMIDEL	1	0.000338	0.000338	7.22	0.0138*
MALEM	1	0.001108	0.001108	23.61	0.0001
Error	21	0.000985	0.000047		
Corrected total	23	0.002550			
Hermits					
Threnetes ruckeri					
RMIDEL	1	0.003456	0.003456	7.44	0.0126
MALEM	1	0.010913	0.010913	23.48	0.0001
Error	21	0.009760	0.000465		
Corrected total	23	0.025323			
Eutoxeres aquila					
RMIDEL	1	0.003998	0.003998	7.43	0.0126
MALEM	1	0.012603	0.012603	23.44	0.0001
Error	21	0.011293	0.000538		
Corrected total	23	0.029274			

For all models, interaction terms of covariates with lineage membership included in error term. Separate analyses were conducted for each outgroup to maintain independence of the tests, which all are based on the same set of ingroup species. Alternative measures of elevational occurrence (Table 1) and body mass (female, species average; ref. 14) gave the same qualitative results. All data are $\log_e$-transformed.

*Analyses were performed in SAS for UNIX on a SPARC station 20. All *P* were less than 0.05 after Bonferroni correction for $n = 3$ simultaneous comparisons.

high-elevation clades relative to ones found at lower elevations. The relationship between the amount of genetic and phenotypic change may be altered as well. Recognition of these biases is important especially for studies of speciation in montane regions, which typically emphasize the relative youthfulness and explosive diversification of high montane forms. Thus, evolutionary studies must consider that genetic dynamics at high elevations may operate differently than in other geographic settings.

The present study adds elevation to the growing list of influences on rates of molecular evolution. However, it is unclear to what extent the underlying mechanisms differ among these many correlates of rate. The negative association between rate of molecular evolution and elevation is counter to expectations that increased subdivision and geographic speciation among high-elevation populations should translate into greater genetic differentiation (4, 18). Moreover, as all hummingbirds breed in their first year, variation in generation time is not likely to be the cause of rate variation in these birds (14). On the other hand, DNA hybridization distances probably reflect mutation rate because they derive from the entire single-copy genome. Thus, the decrease in rate with elevation could reflect a reduced mutation rate caused by physical conditions at high elevations.

The extraordinary physiologies of hummingbirds suggest that metabolic factors could affect mutation rates. Indeed, the negative association between body mass and molecular evolutionary rate documented herein has been explained for mitochondrial DNA as a response to metabolic rate via the mutagenic effects of oxygen (5–7). Although most of the DNA indexed by DNA hybridization represents the nuclear fraction, mitochondria are the primary source of free radicals that damage DNA everywhere in the body (3), and they are present at extraordinary densities in the striated muscles that form the bulk of a hummingbird's mass (19). Thus, free-radical flow caused by mitochondrial activity could increase mutation rates in the nuclear genomes of hummingbirds.

As a starting point for future studies, I suggest that the changed physical conditions at higher elevations (20) could lower mutation rates in resident hummingbirds either because lower partial pressures of oxygen limit maximum oxygen consumption (21) or because lower temperatures require hummingbirds to enter a state of physiological torpor more frequently and at a lower body temperature for a given body mass (20), or both. Lower oxidative stress also might arise as a consequence of reduced caloric intake (22) through the consumption of more dilute nectars typical of higher-elevation bird-pollinated plants (23). An additional factor to consider is that many montane hummingbirds cling rather than hover while feeding at flowers (24, 25). This behavioral response at high elevations also may reduce overall oxygen consumption. However, typical hover feeders occur at moderate to high elevations (*Doryfera ludovicae* among mangoes and *Coeligena torquata* among brilliants) and even these species are evolving more slowly than related low-elevation forms (Figs. 1 and 2). Thus, the molecular response to high elevations occurs independent of flight methods.

Whatever its cause, environmental correlates of molecular evolutionary rates may prove widespread, because birds and other homeotherms living at higher latitude often have greater body mass (15), which as documented herein and in a variety of organisms, demonstrates a negative relationships with rate. Moreover, the influence of mutation rates on demographic processes of aging and mortality (26) may impose characteristic evolutionary dynamics in populations living at different elevations and/or latitudes or in different atmospheres (paleoenvironments with more or less oxygen). Further study of the interaction of population and molecular processes in different environments may reveal previously unsuspected phenomena.

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