

**SYSTEMATIC PLACEMENT OF THE BEE HUMMINGBIRD (*MELLISUGA HELENAE*) (AVES: TROCHILIDAE) AND POTENTIAL CONSEQUENCES FOR NOMENCLATURE OF THE MELLISUGINI**Yvonne Lawrie^{1†} · Thomas J. Shannon¹ · Arturo Kirkconnell² · Christopher J. Clark³ · J. Martin Collinson¹ · Guy M. Kirwan^{4*}¹University of Aberdeen, School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, Aberdeen AB25 2ZD, UK.²23520 SW, 107 CT, Homestead, FL 33032, USA.³Department of Evolution, Ecology and Organismal Biology, University of California, Riverside, California 92521, USA.⁴Research Associate, Field Museum of Natural History, 1400 South Lakeshore Drive, Chicago, IL 60605, USA.[†]Current address: Terrestrial Ecology Unit, Department of Biology, Ghent University, 9000 Ghent, Belgium.E-mail: Guy M. Kirwan · GMKirwan@aol.com

Abstract · The Bee Hummingbird (*Mellisuga helenae*), a Near Threatened Cuban endemic, is iconic for its tiny size, with the male being the smallest bird in the world. In this study, one mitochondrial gene (ND2) and introns of two nuclear genes (encoding adenylate kinase and beta-fibrinogen) were sequenced and aligned to homologous sequences from other hummingbird species. With high statistical support, both Maximum Likelihood and Bayesian analyses resolved the Bee Hummingbird as sister to the Bahama Woodstar (*Calliphlox evelynae* or *Nesophlox evelynae*) and the Inagua Hummingbird (*C. lyrura*), rather than the congeneric Vervain Hummingbird (*M. minima*). This finding highlights the need for a nomenclatural rearrangement of several hummingbird species, in line with the results of recent molecular phylogenies.

Resumen · **Sistemática del colibrí abeja (*Mellisuga helenae*) (Aves: Trochilidae) y posibles consecuencias para la nomenclatura de Mellisugini**

El zunzuncito (*Mellisuga helenae*), casi amenazado y endémico de Cuba, es icónico por su pequeño tamaño, pues el macho es el ave más pequeña del mundo. En este estudio, un gen mitocondrial (ND2) e intrones de dos genes nucleares (que codifican la adenilato quinasa y el beta-fibrinógeno), fueron secuenciados y alineados con secuencias homólogas de otras especies de colibríes. Con alta significancia estadística, los análisis de máxima verosimilitud y bayesiano ubicaron al colibrí abeja como especie hermana del colibrí de las Bahamas (*Calliphlox evelynae* o *Nesophlox evelynae*) y del colibrí de Inagua (*C. lyrura*), en lugar del congénico colibrí zumbadorcito (*Mellisuga minima*). Este hallazgo pone de manifiesto, en línea con los resultados de filogenias moleculares recientes, la necesidad del reordenamiento de la nomenclatura de diversas especies de colibríes.

Key words: *Calliphlox* phylogenetics · Ciénaga de Zapata · Cuba · DNA · *Nesophlox*

INTRODUCTION

The Bee Hummingbird (*Mellisuga helenae*) was discovered in 1838 and described by Gundlach (in Lembeye 1850). The male is apparently the smallest bird in the world, with an average body mass of just 1.74 ± 0.06 g (mean \pm SD, $N = 8$ adult males) and overall length of 5–6 cm, marginally smaller than the Vervain Hummingbird (*Mellisuga minima*) (male mass: 2.0 g, $N = 1$, CJC unpubl. data) and woodstars in the genus *Chaetocercus* (Slender-tailed Woodstar *C. burmeisteri*: 1.92 g, $N = 1$; Esmeraldas Woodstar *C. berlepschi*: 1.89 ± 0.2 g, $N = 5$). Monotypic *M. helenae* is endemic to the main island of Cuba and the Isle of Pines (nowadays Isle of Youth), where it inhabits semi-deciduous, evergreen and swamp woodland, second growth woods bordering swampy areas, coastal thickets, pine forest (*Pinus cubensis*), and well-treed gardens, from sea level to almost 1800 m (Garrido & Kirkconnell 2000, Kirwan et al. 2019, Kirkconnell et al. 2020). Both its range and population size have contracted due to habitat loss, and the Bee Hummingbird is now classified as Near Threatened (BirdLife International 2019).

Morphological classifications of hummingbirds have traditionally divided the group into two subfamilies: Phaethornithinae (hermits) and the more speciose Trochilinae (typical hummingbirds). However, relationships within the Trochilinae and their generic-level taxonomy remained poorly resolved historically, with a disproportionately large number of monospecific genera, many of which are named for their flamboyant tail ornamentation or bill morphology (Ridgway 1892). The specialized and evolutionarily constrained morphology and physiology of hummingbirds, together with intense levels of sexual selection in many

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species and high degree of co-adaptation to food plants, has created convergences and divergences that confound traditional taxonomic arrangements (Schuchmann 1999).

Recent molecular analyses based on two mitochondrial (ND2 and ND4) and four nuclear genes (beta-fibrinogen intron 7, adenylate kinase intron 5, ornithine decarboxylase, and muscle skeletal receptor tyrosine) have led to a revised taxonomy of hummingbirds, resolving relationships and, in the process, revealing many examples of paraphyly at the genus level (McGuire et al. 2007, 2009, 2014; Abrahamczyk et al. 2015, Feo et al. 2015, Licona-Vera & Ornelas 2017, Clark et al. 2018). These molecular studies suggest that two genera previously considered part of the Trochilinae (*Topaza* and *Florisuga*—the ‘topazes’) are the sister clade of hermits, and together these two are sister to all other hummingbirds, necessitating recognition of a third subfamily, the Topazinae (see Chesser et al. 2012). The remaining trochilids cluster into seven major well-supported clades, all of which might be recognized as additional subfamilies (‘mangos’, ‘brilliant’, ‘coquettes’, *Patagona*, ‘mountain gems’, ‘bees’, and ‘emeralds’) (McGuire et al. 2007, 2009; Dickinson & Renssen 2013).

The genus *Mellisuga* (Brisson, 1760) comprises two species: the Bee Hummingbird and the Vervain Hummingbird of Jamaica and Hispaniola (Schuchmann 1999, Dickinson & Renssen 2013, del Hoyo & Collar 2014). Until the mid-20th century, *M. helena* was placed in *Calypte* (Gould, 1856) (Ridgway 1911, Cory 1918, Barbour 1923, Bond 1940), until Peters (1945: 137) suggested that it might be more closely aligned with *M. minima*. Genetic analyses previously showed that the Vervain Hummingbird belongs in a strongly supported clade with the Black-chinned Hummingbird (*Archilochus alexandri*, distributed from southwest Canada to northern Mexico), the Ruby-throated Hummingbird (*A. colubris*, a widespread breeder over southern Canada and the eastern USA, and wintering in Middle America), the Bahama Hummingbird (*Calliphlox evelynae*, endemic to the Bahamas and the Caicos Islands), and the Inagua Hummingbird (*C. lyrura*) (McGuire et al. 2007, 2014). The latter is a recent split from *C. evelynae* and is confined to Great and Little Inagua islands (Feo et al. 2015).

The genus *Calliphlox* (Boie, 1831) highlights the incongruence between molecular relationships and traditional taxonomic arrangements. Five species are regularly placed in *Calliphlox*: the Amethyst Woodstar (*C. amethystina*), the Magenta-throated Woodstar (*C. bryantae*), the Purple-throated Woodstar (*C. mitchellii*), the Bahama Hummingbird, and the Inagua Hummingbird (Gill & Donsker 2019). *Calliphlox* is one of two genera (the other being *Chaetocercus*) that share a derived character of ‘highly advanced’ *musculus tensor propatagialis pars brevis*, the wing muscle (Zusi & Bentz 1982, Zusi 2013). Species placed in *Calliphlox* share certain similar morphological features: their size (6–9 cm) and mass (2.3–3.3 g), a long straight or slightly curved bill, green upperparts with a variable amount of rufous, belly variably patterned with white, orange-buff, and green, in adult males an iridescent violet-purple throat and a deeply-forked tail with green central feathers and partially cinnamon-rufous inner webs, and in females a rounded tail with green central rectrices and the other feathers of the tail cinnamon with a broad black subterminal band. However, these general patterns are also exhibited by multiple other species in other

genera—that is, many of these characters are plesiomorphic. Based on molecular data, *Calliphlox* is clearly paraphyletic and generic rearrangement is required (McGuire et al. 2014, Licona-Vera & Ornelas 2017). Of the five *Calliphlox* species, not only are *evelynae* and *lyrura* genetically distant from *amethystina*, *bryantae*, and *mitchellii*, but their courtship displays are dissimilar too (Clark et al. 2018). The generic name *Nesophlox* (Ridgway 1910) is available for *evelynae* and *lyrura* (Schuchmann et al. 2019), a classification recently adopted by BirdLife International (2019) and the American Ornithological Society (Chesser et al. 2019).

The close morphological similarity between Bee and Vervain hummingbirds was invoked to place the Bee Hummingbird in the genus *Mellisuga* (AOU 1983, 1998), but there are some differences between them. *M. minima* is 5–6 cm long, with a mass of 2.0 g (males) and 2.15 g (females) (CJC unpubl. data). It has a straight, short and dull black bill, and metallic green upperparts. The adult male has a slightly concave tail, the female and young male have a rounded tail with the two outermost rectrices broadly tipped with white. Both male and female display greenish flecks on the throat (no gorget), and the male has extensive greenish on the sides. The Bee Hummingbird’s size is 5–6 cm, it has a mass of 1.7 g (males) or 2.2 g (females), and the bill is dull black and very slightly decurved. Adult males have an iridescent red head with elongated lateral plumes, including coronal iridescence otherwise observed only in the genus *Calypte*, deep blue upperparts, darker tail, greyish-white underparts, and a slightly concave tail. Females have upperparts green to greenish blue or intense blue, and a rounded tail with two broad white spots on the outer feathers, a feature which is also present in young males. Consequently, although many of the just-mentioned characters are plesiomorphic, the relationship between Bee and Vervain hummingbirds cannot be assumed on the basis of morphology alone without a complementary genetic analysis. Clark et al. (2018) suggested that the Bee Hummingbird is sister to the Bahama and Inagua hummingbirds, but this result was based on a fragment of one mitochondrial gene (ND2) obtained from a single toe pad (belonging to MCZ 80780, collected at Santa Bárbara, Isle of Pines, held at the Museum of Comparative Zoology, Harvard University, Cambridge, MA). Here, we report sequencing of additional genes from two new Bee Hummingbird individuals and demonstrate that, while it is closely related to the Vervain Hummingbird, the Bee Hummingbird is indeed a sister species to Bahama and Inagua hummingbirds.

METHODS

Sampling. In order to determine the taxonomic affinity of the Bee Hummingbird, muscle tissue samples from two individuals were analyzed. The first (‘MH01’) was a bird mist-netted at Bermejas, in the Ciénaga de Zapata, Matanzas province, Cuba (22°08′00″N, 80°58′00″W, July 2014) that died during processing, but was not saved as a study specimen. The second specimen (‘MH02’) was a female collected by CJC at Bermejas on 5 May 2015, and subsequently deposited in the Museo Nacional de Historia Natural Cubana, La Habana (MNHNC 24.001420). Genomic DNA was isolated from these tissues using the DNA Micro Kit (QIAGEN, UK), following the manufacturer’s instructions, with the addition of 0.01 M dithiothreitol to the digestion mix and elution in

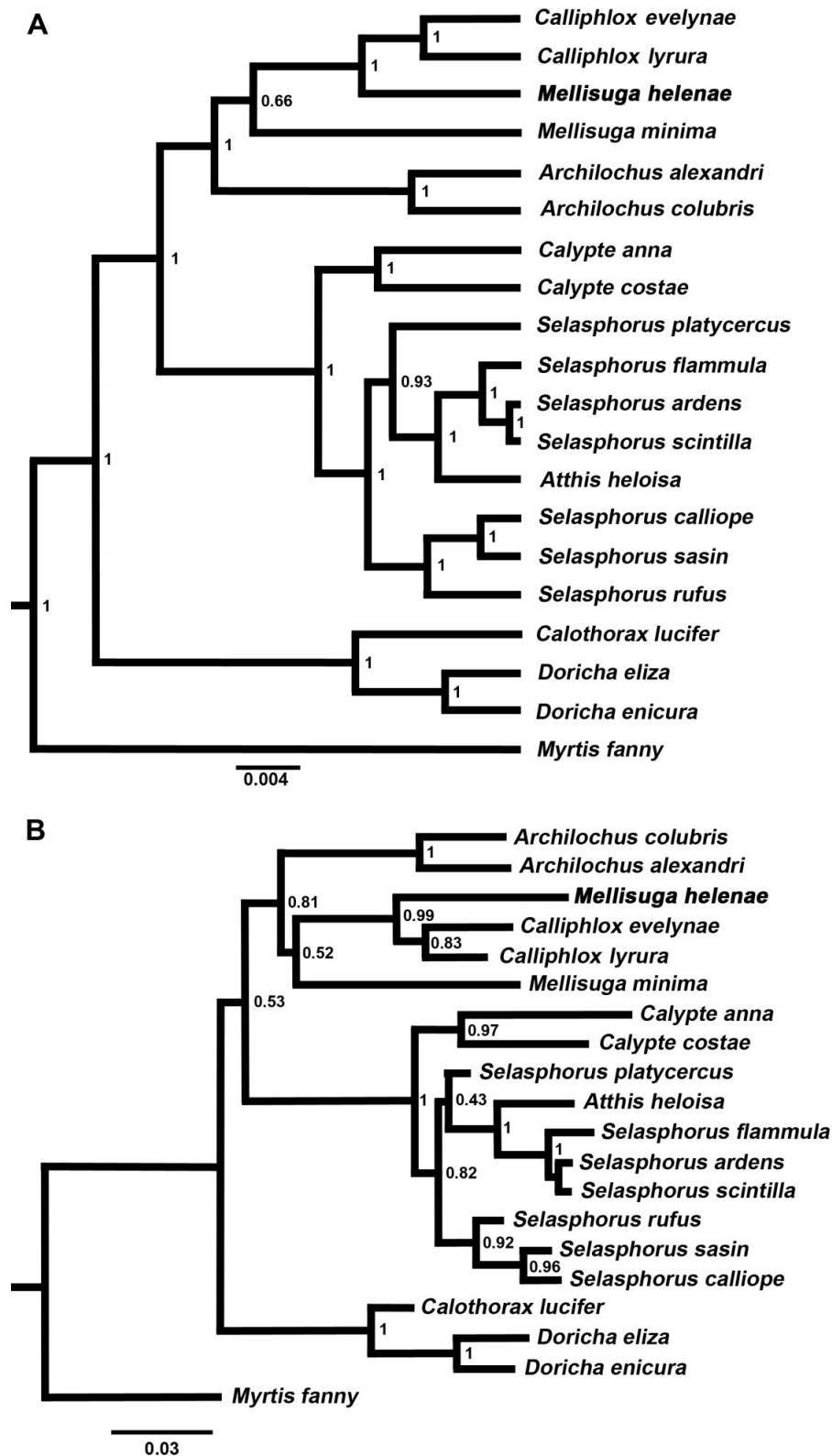


Figure 1. Phylogenetic placement of the Bee Hummingbird (*Mellisuga helenae*). (A) Bayesian and (B) Maximum Likelihood-based phylogenies including the Bee Hummingbird based on 2453–2466 bp of concatenated mitochondrial and nuclear DNA sequence of new data from this study and previously published sequences. Posterior probability and Bootstrap support for key nodes is indicated. See text for details. Bahama and Inagua hummingbirds (woodstars) are split and listed as *Calliphlox evelynae* and *C. lyrura* following Gill & Donsker (2019).

80 μ l of QIAGEN buffer AE. Samples were handled in a clean environment to prevent contamination, and no other hummingbird samples have been processed in the Aberdeen facility. A combination of universal PCR primers L5216 and H6313 (Shannon et al. 2014), and bespoke primers MH01F4 (TTCACTTCTGATTCCCCGA) and MH01R2 (TGAGTAGTAGGC-TAGTCGAG) were used to amplify the full length of the mi-

tochondrial ND2 gene from the samples. Amplification of part of intron 5 of the beta-fibrinogen (FIB5) and of adenylylate kinase (AK1) genes (Shapiro & Dumbacher 2001) was achieved using primers described in McGuire et al. (2007, 2014).

These markers (one mitochondrial and two nuclear genes) were among those sampled by both McGuire et al.

(2014) and Clark et al. (2018), enabling ready comparison of relevant findings. Each 50 µl PCR reaction contained 28.5 µl of ddH₂O, 5 µl of 10x Optibuffer, 1 µl of 50 mM MgCl₂ solution, 3 µl of dNTPs (2 mM each), 5 µl of forward and reverse primers (10 mM each), 2 units (0.5 µl) of BIO-X-ACT Short thermostable DNA Polymerase (Bioline, UK), and 2.5 µl of template DNA. An annealing temperature of 55°C and 30 s extension was used in all cases. PCR products were separated by electrophoresis on 1.5% agarose gels. The DNA from each gel fragment was then isolated using the QIAquick Gel Extraction Kit (Qiagen, UK) according to the manufacturer's protocols. Gel-extracted PCR products were sequenced by Source BioScience (Nottingham, UK). Both Bee Hummingbird samples shared identical alleles of all genes and are treated as a single entity in analyses. All new sequences have been deposited in the European Nucleotide Archive with accession numbers: 'MH01': LR983915 (ND2), LR983917 (AK1) and LR983919 (BFIB7), and 'MH02': LR983916 (ND2), LR983918 (AK1) and LR983920 (BFIB7).

Phylogenetic analysis. To reconstruct phylogenetic relationships, sequences were aligned using ClustalW multiple alignment with MEGA7.0 (Tamura et al. 2013), and the AK1, FIB7, and ND2 genes were concatenated using CLC Sequence Viewer (<http://www.clcbio.com/products/clc-sequence-viewer/>). Sequences for hummingbird comparison groups were sourced from GenBank (accession numbers provided in Supplementary Table 1). The Purple-collared Woodstar (*Myrtis fanny*) was used as an outgroup (McGuire et al. 2014). Phylogenetic trees were constructed using Bayesian Inference in BEAST v2.5.2 (Bouckaert et al. 2014), employing the best-fitting nucleotide substitution model (HKY+G+I) for the concatenated dataset with an empirical base frequency. We employed an uncorrelated relaxed clock with a log-normal distribution, using a Yule speciation prior (Yule 1925) and a random starting tree as the start point. Data were unpartitioned. Two independent Markov Chain Monte Carlo (MCMC) chains were run for 10 million generations for the calibrated concatenated data set, sampling the estimated parameters every 1000 generations. The convergence of each MCMC chains was evaluated in TRACER v1.7.1 (Rambaut et al. 2014), with Effective Sample Size (ESS) values reaching above 200 for all the sampled parameters. Branch support was evaluated using Bayesian Posterior Probabilities (BPP). A Maximum Clade Credibility (MCC) tree was generated in TreeAnnotator v1.10.4 (Drummond et al. 2012), with a burn-in of 10%. We visualized trees in FIGTREE v1.4.4 (Rambaut et al. 2014). Running the analysis with a strict clock yielded the same topology. For Maximum Likelihood analyses, alignments were performed with MEGA7 or CLC Sequence Viewer (<http://www.clcbio.com/products/clc-sequence-viewer/>), and phylogenetic reconstructions with PhyML (Dereeper et al. 2008) and TreeDyn (Chevenet et al. 2006). Analyses were done online using the South of France Bioinformatics Platform (<http://www.atgc-montpellier.fr/index.php?type=pg>), a best fit substitution model derived by software, and 100 Bootstrap replicates to indicate statistical support for nodes. A preliminary Maximum Likelihood (ML) analysis was performed using only the full-length 1041 bp ND2 sequence across a broad range of hummingbird taxa (including many taxa considered to be only distantly related to the Bee Hummingbird) (Supplementary Figure 1).

RESULTS

Our ML analysis placed the Bee Hummingbird with high statistical support (ML = 0.99) in a clade that includes the Vervain, Bahama, and Inagua hummingbirds, as well as the Black-chinned and Ruby-throated hummingbirds (*Archilochus*). This clade is closely related to several other species in the genera *Selasphorus*, *Calypte*, *Atthis*, *Calothorax*, and *Doricha*. These data suggested, albeit based on only one gene, that the Bee Hummingbird is sister to the Bahama Hummingbird (95% bootstrap support). Uncorrected sequence similarity was 92.1% between the Bee and Vervain hummingbirds, less than the 94.7–95.3% between the Bee Hummingbird and Bahama and Inagua hummingbirds.

After 2453–2466 bp of concatenated nuclear and mitochondrial sequence were obtained and assembled from the Bee Hummingbird, we aligned them to homologous sequences from all species in the 'Bee Hummingbird' subclade identified above. Variation in sequence length among the different taxa was due to the presence of six small (1–12 bp) indels in intronic nuclear sequences of at least one of the species in the alignment.

ML and Bayesian trees (Figure 1) were congruent with each other and resolved a strongly supported clade containing *Archilocus alexandri*, *A. colubris*, *Mellisuga helenae*, *M. minima*, *Calliphlox/Nesophlox evelynae*, and *Calliphlox/Nesophlox lyrura*. Within that clade there was only poor support for relative placement of *M. minima* (BI: 66, ML = 0.52), as in previous studies. A sister relationship was apparent, with 1.0 posterior probability (Bayesian tree) and 99% bootstrap support (ML tree), between the Bee Hummingbird and the Bahama and Inagua hummingbirds.

DISCUSSION

Our genetic data are not congruent with current taxonomy, but do show much overlap with the phylogenies presented in McGuire et al. (2014) and Licona-Vera & Ornelas (2017), which did not include the Bee Hummingbird, as well as with the more recently published Clark et al. (2018) study, which did. The hypothesis that *M. helenae* and *M. minima* are sister species is rejected, and the genus *Mellisuga* as currently defined is almost certainly paraphyletic. Possible generic rearrangements in light of our independent data and previous studies are now discussed.

Although hummingbirds originated in Eurasia, diverging from swifts around 42 million years ago, all extant taxa can be traced to a radiation from the lowlands of South America within the last 20–25 million years (Mayr 2007, Bochenski & Bochenski 2008, McGuire et al. 2014). Furthermore, of the nine genetically distinguished major clades of hummingbird (see Introduction), the 'bees' (an assemblage of 16 genera mainly confined to the Caribbean Basin and its periphery, including *Calliphlox*, *Myrtis*, *Chaetocercus*, *Calothorax*, *Doricha*, *Archilochus*, *Mellisuga*, *Calypte*, *Atthis*, and *Selasphorus*) have a high rate of species diversification since their origination within the last c. 5 million years (McGuire et al. 2014). Rapid radiation into vacant niches has obscured true phylogenies in the absence of genetic data, and it is now apparent that large-scale revision of generic nomenclature is required, especially within the 'bees' and the 'emeralds'. To date, broad-scale nomenclatural revision of the family has

been attempted only for the Polytminae, or the ‘mangos’ (Remsen et al. 2015) and the Trochilini, or the ‘emeralds’ (Stiles et al. 2017a,b, Bruce & Stiles 2021).

Our data suggest that the Bee Hummingbird is sister to the Bahama and Inagua hummingbirds. A recent parallel genetic study based on a single sample from the Isle of Pines (rather than from mainland Cuba) and a different, overlapping but independently derived and analyzed dataset produced the same topology. It also concluded that *Mellisuga* is paraphyletic, and that the Bee Hummingbird is sister to the Bahama and Inagua hummingbirds (Clark et al. 2018). Although our study is not as large as those by Clark et al. (2018), Licona-Vera & Ornelas (2017), or McGuire et al. (2014), the topology of our phylogenetic trees and the likely relationships between species are consistent with those studies, providing additional confidence in the placement of the Bee Hummingbird. Furthermore, our findings underline those of Clark et al. (2018) in relation to the latter taxon, which was poorly screened (Clark et al. sampled only ND2 from a single museum specimen, compared to three fresh samples and six genes from the Vervain Hummingbird). The clade we resolved with 100% posterior probability support, containing all of the Bee, Vervain, Bahama, Inagua, Black-chinned, and Ruby-throated hummingbirds, also has 100% posterior probability support in the analyses of Licona-Vera & Ornelas (2017), McGuire et al. (2014) and Clark et al. (2018). We therefore believe that generic level revision is required. However, none of the existing studies strongly resolves the position of the Vervain Hummingbird within this clade.

One possible rearrangement, with limited ramifications for taxonomic stability, is to accept, on the basis of genetics and morphology (this study; Clark et al. 2018), that the most likely placement of the Vervain Hummingbird (*Mellisuga*) is as sister to a clade containing the Bee Hummingbird (*Mellisuga*) and the Bahama and Inagua hummingbirds (*Calliphlox* or *Nesophlox*), excluding the two species of *Archilochus*, the Black-chinned and Ruby-throated hummingbirds. Under this scenario, an acceptable solution is to maintain *Archilochus* (Reichenbach, 1854), but to place the other four species in a single genus, for which *Mellisuga* (Brisson, 1760) would have priority.

A more conservative solution, until the placement of the Vervain Hummingbird is resolved through the acquisition of more data, would be to place the Bee, Vervain, Black-chinned, Ruby-throated, Bahama, and Inagua hummingbirds in a single genus, for which *Mellisuga* would have priority. Additional work, involving larger sample sizes and more markers (both mitochondrial and nuclear), presumably will be necessary to more clearly resolve the relationships among these taxa, and consequently the most appropriate nomenclature. The use of *Nesophlox* (Ridgway, 1910) for the Bahama and Inagua hummingbirds is not strongly supported by genetics alone, and will need robust justification on morphological, vocal or behavioral criteria, if it is to be more widely adopted.

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