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DNA-DNA HYBRIDIZATION EVIDENCE FOR SUBFAMILY STRUCTURE AMONG HUMMINGBIRDS

ROBERT BLEIWEISS,[1] JOHN A. W. KIRSCH,[1] AND JUAN CARLOS MATHEUS[2,3]

[1]*Department of Zoology and the Zoological Museum, University of Wisconsin, Madison, Wisconsin 53706, USA; and*

[2]*Museo Ecuatoriano de Ciencias Naturales, Quito, Ecuador*

ABSTRACT.—We used DNA-DNA hybridization to determine large-scale phylogenetic structure among hummingbirds (Trochilidae). Analyses of complete matrices of ΔT_m and ΔT_{mode} statistics among eight hummingbird genera and a swift generated the same fully resolved topology, which bootstrapping and jackknifing analyses supported at the 100% level. The data are consistent with monophyly for the traditional hermit (Phaethornithinae) and nonhermit (Trochilinae) subfamilies, and with placement of the hermitlike Tooth-billed Hummingbird (*Androdon aequatorialis*) and Green-fronted Lancebill (*Doryfera ludoviciae*) among trochilines. Among the trochilines examined, *D. ludoviciae* is more closely related to the Sparkling Violet-ear (*Colibri coruscans*) than to *A. aequatorialis*, and the Collared Inca (*Coeligena torquata*) is the sister group to these three. Among the hermits examined, the White-tipped Sicklebill (*Eutoxeres aquila*) represents the first branch, followed by the White-whiskered Hermit (*Phaethornis yaruqui*), and the closely related Bronzy Hermit (*Glaucis aenea*) and Band-tailed Barbthroat (*Threnetes ruckeri*). Evolutionary rate estimates from ΔT_m trees corrected for nonadditivity indicate significant rate variation among lineages. Calibration of divergence times with the earliest-known fossil swift suggests that the diverse Andean radiation of trochilines is comprised of at least two lineages (*C. torquata, D. ludoviciae/C. coruscans*) whose origins date to a period of uplift during the mid-Miocene. *Received 10 September 1992, accepted 17 December 1993.*

FAMOUS FOR THEIR characteristic adaptations to feeding at flowers, the more than 330 hummingbird species comprise one of the principal evolutionary radiations among birds. Current efforts to understand the diversification of hummingbirds are limited by lack of the historical framework necessary for the study of evolutionary pattern and process. There is little doubt that hummingbirds constitute a monophyletic group. However, current hummingbird classifications are still based largely on 19th-century studies that relied on bill and plumage characters (Elliot 1879, Boucard 1895, Hartert 1900, Ridgway 1911, Simon 1921), which are now known to be influenced greatly by feeding and social behaviors (Feinsinger and Colwell 1978). Construction of a hummingbird phylogeny is handicapped by the paucity of fossils, and by the extensive and varied modifications to trochilid locomotor and feeding systems that obscure morphological homologies (Cohn 1968). For these reasons, biochemical methods for phylogeny construction should provide essential clues to hummingbird evolution. These techniques allow comparison across a wide taxonomic range in measures that are independent of anatomy. In this paper, we examine the large-scale structure of the hummingbird radiation with DNA-DNA hybridization, a technique that provides an objective measure of overall genomic similarity.

The long-standing distinction between the hermit (Phaethornithinae) and nonhermit (Trochilinae) subfamilies (Reichenbach 1854, Cabanis and Heine 1860, Gould 1861, Ridgway 1911) provides the starting point for insights into hummingbird phylogeny. As currently recognized, the approximately 30 hermit hummingbirds typically are forest dwellers with decurved bills and dull monomorphic plumage, whereas most of the about 300 nonhermits have broader ecological ranges, straight bills, and often iridescent, sexually dichromatic plumages (Stiles 1981, Collins and Paton 1989, Paton and Collins 1989, Bleiweiss 1990). Our primary objective was to test monophyly for the subfamilies. In addition, we sought to determine the subfamilial placement of the problematic gen-

[3] Current address: Corporación Ornitológica del Ecuador (CECIA), P.O. Box 17-17-906 Quito, Ecuador.

TABLE 1. Summary of species and specimens used, with each individual identified by its DNA extraction number. Voucher specimens deposited as study skins or skeletons in collections of University of Wisconsin Zoological Museum or Museo Ecuatoriano de Ciencias Naturales.

Glaucis aenea. Encampamento de CODESA, 21.6 road km from Pedro Vicente Maldonado, Provincia Pichincha, Ecuador (1135, 1136, 1137, 1138).

Threnetes ruckeri. Centro Científico Río Palenque, 56 km SW Santo Domingo de los Colorados, on Río Babo, Provincia de Los Ríos (1277, 1278); Encampamento de CODESA, 21.6 road km from Pedro Vicente Maldonado, Provincia de Pichincha (1355, 1356), Ecuador.

Phaethornis yaruqui. Hacienda Santa Isabel, 2.6 km above Toache along Río Pilatón (1158, 1159); Encampamento de CODESA, 21.6 road km from Pedro Vicente Maldonado, Provincia de Pichincha (1351, 1352), Ecuador.

Eutoxeres aquila. Encampamento de CODESA, 21.6 road km from Pedro Vicente Maldonado, Provincia de Pichincha, Ecuador (1162, 1163, 1401, 1402).

Androdon aequatorialis. Encampamento de CODESA, 21.6 road km from Pedro Vicente Maldonado, Provincia de Pichincha, Ecuador (1397, 1398, 1435, 1436).

Doryfera ludoviciae. Below Hacienda Santa Rosa on Río Cinto, Provincia de Pichincha, Ecuador (1353, 1354, 1437, 1438).

Colibri coruscans. Tumbaco, Provincia de Pichincha, Ecuador (1357, 1358, 1403, 1404).

Coeligena torquata. Below Hacienda Santa Rosa on Río Cinto, Provincia de Pichincha, Ecuador (1279, 1280, 1399, 1400).

Chaetura pelagica. Town of McFarland, Dane County, Wisconsin (1049, 1050, 1148, 1441).

era *Androdon* and *Doryfera*; their superficial resemblance to hermits has led some to place them within that assemblage (Peters 1945, Meyer de Schauensee 1966), whereas others have placed one or both of these genera among nonhermits (Ridgway 1911, Wetmore 1968, Zusi and Bentz 1982, Stiles and Skutch 1989).

METHODS

Choice of taxa.—We collected all hummingbirds on field trips to Ecuador between 1987 and 1991 (Table 1). We obtained a representative species from four of the five traditionally recognized hermit genera: Bronzy Hermit (*Glaucis aenea*), Band-tailed Barbthroat (*Threnetes ruckeri*), White-whiskered Hermit (*Phaethornis yaruqui*), and White-tipped Sicklebill (*Eutoxeres aquila*). The hermit genus *Ramphodon* is endemic to Brazil and was not obtained. In addition to *Androdon aequatorialis* and *Doryfera ludoviciae*, we included two putative trochilines, the Sparkling Violet-ear (*Colibri coruscans*) and the Collared Inca (*Coeligena torquata*). Together, these eight genera also allowed us to examine whether hypotheses of relationship among the principal hummingbird clades are concordant across molecular (allozyme; Gill and Gerwin 1989) and morphological (condition of the tensor patagii brevis muscle, TPB; Zusi and Bentz 1982) characters. We used the Chimney Swift (*Chaetura pelagica*, Apodidae) as an unambiguous outgroup to root the trees.

Biochemical methods.—All tissues (soft organs and muscle) were preserved in either 95% ethanol or liquid nitrogen shortly after collection, normally within 15 min of death. General laboratory procedures for the purification and sizing of nuclear driver DNA, and isolation and iodination of single-copy tracer DNA followed the methods described in Kirsch et al. (1990b) with the following modifications. Raw extracts were sized on 1% agarose gels before as well as after sonication to estimate fragment lengths; all samples used in experiments had a mean fragment size of 500 to 700 bp. Based upon reassociation kinetic studies of birds generally, and of hummingbirds in particular (unpubl. data), we used a C_0t value of 200 (eC_0t 1,130) to prepare all single-copy extracts. We adjusted the iodination reaction mixture's pH to the range of 4.4 to 4.7 by color matching samples with ColorpHast Indicator Strips (pH 4.0–7.0; EM Science, Cherry Hill, New Jersey).

Each hybrid was fabricated in a 1-ml septum vial by combining 100 μg of sheared nuclear DNA at a standard concentration of 4 μg/μl in 0.48 M sodium phosphate buffer (PB) with approximately 1.0 μg of radiolabeled single-copy DNA. This low tracer : driver ratio was chosen to assure accurate quantitation and did not appear to cause undue distortion of melting statistics due to tracer : tracer hybridization; our elution curves lack the secondary high-temperature bump that indicates severe tracer : tracer hybridization. Hybrids destined for the same run were boiled simultaneously for 10 min and incubated at 60°C to a C_0t of 6,000 (eC_0t 33,900). We used hydroxyapatite (HAP) chromatography in 0.12 M PB to obtain thermal-elution profiles, which requires that each hybrid be diluted to 0.12 M PB before loading it onto HAP columns in the thermal elution device (TED; Kirsch et al. 1990a). We previously documented that nonspecific reassociation of DNAs may occur if such diluted hybrids are allowed to sit at room temperature for more than 45 min (Bleiweiss and Kirsch 1993a), the time it usually takes to load the TED. To avoid this effect, each hybrid was diluted in turn and loaded onto a randomly selected column in the TED. Each

run began with two 10-ml room-temperature washes followed by one 8-ml elution at 52°C, and 22 additional 5-ml elutions 2° apart from 54° to 96° inclusive.

Matrix design.—Each taxon in turn served as a labeled homologous standard, and was used to generate a complete matrix of pairwise distances. The matrix was designed to maximize intraspecific genetic variation among replicate distances between taxa, a procedure that should provide a conservative measure of intertaxon separation. Previous experiments indicate that different individuals contribute significant additive variance to some melting statistics (Bleiweiss and Kirsch 1993b). Therefore, we used driver DNA from a different individual for each of the four replicate heterologous comparisons per cell in the matrix. Each of two TED runs per label included two randomly selected replicate drivers per taxon, for a total of 18 experimental hybrids per run.

In addition, we took experimental precautions to limit systematic biases (enumerated in Bleiweiss and Kirsch 1993a), including compression of distances measured from some homologous standards to heterologous hybrids (Springer and Kirsch 1991). Such compression is caused by low-melting standards, which may arise either from degraded DNA used to make tracers or from preparation error during iodination (Bleiweiss and Kirsch 1993b). We minimized this problem by: (1) choosing to iodinate well-preserved undegraded DNAs; and (2) selecting iodinated tracers that demonstrated high homologous melting temperatures ($T_{mode} > 83.0$°C) in a "screening" run of TED. Residual compression was further reduced through application of a scalar correction for asymmetry (Sarich and Cronin 1976).

We treated interrun variation as a source of random error added to individual variation because melting temperatures of standards differed little among runs (see Dickerman 1991). Although replicate tracers would add random variation to replicate measures, the small quantity of DNA obtainable from individual hummingbirds makes this level of replication impractical.

Calculation and correction of stability indices.—We expressed the usual thermal-stability measures of T_{mode}, T_m (T_{median}) and NPH (normalized percentage hybridization; Sheldon and Bledsoe 1989, Kirsch et al. 1990b) as Δ-values: the distance between a heterolog and the mean value of the homologous standard comprised of DNAs from the same individual. Thermal stability of hybrid duplexes is partly a function of buffer concentration, as well as temperature. As a result, hybrids that are stable at 60°C in 0.48 M PB (incubation conditions) may dissociate at slightly lower temperatures in 0.12 M PB (chromatography conditions; Werman et al. 1990). Therefore, we calculated T_{mode} and T_m starting from 56°C. The modal melting temperature (T_{mode}) was estimated by fitting a downward-opening parabola to five points in the region of the observed modal elution temperature; the fitted mode was then set equal to the parabolic vertex. The median melting temperature (T_m), the temperature at which 50% of hybrid strands are dissociated, was estimated by linear interpolation when hybrid strands attained 50% dissociation between observed elution temperatures. $T_{50}H$ was obtained by normalizing each fraction of radioactive counts for reduced NPH, the percentage reassociation of heteroduplex compared to homoduplex, and proceeding as for T_m. Counts eluted from 52° to 54°C contribute to the fraction of total hybridizable DNA and serve as the basis for normalizing the percentage hybridization. We excluded the two room temperature washes from calculation of NPH, as these probably include fragments that are incapable of forming hybrids at the criterion temperature, as well as much free iodine.

The high variability among replicate NPH values limits use of this measure for phylogeny construction (except for very distant comparisons of well-separated taxa; see Kirsch et al. 1991). However, reduced NPH (less than 100%) compresses T_m distances because the genomic fraction that is too divergent to form stable hybrids at the criterion temperature is not incorporated into the measure of divergence. Better estimates of branch lengths for rate tests can be obtained by correcting T_m for reduced NPH ($T_{50}H$). We used observed NPH to adjust each T_m rather than estimating $T_{50}H$ from an empirical regression on T_m. All indices were then corrected for asymmetry arising from variation in the melting temperature of homologous standards by application of A. W. Dickerman's program SYMMETRY, which calculates a modified percent nonreciprocity correction (100 [reciprocal differences/reciprocal sums]) scaled to the average homologous melting temperature (Sarich and Cronin 1976). Finally, $\Delta T_{50}H$ values were further adjusted for multiple hits by application of the Jukes-Cantor (1969) correction of homoplasy (convergence, parallelism, and multiple mutations at single-base sites). This admittedly crude correction assumes equal AT:GC ratios, but is the only one easily applicable to distance data.

Tree construction.—Best-fit least-squares algorithms available in Felsenstein's (1990; version 3.3) PHYLIP package were used to construct phylogenetic trees. We conducted parallel analyses on uncorrected and corrected matrices for all indices without (program FITCH) and with (program KITSCH) the assumption of a molecular clock. Analyses were done on square matrices in which reciprocal values were retained as distinct, and with the global optimization (G), and subreplicate (S) options enabled. Both FITCH and KITSCH search for the best-fit tree based on the criterion that the unexplained sums-of-squares are minimized. We used the unweighted least-squares method (power option set to $P = 0.0$; Cavalli-Sforza and Edwards 1967) to obtain best-fit trees because there was no apparent increase in variance with distance in our data (as noted below).

TABLE 2. Summary statistics for ΔT_{mode} values: mean melting temperature followed by standard deviation/number of replicates. Percent nonreciprocity improved from 2.593 to 2.114 with application of scalar multiplier corrections at bottom of each column. In Tables 2–4, diagonals expressed as actual melting temperatures or index values, not as relative values.

	Taxon								
Taxon	1	2	3	4	5	6	7	8	9
1 *Chaetura pelagica*	85.77 0.26/6	17.94 0.88/4	19.72 1.06/4	16.69 0.36/4	19.11 0.45/4	18.16 0.61/4	16.16 0.44/4	19.34 0.79/4	18.47 0.31/4
2 *Phaethornis yaruqui*	17.51 0.45/4	84.02 0.09/4	5.31 0.37/4	3.38 0.12/4	3.59 0.36/4	7.16 0.30/4	7.23 0.18/4	6.83 0.48/4	6.93 0.22/4
3 *Eutoxeres aquila*	17.37 0.42/4	5.28 0.37/4	86.18 0.13/4	5.21 0.27/4	5.37 0.56/4	6.70 0.20/4	6.71 0.27/4	6.49 0.51/4	6.78 0.25/4
4 *Threnetes ruckeri*	17.45 0.57/4	3.64 0.09/4	5.16 0.13/4	84.14 0.21/4	2.25 0.27/4	7.17 0.24/4	6.97 0.12/4	6.61 0.24/4	7.12 0.40/4
5 *Glaucis aenea*	17.46 0.38/4	3.67 0.12/4	5.31 0.22/4	2.35 0.30/4	84.34 0.27/4	7.14 0.22/4	7.44 0.47/4	6.68 0.18/4	7.06 0.20/4
6 *Androdon aequatorialis*	17.55 0.54/4	6.82 0.25/4	6.58 0.30/4	6.91 0.22/4	6.80 0.06/4	84.27 0.15/4	5.43 0.14/4	4.65 0.07/4	6.03 0.16/4
7 *Doryfera ludoviciae*	17.69 0.69/4	6.73 0.30/4	6.24 0.44/4	6.60 0.26/4	6.57 0.22/4	4.96 0.26/4	83.41 0.22/6	3.53 0.07/4	5.56 0.18/4
8 *Colibri coruscans*	17.46 0.49/4	6.76 0.19/4	6.15 0.30/4	6.52 0.29/4	6.41 0.13/4	5.29 0.36/4	3.78 0.13/4	83.90 0.10/4	5.33 0.46/4
9 *Coeligena torquata*	17.62 0.39/4	6.85 0.23/4	6.46 0.28/4	6.80 0.11/4	6.84 0.31/4	6.01 0.23/4	6.00 0.12/4	5.61 0.43/4	84.49 0.21/4
Correction	1.034	1.007	0.989	1.036	1.005	0.980	0.980	0.976	0.991

We used several methods to test the robustness of the resulting phylogenetic trees. We applied a modified Mantel test (Archie 1990) to determine if the FITCH matrix departed significantly from the null hypothesis of no structure. Column (tracer) values were randomized 100 times, with the constraint that the diagonal (zero) elements and outgroup (*Chaetura pelagica*) column raw Δ-values were held constant to provide a conservative test of relationships among ingroup taxa. We then tested the significance of the resulting Z-scores:

$$(\text{mean SS matrix}_{\text{randomized}} - \text{SS matrix}_{\text{structured}}) \div (\text{SD SS matrix}_{\text{randomized}}),$$

where SS equals the sum-of-squares for the corresponding matrix.

Subsequently, each matrix of uncorrected and corrected data was bootstrapped (see Krajewski and Dickerman 1990) to evaluate the robustness of trees to measurement error, and jackknifed (see Lanyon 1985) to evaluate the sensitivity of trees to internal inconsistencies due to inclusion of a specific taxon. Bootstrap analysis of distance data measures the stability of each node in the tree to the effects of random sampling among the actual replicate distance measures in each cell of the original matrix; stability is expressed as the proportion of replicate matrices that recover that branch point. We used Felsenstein's (1990) program CONSENSE to obtain the majority-rule consensus topology among FITCH analyses on bootstrap pseudoreplicate matrices created by A. W. Dickerman's program BOOTTEMP, which samples the original matrix with replacement. We subjected each pseudoreplicate matrix to the same FITCH options noted above, and also randomized the input order of taxa prior to each FITCH run. We jackknifed each matrix by removing each taxon in turn from the full 9 × 9 matrix and then finding, by inspection, the strict-consensus topology among the reduced FITCH trees. Taxon-specific distortions of the topology can be inferred from lack of support for any node when that taxon is omitted. Finally, rate variation was tested by comparing the sums-of-squares for FITCH and KITSCH trees for the Jukes-Cantor-corrected $\Delta T_{50}H$ data by the *F*-ratio test of Felsenstein (1986, 1990). This test evaluates whether the sums-of-squares of the best-fit tree without assumption of a clock (FITCH) is significantly smaller than the sums-of-squares for the best-fit tree obtained under the clock model (KITSCH). The *F*-ratio is calculated as:

$$(SS_{KITSCH} - SS_{FITCH}/df_{KITSCH} - df_{FITCH})/(SS_{FITCH}/df_{FITCH}),$$

where SS equals the sum-of-squares and df the degrees of freedom for the corresponding matrix.

RESULTS

Distance matrix.—The 9 × 9 square matrices of ΔT_m, ΔT_{mode} and ΔNPH are comprised of 328

TABLE 3. Summary statistics for ΔT_m values: mean melting temperature followed by standard deviation/number of replicates. Percent nonreciprocity improved from 3.274 to 1.424 with application of scalar multiplier corrections at bottom of each column.

Taxon	Taxon								
	1	2	3	4	5	6	7	8	9
1 *Chaetura pelagica*	83.26 0.40/6	13.67 0.66/4	14.83 0.96/4	13.31 0.42/4	13.50 0.60/4	14.04 0.26/4	12.30 0.54/4	13.33 1.08/4	14.26 0.27/4
2 *Phaethornis yaruqui*	14.41 0.40/4	80.84 0.07/4	5.92 0.49/4	3.39 0.16/4	3.45 0.15/4	6.85 0.39/4	6.39 0.12/4	6.70 0.65/4	6.94 0.21/4
3 *Eutoxeres aquila*	14.52 0.53/4	5.07 0.13/4	84.10 0.21/4	5.25 0.11/4	5.23 0.54/4	6.43 0.21/4	6.00 0.12/4	6.37 0.40/4	6.69 0.34/4
4 *Threnetes ruckeri*	14.30 0.60/4	3.57 0.07/4	5.80 0.25/4	81.41 0.41/4	2.14 0.36/4	6.80 0.18/4	6.32 0.03/4	6.29 0.31/4	7.00 0.36/4
5 *Glaucis aenea*	14.40 0.33/4	3.68 0.23/4	5.97 0.25/4	2.41 0.44/4	81.11 0.49/4	6.82 0.17/4	6.76 0.53/4	6.46 0.13/4	7.07 0.33/4
6 *Androdon aequatorialis*	14.43 0.64/4	6.42 0.13/4	7.20 0.33/4	6.57 0.23/4	6.26 0.21/4	81.17 0.36/4	4.98 0.08/4	4.78 0.06/4	6.12 0.16/4
7 *Doryfera ludoviciae*	14.47 0.46/4	6.44 0.09/4	6.82 0.50/4	6.33 0.26/4	6.34 0.24/4	4.92 0.27/4	80.50 0.24/6	3.52 0.13/4	5.77 0.16/4
8 *Colibri coruscans*	14.43 0.60/4	6.46 0.02/4	6.71 0.49/4	6.20 0.28/4	6.08 0.21/4	5.41 0.51/4	3.71 0.12/4	80.62 0.10/4	5.61 0.33/4
9 *Coeligena torquata*	14.65 0.39/4	6.51 0.24/4	7.04 0.33/4	6.51 0.12/4	6.56 0.34/4	5.89 0.39/4	5.37 0.09/4	5.65 0.45/4	81.64 0.46/4
Correction	0.949	1.027	0.920	1.027	1.061	0.985	1.040	1.016	0.972

experimental hybrids (Tables 2–4). Six hybrids (<2.0% of total) were re-run because their first melts gave anomalously low NPH values: two with *Doryfera ludoviciae* as tracer, and four with *Chaetura pelagica* as tracer. Homologous standards for these replacement hybrids were retained, as indicated by six rather than four replicates in the corresponding diagonal cell. Typical thermal-elution profiles for hybrid DNAs with hummingbird labels indicate that average ΔT_m and ΔT_{mode} are in the range of from 2.0° to 9.0°C (Fig. 1). Thus, DNA-DNA hybridization indicates substantial genetic divergence among major hummingbird lineages. Distances from hummingbirds to the swift are at least 15°C (for ΔT_m), reflecting a distant sister-group relationship (Sibley and Ahlquist 1990, Bleiweiss et al. unpubl. data).

The average heteroduplex variances for ΔT_m and ΔT_{mode} (Tables 2–4) fall between ones obtained in other hybridization studies with avian tissues, being higher than those reported for herons, nine-primaried oscines, and tits (Sheldon 1987, Sheldon and Bledsoe 1989, Sheldon et al. 1992), and lower than those reported for cranes (Krajewski 1989). The intermediacy of our values may arise from differences in experimental design. Our strict adherence both to replication of heteroduplex drivers with different individuals and to inclusion of lower temperatures for the calculation of indices may increase replicate variance (as compared to Sheldon 1987, Sheldon and Bledsoe 1989, Sheldon et al. 1992). Conversely, our use of one tracer preparation for each taxon may decrease replicate variance (as compared to Krajewski 1989). As documented previously, the precision of replicate measures is highest for ΔT_{mode} (SD = 0.305), intermediate for ΔT_m (SD = 0.317) and lowest for NPH (SD = 1.631; Sheldon and Bledsoe 1989, Bleiweiss and Kirsch 1993b). However, the superior precision of ΔT_{mode} compared to ΔT_m is not as great as sometimes claimed (Sarich et al. 1989). Regressions of standard deviation on genetic distance from the homoduplex standard demonstrated no trend with distance for the various uncorrected and corrected matrices. Therefore, we analyzed all matrices with the Cavalli-Sforza and Edwards (1967) method of unweighted least-squares regression.

Phylogeny.—The best-fit trees obtained by FITCH and KITSCH for unsymmetrized and symmetrized ΔT_{mode} and ΔT_m matrices support the same topology (Fig. 2). The excellent fit of this topology to the matrices of observed distances is indicated by the small residual (unex-

TABLE 4. Summary statistics for decrease in Δ-normalized percentage hybridization (NPH): mean ΔNPH followed by standard deviation/number of replicates. Percentage nonreciprocity improved from 21.664 to 16.747 with application of scalar multiplier corrections at bottom of each column.

Taxon	Taxon								
	1	2	3	4	5	6	7	8	9
1 *Chaetura pelagica*	100.00	47.55	24.48	51.43	35.10	40.33	52.73	33.97	41.83
	0.30/6	1.77/4	2.23/4	2.11/4	8.26/4	1.67/4	3.30/4	10.66/4	2.61/4
2 *Phaethornis yaruqui*	41.30	100.00	3.90	4.97	0.18	9.05	12.00	6.93	10.07
	3.68/4	0.82/4	0.77/4	1.19/4	1.46/4	1.19/4	0.16/4	1.24/4	0.94/4
3 *Eutoxeres aquila*	43.45	7.88	100.00	9.02	5.85	7.70	11.60	7.53	10.00
	2.48/4	0.94/4	0.23/4	1.09/4	1.88/4	1.56/4	1.01/4	2.96/4	1.68/4
4 *Threnetes ruckeri*	44.33	4.07	4.43	100.00	−1.00	8.78	13.03	6.53	10.10
	2.07/4	0.52/4	1.10/4	0.90/4	2.01/4	0.53/4	2.27/4	0.92/4	0.62/4
5 *Glaucis aenea*	42.85	3.67	3.63	4.00	100.00	9.73	8.68	9.17	11.15
	2.93/4	0.28/4	0.42/4	1.65/4	1.14/4	1.31/4	5.31/4	3.01/4	2.04/4
6 *Androdon aequatorialis*	44.18	11.90	6.28	12.92	4.03	100.00	9.83	4.92	9.22
	1.30/4	0.88/4	1.52/4	0.96/4	1.82/4	0.52/4	0.53/4	1.19/4	0.21/4
7 *Doryfera ludoviciae*	43.98	10.70	5.05	11.12	7.95	6.53	100.00	5.37	8.55
	1.06/4	0.76/4	0.53/4	1.11/4	2.11/4	0.92/4	0.49/6	1.82/4	0.73/4
8 *Colibri coruscans*	41.05	10.40	6.53	10.87	3.53	6.20	5.33	100.00	7.27
	1.45/4	0.71/4	2.74/4	1.05/4	2.26/4	0.24/4	1.31/4	0.44/4	0.75/4
9 *Coeligena torquata*	42.63	10.70	5.00	10.90	8.15	6.55	8.55	7.50	100.00
	2.97/4	1.10/4	1.15/4	0.55/4	1.75/4	0.48/4	1.17/4	3.07/4	0.58/4
Correction	0.908	0.767	1.570	0.762	1.283	1.000	0.775	1.037	0.873

plained) sums-of-squares (Table 5), which are further reduced by the asymmetry correction. The Mantel test indicated significant ($P < 0.05$) structure in the FITCH matrix for all ΔT_{mode} (unsymmetrized $Z = 7.65$, symmetrized $Z = 7.78$) and ΔT_m (unsymmetrized $Z = 7.40$, symmetrized $Z = 8.39$) matrices. In addition, both jackknifing and bootstrapping (1,000 times) of all ΔT_{mode} and ΔT_m matrices supported each node 100% of the time.

In the phylogeny, the basal dichotomy clearly is consistent with the traditional distinction between the subfamilies Phaethornithinae (*Glaucis, Threnetes, Phaethornis, Eutoxeres*) and Trochilinae. Additionally, both *Androdon aequatorialis* and *Doryfera ludoviciae* are placed in the latter clade. Among the four trochilines, our results support a sister-group relationship between the High Andean *Coeligena torquata* and the trio of *A. aequatorialis, D. ludoviciae,* and *Coli-*

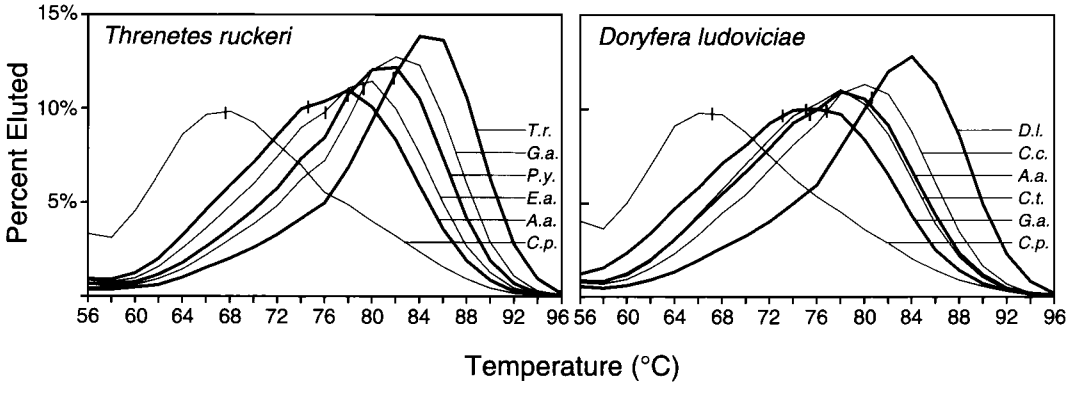

Fig. 1. Representative thermal-elution curves for hummingbirds and Chimney Swift. Curves distinguished by alternating bold (starting with standard at right) and fine lines: T.r., *Threnetes ruckeri*; G.a., *Glaucis aenea*; P.y., *Phaethornis yaruqui*; E.a., *Eutoxeres aquila*; A.a., *Androdon aequatorialis*; C.p., *Chaetura pelagica*; D.l., *Doryfera ludoviciae*; C.c., *Colibri coruscans*; C.t., *Coeligena torquata*.

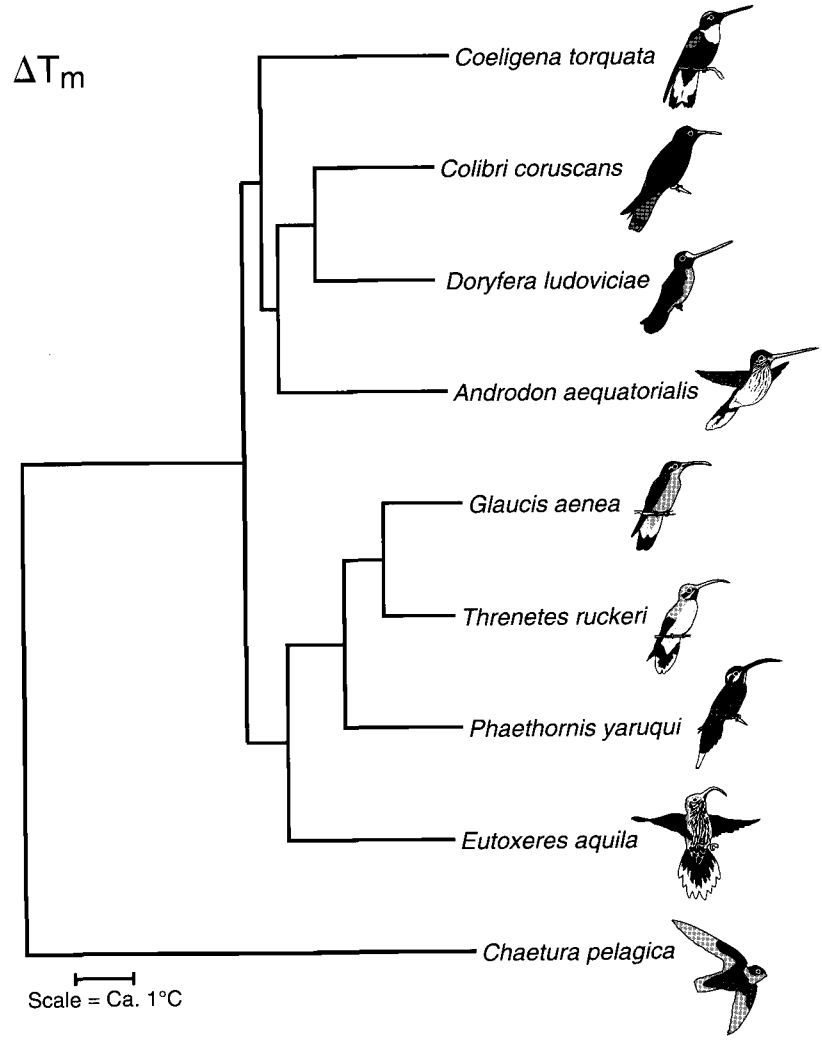

Fig. 2. Best-fit topology generated by FITCH program in PHYLIP from symmetrized ΔT_m matrix. Same topology obtained for all other combinations of index and uncorrected or corrected data. Bird drawings based on plates by J. A. Gwynne, Jr. (*Glaucis, Threnetes, Phaethornis, Eutoxeres, Androdon, Doryfera*) and G. Tudor (*Colibri, Coeligena*) in Hilty and Brown (1986).

bri coruscans. Within the latter clade, *D. ludoviciae* shares more recent ancestry with *C. coruscans* than with *A. aequatorialis*. Among the four phaethornithines, *Glaucis aenea* and *Threnetes ruckeri* together form the sister group to *Phaethornis yaruqui*, while *Eutoxeres aquila* is the sister group to these three. The outgroup swift is very distant from all of these, and uniformly roots the tree between the two hummingbird subfamilies.

Rates.—Heteroduplex NPH values were reduced 0.18% to 13.03% among hummingbirds, and 52.73% between hummingbirds and the swift. Thus, some correction of ΔT_m for reduced NPH is warranted prior to conducting rate tests. The best-fit FITCH tree generated from the symmetrized $\Delta T_{50}H$ matrix further corrected for nonadditivity with the Jukes-Cantor correction agrees with that obtained with ΔT_m and ΔT_{mode} and reveals some apparent rate differences among hummingbird lineages (Fig. 3). As FITCH and KITSCH gave the same best-fit topology for the $\Delta T_{50}H$ matrix, the significance of the rate differences among hummingbird lineages (Fig. 3) could be assessed directly (without specification of a user tree for KITSCH) using Felsen-

TABLE 5. Summary of sums-of-squares for all topologies (na = not applicable).

	Total correction		Fitch correction		Kitsch correction	
Index	None	Symmetry	None	Symmetry	None	Symmetry
ΔT_{mode}	10,145	na	61.25	46.06	65.97	53.24
ΔT_{m}	5,831	na	33.30	7.69	43.15	13.01

stein's (1986) *F*-ratio test. For our trees: $SS_{KITSCH} = 425.67$, $SS_{FITCH} = 391.22$, $df_{KITSCH} = 321$, $df_{FITCH} = 313$. This gives an *F*-ratio of 3.40 (df = 8 and 313; calculations based on those for subreplicate option), which is significant at $P < 0.005$.

DISCUSSION

The robustness of our phylogenetic tree is enhanced by our experimental design, which: (1) employed a complete suite of reciprocal comparisons to distinguish rate variation from relationship; (2) used a different individual for each replicate driver to provide an internal control for hypotheses of relationship among taxa; and (3) limited compression among reference standards. Here we compare our results with those obtained in earlier studies based on the same and other characters. We then use our phylogeny to explore evolutionary rates and patterns of historical biogeography among these hummingbird lineages.

COMPARISON WITH OTHER PHYLOGENETIC STUDIES

Comparison among techniques.—Our results support previous evidence consistent with monophyly of the traditional Phaethornithinae (hermit) and Trochilinae (nonhermit) subfamilies, and for placement of the hermitlike *Androdon aequatorialis* and *Doryfera ludoviciae* among the trochilines. The earlier DNA-DNA hybridization study by Sibley and Ahlquist (1990) used 4 hermit and 13 nonhermit genera, but labeled only two trochilines. Nevertheless, their UPGMA analysis indicated that the hermit genera are all excluded from the trochilines, which included *A. aequatorialis*. Allozymes also support the major features of our phylogeny, including cohesion of the traditional hermit genera, their distant separation from *D. ludoviciae* and *A. aequatorialis* (Gill and Gerwin 1989), and the placement of the latter two among the trochilines (Gerwin 1985, pers. comm.). While a cladistic analysis of hummingbird phylogeny based on anatomy is lacking, features of the nasal operculum, skull, humerus, and tensor patagii brevis (TPB) muscle all support the association of traditional hermit genera (Zusi unpubl. abstract) and placement of *A. aequatorialis* and *D. ludoviciae* among the trochilines (Zusi and Bentz 1982). Thus, a broad spectrum of characters are concordant on the issues of subfamily structure and membership therein.

Although we included exemplars of only 4 of the more than 100 trochiline genera, even these few comparisons revealed some surprising associations, not all of which agree with hypotheses of relationship based on other characters. Some linear classifications place *Androdon aequatorialis* at the end of the hermits and before the nonhermits. This arrangement implies that resemblance of *A. aequatorialis* to hermits might be due to shared plesiomorphy. Although allozyme data place *A. aequatorialis* most often at the base of the trochilines (Gerwin 1985,

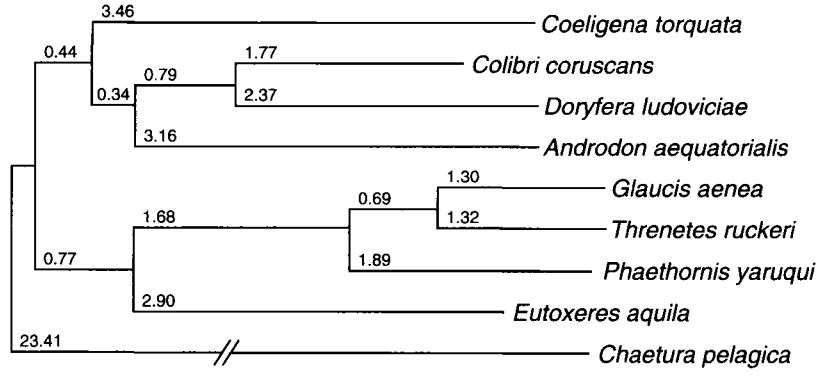

Fig. 3. Fitted branch lengths for symmetrized and Jukes-Cantor-corrected $\Delta T_{50}H$ matrix.

pers. comm.), our DNA-DNA hybridization data indicate that *A. aequatorialis* is a derived clade that is not even the closest relative of *Doryfera ludoviciae*, the other hermitlike trochiline in our phylogeny. For the same reasons, the sister relationship between the Andean *Coeligena torquata* clade and the trio of *A. aequatorialis, D. ludoviciae* and *Colibri coruscans* is also noteworthy. Although both morphology and allozymes suggest these same two groups, the latter is interpreted as "primitive" and *C. torquata* as "advanced" (Peters 1945, Zusi and Bentz 1982).

There are few existing hypotheses of higher-level relationships among hermits, in part because of the rather similar appearance of the constituent genera. Sibley and Ahlquist's (1990) DNA-DNA hybridization study did not include a labeled hermit, so they resolved no structure within this clade. Allozymes indicated three groups, *Eutoxeres, Phaethornis,* and *Glaucis-Threnetes,* but their linkages depended on the distance measure and algorithm employed, nor was any group supported by a single synapomorphy (Gill and Gerwin 1989). Our data resolve these same three groups, but also reveal their hierarchical relationships: *Eutoxeres aquila* is basal to the assemblage, followed in order by *Phaethornis yaruqui* and then *Glaucis aenea-Threnetes ruckeri.*

Phylogeny vs. adaptation.—Differences in the resolving power or hypothesized relationships inferred from different methods of phylogeny construction naturally lead to questions about the reliability of these methods as phylogenetic tools. Although DNA-DNA hybridization provides the only available method for comparing entire (single-copy) genomes, the method's application to phylogenetics remains controversial because of questions about the reliability of previous studies. The agreement between our results and those of Sibley and Ahlquist (1990) suggests that those authors obtained robust phylogenetic hypotheses for hummingbirds.

In contrast to DNA-DNA hybridization data, which lends very strong support for a particular topology, allozymes have yielded unstable phylogenetic hypotheses that are sensitive to the genetic-distance measure and to the algorithm applied (Gerwin and Zink 1989, Gill and Gerwin 1989). The superior performance of hybridization may reflect the level of our comparisons, which are in the range in which distance measures such as ΔT_{mode} and ΔT_m perform most effectively. However, we believe that the application of electrophoresis to hummingbird relationships also involves certain idiosyncratic difficulties that stem from biological considerations. One potential shortcoming is that many of the proteins used in allozyme studies are involved in sugar metabolism and may not be neutrally evolving characters in nectarivores. Furthermore, some genetic-distance measures used to analyze allozyme frequency data are sensitive to levels of within-taxon heterozygosity (Swofford and Olsen 1990), which appear to be substantially lower within species of hummingbirds than within other taxa (Gerwin and Zink 1989). This may explain the instability of the trees with respect to different measures and analyses. Our preliminary data indicate that the rate of molecular evolution in hummingbirds is more rapid than in many other birds (Bleiweiss and Kirsch unpubl. data). The coupling of rapid divergence with low levels of heterozygosity could lead to widespread fixation of unique alleles in different taxa, which is consistent with the high percentage of autapomorphic characters observed for many hummingbird genera (Gill and Gerwin 1989). The paucity of synapomorphies would then yield little resolution in the trees.

Morphological characters especially can be sensitive to adaptive modifications that obscure relationships. For example, the distinction between hermit and nonhermit hummingbirds constitutes an ecological dichotomy (as discussed in the introduction) that could reflect ecological convergence among unrelated forms. Despite this potential difficulty, hypotheses of subfamily structure and major groupings therein based on internal morphology (Zusi unpubl. abstract, Zusi and Bentz 1982) agree quite well with those from DNA-DNA hybridization, although a more explicit cladistic analysis of morphology is needed to determine the extent to which morphology and DNA agree about actual branching patterns within subfamilies.

It is less surprising that external bill and plumage characters are misleading indicators of higher-level relationships, as these characters vary within hummingbird genera (Hilty and Brown 1986). The broad concordance of evidence placing *Androdon aequatorialis* and *Doryfera ludoviciae* within the Trochilinae gives us confidence that their hermitlike bills and plumages do not reflect relationship to hermits. Indeed, the other trochiline genera represented in our phylogeny also contain dull monomor-

phic species (*Colibri delphinae, Coeligena coeligena, Coeligena wilsoni*). The widespread occurrence of dull monomorphic plumage among the Trochilinae suggests a complicated pattern for the evolution of this unusual plumage. The most plausible hypothesis is that dull monomorphic plumage evolved convergently several times in the subfamily.

Historical Biogeography

Timing of divergence.—Although clocklike molecular evolution potentially could be used to determine the timing of major splitting events from branch lengths, the hummingbird clock is difficult to calibrate for several reasons. First, their fossil record is limited in time and scope, being comprised exclusively of a few Pleistocene and Recent remains (Olson and Hilgartner 1982, Graves and Olson 1987). Second, the increase in rate of molecular evolution among hummingbirds (see above) may inflate the clock's estimates for divergence times. Finally, as demonstrated here, lineages differ in their rate of molecular evolution. Despite these limitations, we believe that a heuristic evolutionary time scale for hummingbirds can be obtained in the conventional manner with a calibration point provided by the much better fossil record of their sister group, the swifts (Sibley and Ahlquist 1990, Bleiweiss et al. unpubl. data).

The oldest known fossil swifts are members of the extinct taxon *Eocypselus vincenti* from the early Eocene of Great Britain, dating from approximately 50 m.y.a. (Harrison 1985). This true swift undoubtedly postdates the actual time when hummingbirds and swifts diverged from their common ancestor. Thus, the calibration must be a minimum estimate of divergence time. Nevertheless, the estimated timings of environmental events are themselves not so finely resolved as to invalidate rough comparisons. Taking the swift fossil date as the calibration point for the divergence of swifts and hummingbirds, the rate of molecular divergence is estimated as 55 m.y. divided into the average tip-to-tip distance from the swifts (*Chaetura pelagica*) to all hummingbird lineages in the Jukes-Cantor corrected $\Delta T_{50}H$ tree (27.5°C); this yields an average divergence rate of 0.55°C per million years.

History of diversification.—Dividing the average branch length between *C. torquata* and the clade containing the other three trochilines (7.02°C) by the rate calculated above places the divergence between these two clades at approximately 12.76 m.y.a. This mid-Miocene date coincides approximately with a phase of uplift in which the Andes attained elevations above 2,000 m (Reig 1986, Van der Hammen and Cleef 1986). Coincidentally, members of *Coeligena* are endemic to the Andes at elevations above about 1,500 m. The divergence of the *Coeligena* clade during an important episode of mountain-building suggests that some principal trochiline clades arose coincident with the creation of the high elevations they now inhabit. The great relative age and parallel diversification of at least two sympatric lineages associated with the Andes today—those identified by *Coeligena torquata* and *Doryfera ludoviciae*/*Colibri coruscans* in our tree—appear to be two historical factors contributing to the remarkable diversity of Andean hummingbird communities. Nevertheless, the relatively short internodes between the principal hummingbird clades (ca. 1.21°C between the subfamilies, for example) suggest that some major clades diverged over a relatively brief time period.

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